Award Number:
W81XWH-09-1-0083

TITLE: Chemotherapeutic Targeting of Fibulin-5 to Suppress Breast Cancer Invasion and Metastasis Stimulated by Transforming Growth Factor-Beta

PRINCIPAL INVESTIGATOR:
William P. Schiemann, Ph.D

CONTRACTING ORGANIZATION:
Case Western Reserve University, Cleveland, OH 44106

REPORT DATE:
October 2012

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
10-22-2012	Final Report	13 Dec 2011 – 13 Oct 2012

4. TITLE AND SUBTITLE
Chemotherapeutic Targeting of Fibulin-5 to Suppress Breast Cancer Invasion and Metastasis Stimulated by TGF-beta

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0083

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. William P. Schiemann

E-Mail: wps20@case.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Case Western Reserve University
School of Medicine
10900 Euclid Avenue
Cleveland, OH 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary objective of this application is to establish how Fibulin-5 enhances the oncogenic activities of TGF-beta, particularly its ability to stimulate breast cancer invasion and metastasis. We hypothesized that inactivating Fibulin-5 function will prevent the conversion of TGF-beta from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-beta. Major findings of the past funding cycle include the ability of Fibulin-5 to (i) bind integrins on mammary epithelial cells (MECs) independent of its integrin-binding RGD motif; (ii) interact physically with TGF-beta and enhance its presentation to TGF-beta receptors; (iii) promote epithelial-mesenchymal transition in an integrin-independent manner in part by stimulating the expression of Cox-2, PAI-1, and MMP-9; (iv) enhance MEC proliferation by activating FAK and ERK1/2; and (v) induce MEC resistance to apoptosis and anoikis by stimulating NF-kappaB activation, by inducing survivin and xIAP expression, and by repressing TNF-alpha expression. Finally, we determined that Fibulin-5 expression is greatly augmented during breast cancer progression, particularly at the point when malignant MECs acquire metastatic phenotypes. This important finding implicates Fibulin-5 as a potential marker for breast cancer metastasis and reinforces the need to target Fibulin-5 chemotherapeutically in patients with metastatic disease.

15. SUBJECT TERMS
Transforming Growth Factor-beta; Fibulin-5; Breast Cancer; Metastasis; Invasion; Chemotherapy

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU		**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction…….. 4

Body…….. 4

Key Research Accomplishments…….. 11

Reportable Outcomes……. 11

Conclusion……. 14

References……. 1

Appendices……. 17

INTRODUCTION:

Breast cancer is the second leading cause of cancer death in women in the United States. Invasion and metastasis are the most lethal characteristics of breast cancer and the leading cause of breast cancer-related death. TGF-β normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, or by inducing MEC apoptosis. Mammary tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate breast cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant MECs overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the development and progression of mammary tumors. These knowledge gaps have prevented science and medicine from implementing treatments effective in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. We recently discovered that the expression and activity of the TGF-β gene target, Fibulin-5 (FBLN5), potentiates TGF-β stimulation of invasion and epithelial-mesenchymal transition (EMT) in normal and malignant MECs *in vitro*, and more importantly, enhances the growth and pulmonary metastasis of mammary tumors in mice. Interestingly, we find that FBLN5 incorporates into active TGF-β receptor complexes in a β3 integrin-dependent manner, an event associated with the activation of intracellular signaling by TGF-β. Based on these and other compelling findings, we hypothesized that inactivating FBLN5 function will prevent the conversion of TGF-β from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-β. The goals of this project are to determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and to determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs. Finally, we will determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression *in vivo*. These studies are important because they will *(i)* provide valuable information on how breast cancers develop and progress, and on how TGF-β promotes these processes; *(ii)* identify the signaling mechanisms and systems that mediate the oncogenic nature of TGF-β; and *(iii)* identify FBLN5 antagonists capable of alleviating the oncogenic activities of TGF-β, as well as establish their effectiveness in preventing breast cancer progression stimulated by TGF-β. Moreover, application of our findings will enable science and medicine to one day improve the prognosis and treatment of patients with metastatic breast cancer

BODY:

Overview and General Findings: The specific aims of the proposed research have not been modified. Indeed, our recently published manuscript in the journal *Carcinogenesis* [1] clearly established the importance of FBLN5 in promoting epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells (MECS). Equally important, we showed that FBLN5 expression greatly enhanced the ability of TGF-β to stimulate EMT, as well as promoted its oncogenic activities in normal and malignant MECs both *in vitro* and *in vivo*. Clearly, elucidating the molecular mechanisms that enable FBLN5 to enhance oncogenic TGF-β signaling has tremendous potential to neutralize the metastasis promoting activities of this multifunctional cytokine, and as such, to ultimately improve the clinical course of breast cancer patients with metastatic disease.

Data in the scientific literature has recently established the essential role of TGF-β in regulating the activities of breast cancer-associated fibroblasts and stromal components [2-5]. Indeed, mounting evidence indicates that TGF-β promotes breast cancer progression in part *via* its reprogramming of MEC microenvironments and their cellular architectures. Moreover, TGF-β also induces desmoplastic and fibrotic reactions that elicit the formation of tense, rigid tumor microenvironments that *(i)* enhance the selection and expansion of developing mammary neoplasms, particularly that of late-stage metastatic cells, and *(ii)* predict for poor clinical outcomes in breast cancer patients. Our previous published studies established FBLN5 as an important stromal-produced secreted factor that regulates tumor development in mice [6-9]. Thus, we characterized changes in the fibroblast transcriptome elicited by FBLN5, or by FBLN5 plus TGF-β. Microarray analyses identified 1181 genes whose expression is regulated by FBLN5, and an additional 1675 genes whose expression is regulated by

TGF-β. Differential expression of 14 individual genes was verified by semi-quantitative real-time PCR. Downregulated FBLN5 gene targets included *a)* BB503935; *b)* pleckstrin-homology domain-containing family A member; *c)* transglutaminase-2; and *d)* Rho GTPase activating protein 24. Upregulated FBLN5 gene targets included *a)* BB533736; *b)* BB831146; *c)* HoxD9; *d)* thrombospondin-1; *e)* collagen type XI; *f)* angiopoietin-1; *g)* cysteine-rich protein 61; *h)* Dkk3; *i)* fibromodulin; and *j)* HoxD10. Oncomine analyses showed the expression of fibromodulin to be upregulated in human breast cancers, and as such, we further characterized the activities of this novel FBLN5 gene target. In doing so, we found that fibromodulin expression greatly enhanced the coupling of TGF-β to Smad2/3 and AP-1 activation, while simultaneously abrogating both basal and TGF-β-stimulated NF-κB activation in fibroblasts. Importantly, we observed fibromodulin expression to stabilize that of the NF-κB inhibitory protein, IκBα. We further determined that fibromodulin stabilized IκBα expression by activating JNK and CK-II, which inactivate calpain and its proteolytic activity against IκBα. Thus, in addition to inhibiting NF-κB activity in fibroblasts, the activation of this fibromodulin-dependent pathway promotes apoptosis in fibroblasts. Even more strikingly, Oncomine analyses showed the expression of fibromodulin to be reduced at metastatic sites relative to nonmetastatic lesions in gastric cancers, head and neck cancers, and sarcomas. These analyses also found aberrantly low fibromodulin expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood. Our findings related to this novel FBLN5:fibromodulin signaling axis were published in February 2001 by the *Journal of Biological Chemistry* (vol 286, pages 6414-6422). **Clinically, chemotherapeutic targeting of this pathway may offer novel inroads into alleviating the oncogenic activities of TGF-β in breast cancer stroma.**

Over the course of the past funding cycle, we have potentially discovered some spectacular insights into a longstanding paradox that has confounded *TGF-β Biologists* over the last decade – *i.e.,* how do gains [1, 10-21] and losses [5, 22, 23] of TGF-β function both drive breast cancer metastasis within the same late-stage breast cancers? As will be discussed in greater detail below, we recently observed hypoxia to be sufficient in inactivating the TGF-β signaling, presumably as a means to circumvent the ability of TGF-β to induce apoptosis in hypoxic MECs. Along these lines, we also observed hypoxia to significantly upregulate FBLN5 expression, which mediates survival signaling and prevents MEC apoptosis elicited by hypoxia. These findings are potentially paradigm changing and provide novel and innovative insights into the inherent plasticity employed by late-stage breast cancer cells to facilitate their development and metastatic progression. **These important findings are now being prepared to publication.**

Based on our findings presented below, we remain convinced that our analyses of noncanonical and oncogenic effectors targeted by FBLN5 and TGF-β will enable the development of safer, more directed chemotherapies capable of phenotypically normalizing and reverting the malignant behaviors of developing and progressing breast cancers.

PLEASE NOTE: Figures and findings previously presented as part of our BC084651 mid-term and annual reports are indicated in "blue text – e.g., Fig. 1." Figures and findings during December 2010-December 2011 are indicated in "red text – e.g., Fig. 2." Finally, newly generated figures and findings obtained in the last reporting year from December 2011-October 2012 are indicated in "green text – e.g., Fig. 25."

Task-Specific Findings:

Task 1: ***Determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes.*** We previously engineered normal NMuMG and metastatic 4T1 cells to stably express β3 integrin or its inactive mutant, D119A-β3 integrin [10-12]. Our previously published studies demonstrated the function of β3 integrin in promoting oncogenic TGF-β signaling, including its ability to stimulate EMT and pulmonary metastasis of breast cancer cells [10-12]. We recently introduced wild-type FBLN5 and its RGE-mutant, which we demonstrated previously to prevent FBLN5 from ligating integrins on endothelial cells [7]. The functional characteristics of these FBLN5 and β3 integrin manipulations on MEC behavior in response to TGF-β are quite interesting and will be

discussed below (*see Task 2).* With respect to the primary objective of Task 1 – *i.e.,* to identify the molecular determinants that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and more importantly, to determine the impact of disrupting the formation of these complexes on normal and malignant MEC response to TGF-β – our preliminary data indicate that FBLN5 is capable of binding β3 integrin on MECs independent of its integrin-binding RGD motif. Indeed, we find that MEFs derived from FBLN5-deficient embryos respond poorly to TGF-β, and that re-expression of either wild-type FBLN5 or RGE-FBLN5 molecules in these FBLN5-deficient MEFs significantly enhance MEF response to TGF-β. Thus, our findings to date suggest that FBLN5 may incorporate into TGF-β receptor complexes independent of traditional integrin-binding activities. Alternatively, FBLN5 may incorporate into TGF-β receptor complexes in a manner wholly independent of β3 integrin. With respect to the former possibility, we now are optimizing the expression and purification systems necessary to isolate various recombinant FBLN5 mutants, including full-length wild-type and RGE-mutant FBLN5 molecules, as well as those mutants that lack the N-terminal Pro-rich domain (*i.e.,* ΔPro), the entire N-terminal domain (*i.e.,* ΔNT), and the entire globular C-terminal domain (*i.e.,* ΔCT) or those that only contain the N-terminal (*i.e.,* NT-FBLN5) or C-terminal (*i.e.,* CT-FBLN5). FBLN5 mutants found to incorporate into TGF-β receptor complexes will then be subjected to gross- and fine-deletion analyses, followed by Ala-scanning mutagenesis to elucidate the molecular determinants that mediate FBLN5 association with TGF-β receptors. We fully expect to possess engineered FBLN5 molecules that are incapable of supporting oncogenic TGF-β signaling by the completion of Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3.

Because our initial studies of FBLN5 incorporation into TGF-β receptor complexes showed that wild-type and RGE-FBLN5 were both capable of capturing β3 integrin in immunocomplex assays, we began to consider the possibility that FBLN5 may incorporate into TGF-β receptors in an integrin-independent fashion. In support of this notion, we found that FBLN5 bears striking homology to members of LTBP (latent TGF-β-binding proteins) family of proteins, particularly in their calcium-binding EGF-like repeats. Thus, we hypothesized that FBLN5 may bind directly to TGF-β, which then pulls FBLN5 into TGF-β receptor complexes. Accordingly and quite surprisingly, we used three separate and distinct binding protocols to show unambiguously that FBLN5 does indeed interact physically with active TGF-β independent of whether FBLN5 can bind to integrins (*i.e.,* wild-type FBLN5 and RGE-FBLN5 bind indistinguishably to active TGF-β). **This finding represents a major advance for TGF-β and FBLN5 biologists, and may in fact explain why FBLN5-deficient MEFs are unresponsive to TGF-β**. Indeed, our findings indicate that FBLN5 may function in binding directly to TGF-β and facilitating its presentation and/or incorporation to inactive TGF-β receptor complexes, resulting in enhanced transmembrane signaling initiated by TGF-β. Accordingly, MECs engineered to overexpress FBLN5 exhibit significantly elevated levels of Smad2/3 activity as compared to their GFP-expressing counterparts, a finding consistent with FBLN5 functioning to present and enhance autocrine TGF-β signaling in normal and malignant MECs. We have now engineered MECs to produce various FBLN5 mutants to map the domains operant in mediate its interaction with TGF-β. After affirming which regions of FBLN5 bind TGF-β1, we will immediately generate FBLN5 mutants that lack this domain/motif to assess how preventing FBLN5 from binding TGF-β impacts normal and malignant MEC response to TGF-β both *in vitro* and *in vivo.* As above, we fully expect to complete this exciting and important task during Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3

Task 2: ***Determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs.*** The primary objective of Task 2 is to identify FBLN5 effectors operant in mediating oncogenic signaling by TGF-β. In this regard, we have found that wild-type and RGE-FBLN5 are both capable of promoting partial EMT phenotype in normal MECs (**Fig. 1A**). Moreover, we find FBLN5 expression to be significantly upregulated in 4T1 progression series, which is an established mouse model of triple-negative breast cancer (TNBC; **Fig. 1B**). Interestingly, we also observed the combination of FBLN5 and β3 integrin to significantly

enhance the proliferative *(data not shown)* and invasive (**Fig. 1C**) potential of normal MECs, a response that was not recapitulated in MECs co-expressing RGE-FBLN5 and β3 integrin. In addition, the combined expression of FBLN5 and β3 integrin greatly attenuated the sensitivity of MECs to the cytostatic activities of TGF-β. The enhanced response of MECs to FBLN5 also correlated with its ability to significantly augment the activation of FAK and ERK1/2 in these same cells *(data not shown)*. Thus, FBLN5 expression induced by TGF-β in normal and malignant MECs appears to play a significant role in mediating its growth promoting activities in MECs. We recently developed the only *in vitro* assay that wholly recapitulates the phenomena underlying the "TGF-β Paradox" during mammary tumorigenesis [16, 24], which converts the actions of TGF-β from that of a tumor suppressor to a tumor promoter (*see* [25-28]). We exploited this unique 3D-organotypic culture system to further explore the function of FBLN5 in promoting an invasive phenotype in normal MECs. In doing so, we propagated parental NMuMG cells or their derivatives engineered to stably express either FBLN5, β3 integrin, or both transgenes in combination in the absence or presence of TGF-β under compliant or rigid 3D-organotypic culture conditions. **Figure 2** shows that relative to parental (*i.e.,* GFP/YFP) NMuMG cells, those engineered to express β3 integrin formed substantially larger (by 275%) and densely packed acinar structures, while those expressing FBLN5 formed substantially smaller (by 65%) organoids. Interestingly, NMuMG cells that expressed both transgenes formed acinar structures whose size was also substantially larger (by 195%) than their parental counterparts and in many respects resembled those of β3 integrin-expressing NMuMG cells (**Fig. 2**). Thus, β3 integrin stimulates acinar growth, while FBLN5 inhibits this event in a manner that can be neutralized by expression of β3 integrin. Despite the dramatic differences in their relative growth rates, all four NMuMG derivatives readily responded to the cytostatic activities of TGF-β and formed diminutive organoids when propagated in continued presence of this cytokine (**Fig. 2**). When cultured under rigid conditions, all NMuMG derivatives acquired a branched morphology that was potentiated by β3 integrin expression (**Fig. 2**). Quite surprisingly, FBLN-expressing NMuMG cells formed unique linear and irregularly-branched structures indicative of a highly invasive phenotype (**Fig. 2**). Moreover and in contrast to their parental and β3 integrin-expressing expressing counterparts, FBLN5-expressing NMuMG cells were uniquely resistant to the apoptotic inducing activities of TGF-β under rigid microenvironmental conditions (**Fig. 2**). Moreover, the survival promoting activities of FBLN5 are wholly coupled to the ability of FBLN5 to activate Src *(data not shown*). Taken together, these new findings offer some potentially important insights into the role of FBLN5 in MECs, suggesting that FBLN5 may function as an inhibitor of acinar growth and development in compliant microenvironments. However, these findings also suggest that FBLN5 may sense and respond to mechanically rigid and tense microenvironmental conditions, resulting in the acquisition of invasive and survival phenotypes.

Along these lines and in stark contrast to its effects in fibroblasts [9], we find that FBLN5 greatly enhances basal and TGF-β-stimulated NF-κB activity in normal (**Fig. 3A**) and malignant *(data not shown)* MECs in part *via* promoting increased degradation of IκBα (**Fig. 3B**). Moreover, these FBLN5-dependent activities require signaling inputs initiated by Src *(data not shown)*. Indeed, we recently found that EMT induced by TGF-β initiates a pro-survival gene expression profile *(data not shown)*, such that MECs that survive the EMT process are more resistant to apoptosis and anoikis. Given our published work that FBLN5 promotes EMT in normal and malignant MECs [1], we reasoned that FBLN5 expression would also promote survival signaling in these same cells. Accordingly, we now find that FBLN5 greatly suppresses TNF-α expression (by 90%) in normal MECs *(data not shown)*, while simultaneously stimulating that of the *(i)* survival factors, survivin and xIAP *(data not shown)*; *(ii)* angiogenic and EMT molecule, Cox-2 *(data not shown)*; *(iii)* pro-metastatic molecule, PAI-1 *(data not shown)*; and *(iv)* pro-invasion and EMT-molecule, MMPs 2, 3, and 9 *(see below)*. In addition, we further observed FBLN5 expression to be sufficient in inhibiting Caspase-3/7 activation by TNF-α in normal MECs (**Fig. 4**). Along theses lines, neoadjuvants and conventional chemotherapies can accelerate disease progression and metastasis *via* a hypoxia-induced EMT [29-33]. We too have demonstrated the ability of the c-Abl antagonist, Gleevec (Imatinib) to elicit EMT programs and disease progression of TNBCs [24]. Given these apparent associations between hypoxia and metastatic progression, we addressed the question as to whether hypoxia

augmented or attenuated TGF-β signaling in normal and malignant MECs. **Figure 5A** shows that 4T1 cells readily responded to TGF-β when propagated in traditional 2D-cultures under normoxic conditions, but not when there were cultured under hypoxic conditions. These cells are subject to extensive autocrine TGF-β signaling [13-15, 20, 21], which enhances breast cancer development and progression. As shown in **Fig. 5B**, the ability of hypoxia to inhibit gene transcription appeared to be specific for that mediated by TGF-β, as CMV-driven gene expression was unaffected by changes in oxygen tension. Similar effects of normoxia and hypoxia on TGF-β signaling were also observed metastatic breast cancer cells propagated in 3D-organotypic cultures (**Fig. 5C & D**). Interestingly, we also found FBLN5 expression to be significantly induced by hypoxia (**Fig. 6**). A primary goal of our research is to develop novel means to eradicate TNBC. A major limitation to eradicating TNBCs reflects the inability to create a xenograft model that recapitulates TNBC development and metastatic progression. We have recently devised the means to overcome this barrier by transforming normal MECs (*i.e.,* NMuMG cells) *via* their enforced expression of EGFR, which is upregulated in TNBCs and predicts for poor patient prognosis [34, 35]. We refer to these transformed MECs as "NME cells," which readily form nonmetastatic tumors in mice [20]. Importantly, we can induce NME tumors to acquire metastatic phenotypes by first stimulating them to undergo EMT *in vitro* prior to their implantation into the mammary fat pads of mice [20]. In fact, mice injected with pre-EMT NME cells are cured following primary tumor resection, while animals injected with post-EMT NME cells rapidly develop lethal disease recurrence upon removal of their primary tumors. Additionally, following their stimulation with TGF-β, metastatic NME cells acquire a TNBC phenotype by losing expression of ER-α and PR. Importantly, the phenotypes of individual pre- and post-EMT NME cells are stable following *ex vivo* isolation, thereby providing a unique, powerful, and innovative *in vitro* and *in vivo* system to *"deconstruct"* the molecular and spatiotemporal underpinnings that govern TNBC development, metastatic progression, and disease recurrence. **Figure 7A** shows that parental and FBLN5-expressing NME cells exhibit similar growth characteristics and sensitivity to cytostasis mediated by TGF-β. However, culturing these same cells under hypoxic conditions demonstrated the ability of FBLN5 to significantly promote the growth of NME cells as compared to that of their parental counterparts (**Fig. 7B**). Likewise, enforced expression of FBLN5 conferred NMuMG cells resistance to hypoxia-driven apoptosis (**Fig. 8**), presumably by preventing Caspase-3 cleavage and activation (**Fig. 9**), and by stimulating NF-κB (**Fig. 10**). **Collectively, these findings identify FBLN5 as a novel promoter of breast cancer survival in hypoxic tumor conditions, and as such, bolster the notion that chemotherapeutic targeting of FBLN5 may provide a novel means to eliminate metastatic breast cancers prior to their initial exit and dissemination from their primary tumors. We will test this hypothesis, as well as determine the molecule mechanism that elicits transient inactivation of TGF-β signaling in hypoxic mammary tumors.**

In addition, we have begun manipulating the expression of these FBLN5 gene targets in normal and malignant MECs to access their role in regulating MEC response to TGF-β both *in vitro* and *in vivo.* Initial targets are members of the MMP family of proteases. Indeed, we observed FBLN5 to strongly induce the expression of MMPs 2, 3, and 9 in normal (**Fig. 11**) and metastatic (**Fig. 12**). Interestingly, whereas the ability of FBLN5 to activate NF-κB transpires through an integrin-independent pathway (**Fig. 3**), the coupling of FBLN5 to MMP expression clearly requires signaling inputs from integrins. For instance, **Figure 13** shows that FBLN5, but not its mutant RGE counterpart (*i.e.,* cannot ligate integrins), readily and potently induces the expression of MMPs 2, 3, and 9. Surprisingly, expression of β3 integrin inhibited the ability of FBLN5 to promote MMP expression, a reaction that was not recapitulated by expression of the nonfunctional β3 integrin mutant, D119A-β3 integrin. Thus, while FBLN5 clearly binds β3 integrin, this event serves to neutralize MMP expression stimulated by FBLN5. Mechanistically, the ability of FBLN5 to induce MMP expression in normal MECs transpires through the activation of MAP kinases and Src, as well as require Ca^{++}-dependent signaling inputs (**Fig. 14**). Our findings in **Figure 15** wholly support this idea and also show that the integrin effectors, FAK and Pyk2, are necessary for FBLN5 stimulation of MMP expression in NMuMG cells. In addition to binding to αvβ3 integrin, FBLN5 also ligates αvβ5 and α9β1 integrins, suggesting that the coupling of FBLN5 to MMP expression way proceed through other integrin heterodimers operant in binding FBLN5. Along these lines, we have recently determined that FBLN5

induces MMP expression through a β1 integrin- and ERK1/2-dependent pathway (**Fig. 16**). Indeed, neutralizing antibodies against β3 integrin were ineffective in altering the coupling of FBLN5 MMP expression, which contrasts sharply to the dramatic reduction in MMP expression observed following administration of either neutralizing β1 integrin antibodies (**Fig. 16**), or following cellular depletion of β1 integrin expression (**Fig. 17**). Likewise, we also observed FBLN5 to induce MMP expression to be dependent upon the PTK activity of EGFR, as determined by the ability of the EGFR inhibitor, AG1478 to abrogate MMP expression stimulated by FBLN5 (**Fig. 18A**). Similar antagonism of IGF-1R signaling by administration of AG1024 had no effect on MMP expression induced by FBLN5 (**Fig. 18B**). Mechanistically, these FBLN5-specific events transpire through its stabilization of EGFR expression in MECs (**Fig. 18C**), as well as through its ability to enhance the coupling of EGFR to ERK1/2 in these same MECs (**Fig. 19 & 20**). The latter response is also entirely dependent upon signaling inputs arising from β1 integrin (**Fig. 20**), suggesting that FBLN5 coordinates that the oncogenic crosstalk that exists between β1 integrin and EGFR in mammary tumors.

Finally, two recent studies have linked the induction of EMT by TGF-β to its regulation of protein translation, a reaction comprised of a TGF-β:AKT2:hnRNP E1 signaling axis that enables the production of Dab2 and ILEI [36, 37]. Mechanistically, AKT2-mediated phosphorylation of hnRNP E1 dissociates this molecule from transcripts containing the BAT elements, thereby initiating the synthesis of proteins operant in driving EMT. Additionally, rendering NMuMG cells deficient in hnRNP E1 is sufficient to induce an EMT program [36, 37] in a manner reminiscent of that observed by the overexpression of FBLN5 in these same cells [1]. Moreover, MECs that have emerged from EMT programs universally express robust levels of FBLN5 independent of EMT-initiating agent [38], suggesting an essential role for FBLN5 in driving EMT programs. Along these lines, we recently obtained hnRNP E1-deficient NMuMG cells from Dr. Philip H. Howe (MUSC) and find that these MECs house aberrantly elevated (by ~2000-fold) levels of FBLN5 transcript (**Fig. 21A**) and protein *(data not shown)*. Consistent with the ability of FBLN5 to induce MMP-9 expression (**Fig. 11-18**), we observed hnRNP E1-deficient NMuMG cells to express dramatically upregulated levels of MMP-9 (**Fig. 21B**). Finally, **Fig. 22** shows that FBLN5 induces Dab2 expression in part *via* a β1 integrin-dependent manner, thereby implicating FBLN5 as an important player responsible in mediating the actions of hnRNP E1 in transitioning MECs. We are now testing this important hypothesis by depleting FBLN5 expression in hnRNP E1-deficient cells to gauge the extent to which this ECM protein drives EMT and survival signaling, as well as induces MMP expression during the metastatic progression of malignant MECs. **These findings are a major advance to the fibulin field, and we now are rapidly extending these findings to the aforementioned normal and malignant MECs engineered to express all combinations of wild-type and mutant FBLN5 and β3 integrin molecules**.

Consistent with the aforementioned findings, **Fig. 25** clearly demonstrates that the coupling of FBLN5 to the enhanced survival and proliferation of NMuMG cells under hypoxic conditions requires the expression and activity of β1 integrin, as rendering these MECs deficient in β1 integrin expression alleviated the oncogenic activities of FBLN5. Moreover, these oncogenic FBLN5 activities, as well as those driven by hnRNP E1-deficiency and its dramatic upregulation of FBLN5 expression (**Fig. 21**), are also associated with the ability of hypoxia to induce EMT programs in normal (**Fig. 26**) and malignant *(data not shown)* MECs. Accordingly, FBLN5 enhances the motility and closure of wounded NMuMG cell monolayers specifically under hypoxic culture conditions (**Fig. 27B**), as does hnRNP E1-deficiency in normal MECs (**Fig. 27D**). More importantly, these same culture conditions produce far greater enhancements of breast cancer cell motility as compared to their normal MEC counterparts, thereby implicating hypoxia- and FBLN5-driven migration as a major mechanism underlying the egress metastatic cells from the primary tumor microenvironment. It is important to note that the current dogma states that the successful completion of mammary carcinoma metastasis requires disseminated breast cancer cells to undergo mesenchymal-epithelial transition (MET), which reverses the phenotypic manifestations of EMT [26-28]. As such, we observed EMT programs induced by hypoxia to be readily reversed upon reoxygenation of breast cancer cells (**Fig. 28A**), or of hnRNP E1-deficient NMuMG cells (**Fig. 28B**). Along these lines, **Fig. 29** shows that normal MECs readily

undergo apoptosis following chronic exposure to hypoxia, an event that is overcome by their malignant counterparts. More importantly, we find that these prosurvival activities conferred to post-EMT and hypoxic breast cancer cells also extend to protect these cells from anoikis-induced cell death (**Fig. 30A-C**). Thus, we have uncovered a novel FBLN5 and hypoxia signaling pathway operant in driving the metastatic progression of breast cancer cells.

In an effort to establish the underlying mechanism that couples hypoxia to enhanced breast cancer cell survival and metastasis, we observed breast cancer cells to progressively downregulate their expression of the cell cycle inhibitor, p27, as compared to its levels measured in normal MECs (**Fig. 31**). Indeed, whereas p27 expression is stabilized in normal MECs propagated under hypoxic culture conditions, we found p27 levels to be dramatically lower in hypoxic breast cancer cells as compared to their normoxic counterparts (**Fig. 32B**). Alterations in p27 stability appear to reflect the ability of breast cancer cells to actively degrade p27 proteins in hypoxia conditions (**Fig. 33C**) as compared to the delayed degradation kinetics of p27 observed in normal hypoxic MECs (**Fig. 33A, B&D**). Moreover, the overall stability of p27 in normal and malignant MECs was clearly independent of their EMT status, suggesting a novel mechanism and determinant operant in coupling hypoxia to p27 degradation. Interestingly, normal MECs stabilize p27 protein levels, while simultaneously suppressing the synthesis of p27 transcripts (**Fig. 34A&B**), a cellular reaction that is dramatically reversed in malignant MECs (*i.e.,* degrade p27 proteins and produce p27 transcripts; **Fig. 34C&D**). Finally, **Fig. 35** depicts the mesenchymal morphologies that are acquired by post-EMT 4T07 cells in response to hypoxia. More importantly, the acquisition of EMT phenotypes by breast cancer cells redistributes p27 from the nucleus to the cytoplasm (**Fig. 36**) thereby identifying a novel mechanism whereby EMT programs couple to the degradation of p27 in hypoxic breast cancer cells. **Collectively, these new and innovative findings implicate FBLN5 and its coupling to hypoxia as a major driver of breast cancer metastasis, suggesting that *(i)* FBLN5 may serve as a predictive biomarker to identify mammary tumors most susceptible to metastatic progression, and *(ii)* inactivating the oncogenic activities of FBLN5 may offer new inroads to eradicate metastatic disease in breast cancer patients.**

Task 3: ***Determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression in vivo***. The primary objective of Task 3 is to establish the effectiveness of abolishing FBLN5 function and its subsequent incorporation into active TGF-β receptor complexes to prevent breast cancer progression and metastasis induced by TGF-β. As mentioned above, this past year saw us identify a variety of novel FBLN5 gene targets, as well as uncover two potentially important tumor promoting functions for FBLN5, namely its ability to facilitate the presentation of TGF-β to its receptors and its potential to induce survival signaling in normal and malignant MECs. These findings are consistent with a role of FBLN5 in driving EMT (**Fig. 1**; [1]), invasion (**Fig. 1**; [1]), and metastasis in late-stage breast cancers [1]. Accordingly, FBLN5 expression is increased robustly in the murine 4T1 progression series from weakly tumorigenic 67NR cells to fully metastatic 4T1 cells (**Fig. 1**). Along these lines, we recently determined that FBLN5 promotes EMT in MECs *via* a β1 integrin-dependent manner (**Fig. 23**), thereby implicating a novel interplay that governs the balance between β1 and β3 integrins in mediating breast cancer development and metastatic progression stimulated by TGF-β. To further explore this idea, we performed initial pilot studies to determine whether overexpression of FBLN5 in NMuMG cells would be sufficient to induce their formation of mammary tumors in nude mice. Unfortunately, tumor development was not induced by FBLN5 expression, indicating that aberrant expression of the ECM molecule is not sufficient to transform MECs and drive tumor development. However, FBLN5 expression greatly enhanced the development and progression of NME tumors produced in mice (**Fig. 24**). In the next year, we will rapidly test these FBLN5 functions using malignant, nonmetastatic 67NR and malignant, highly metastatic 4T1 cells that will be engineered to stably express FBLN5 mutants that fail to bind and present TGF-β to its receptors, as well as those construct derivatives of these breast cancer cell lines whose expression of FBLN5 target genes has been positively and negatively manipulated. Afterward, the impact of these manipulations on primary tumor growth and metastasis will be assessed in syngeneic Balb/C mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Mammary tumorigenesis upregulates FBLN5 expression, particularly at the point when breast cancer cell acquire metastatic phenotypes
- A novel FBLN5 gene signature has now been identified and established
- The FBLN5 gene target, fibromodulin, suppresses NF-κB activity by stabilizing IκBα expression
- Stabilization of IκBα transpires via JNK and CK-II activation, which conspire to inactivate calpain and its proteolytic activity against IκBα
- Activation of this fibromodulin signaling axis promotes apoptosis
- FBLN5 interacts with β3 integrin in an RGD-independent fashion
- FBLN5 binds TGF-β, leading to its enhanced presentation to TGF-β receptors and elevated autocrine TGF-β signaling in normal and malignant MECs
- FBLN5 and β3 integrin promote normal and malignant MEC proliferation, a cellular response coupled to FAK and ERK1/2 activation by FBLN5
- RGE-FBLN5 and β3 integrin fail to induce MEC proliferation
- FBLN5 induces survival signaling in normal and malignant MECs in part by strongly activating NF-κB
- Survival signaling by FBLN5 is also coupled to its ability to suppress TNF-α expression, and to induce that of survivin and xIAP
- FBLN5 potentially induces breast cancer cell EMT, migration, and invasion by upregulating the expression of Cox-2, PAI-1, and MMPs 2, 3 and 9
- β1 integrin is essential for coupling FBLN5 to ERK1/2 activation, which subsequently induces MMP expression in normal and malignant MECs.
- β1 integrin is also essential in mediating EMT induced by FBLN5.
- FBLN5 promotes normal and malignant MEC survival under hypoxic conditions.
- Hypoxia selectively inactivates the TGF-β signaling system, presumably circumventing the tumor suppressing activities of this cytokine.
- FBLN5 induces MMP expression *via* an EGFR- and ERK1/2-dependent mechanism.
- FBLN5 stabilizes EGFR expression, leading to elevated ERK1/2 signaling and enhanced tumor development in mice.
- FBLN5 promotes breast cancer proliferation under hypoxic conditions *via* the expression and activity of β1 integrin.
- Hypoxia induces reversible EMT programs in breast cancer cells, which also exhibit elevated migratory activity and resistance to apoptotic and anoikis stimuli.
- Breast cancer cells progressively downregulate their expression of the cell cycle regulator, p27.
- Hypoxic breast cancer cells initiate p27 degradation as a means to survive hypoxic conditions and apoptotic stimuli.
- EMT programs and hypoxia redistribute p27 from the nucleus to the cytoplasm, leading to its rapid degradation.

REPORTABLE OUTCOMES:

Schiemann Laboratory Publications Acknowledging Support of BC084651:

Keshamouni, V.G. and **Schiemann, W.P.** (2009) EMT in Tumor Metastasis: A Method to the Madness. **Future Oncology** *5,* 1109-1111.

Wendt, M.K., Allington, T.M. and **Schiemann, W.P.** (2009) Mechanisms of epithelial-mesenchymal transition by TGF-β in normal and malignant cells. **Future Oncology** *5,* 1145-1168.

Tian, M. and **Schiemann, W.P.** (2009) The TGF-β paradox in human cancer: An update. **Future Oncology** *5,* 259-271.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2009) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity.

Journal of Biological Chemistry *284,* 34145-34156 *See Faculty of 1000 (http://f1000biology.com/article/id/1168476)

Taylor, M.A., Parvani, J.G. and **Schiemann, W.P.** (2010) The pathophysiology of EMT stimulated by TGF-β. **J Mammary Gland Biol Neoplasia** *15:* 169-190. *20 consecutive months as a "Top 5" journal download.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2010) TGF-β-induced epithelial-mesenchymal transition facilitates oncogenic epidermal growth factor receptor signaling in breast cancer. **Oncogene** *29:* 6485-6498. *Featured in "Mammary Cell News" volume 2.33, September 2, 2010.

Tian, M., Neil, J.R. and **Schiemann, W.P.** (2010) TGF-β and the hallmarks of cancer. **Cell Signal** *23:* 951-962.

Allington, T.M. and **Schiemann, W.P.** (2011) The Cain and Abl of epithelial-mesenchymal transition and TGF-β in mammary epithelial cells. **Cells Tissues Organs** *193:* 98-113.

Lee, Y-H. and **Schiemann, W.P.** (2010) Fibromodulin suppresses nuclear factor-κB activity by inducing the delayed degradation of IκBα *via* a JNK-dependent pathway coupled to fibroblast apoptosis. **J Biol Chem** *286:* 6414-6422.

Taylor, M.A., Amin, J., Kirschmann, D.A. and **Schiemann, W.P.** (2011) Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor-β signaling in breast cancer cells. **Neoplasia** *13:* 406-418. *Cover and featured article (http://www.neoplasia.com/toc.php?vol=13&no=5)

Parvani, J.G., Taylor, M.A. and **Schiemann, W.P.** (2011) Noncanonical TGF-β signaling during mammary tumorigenesis. **J Mammary Gland Biol Neoplasia** *16:* 127-146.

Wendt, M.K., Molter, J., Flask, C.A. and **Schiemann, W.P.** (2011) *In vivo* dual substrate bioluminescent imaging. **J Vis Exp** *56:* e3245, DOI: 10.3791/3245.

Wendt, M.K., Taylor, M.A., Schiemann, B.J. and **Schiemann, W.P.** (2011) Downregulation of epithelial cadherin is required to initiate the metastatic outgrowth of breast cancer. **Mol Biol Cell** *22:* 2423-2435. *See Faculty of 1000 (http://f1000.com/11598960#evaluations)

Wendt, M.K., Tian, M. and **Schiemann, W.P.** (2011) Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression. **Cell Tissue Res** *344:* 85-101.

Taylor, M.A., Lee, Y-H and **Schiemann, W.P.** (2011) Role of TGF-β and the tumor microenvironment during mammary tumorigenesis. **Gene Expr** *15*: 117-132.

Wendt, M.K., Schiemann, B.J., Lee, Y-H., Kang, Y. and **Schiemann, W.P.** (2012) TGF-β stimulates Pyk2 expression as part of an epithelial-mesenchymal transition program required for metastatic outgrowth of breast cancer. ***Oncogene***. 31: doi: 10.1038/onc.2012.230. PMID: 22710711.

*Top Story in "Mammary Cell News" volume 4.24, June 21, 2012.

*Featured on MDLinx.com (http://www.mdlinx.com/obstetrics-gynecology/news-article.cfm/4111249)

Wendt, M.K. and **Schiemann, W.P.** (2012) *"Microenvironmental regulation of TGF-β signaling during metastatic progression"* in Signalling Pathways and Molecular Mediators in Metastasis. Springer. ISBN: 978-94-0007-2557-7. Edited by Alessandro Fatatis. Chapter 5, 115-141.

Morrison, C.D., Parvani, J.G. and **Schiemann, W.P.** (2012) The relevance of the TGF-β paradox to EMT-MET programs. **Cancer Letts:** in press.

Wendt, M.K. and **Schiemann, W.P.** (2012) *"The multifunctional roles of TGF-β in navigating the metastatic cascade"* in TGF-β in Human Disease. Springer. Edited by Aris Moustakas and Kenji Miyazawa: in press.

Zhang, Y., Janyasupab, M., Liu, C-W, Cai, J., Popa, A., Samia, A.C., Wang, K.W., Xu, J., Wendt, M.K., Schiemann, B.J. Thompson, C.L., **Schiemann, W.P.** and Liu, C.C. (2012) Detection of LOXL2, a biomarker of metastatic breast cancers in blood and urine samples. *(Submitted).*

Lee, Y-H., Wendt, M.K. and **Schiemann, W.P.** (2012) Fibulin-5 (FBLN5) initiates EMT and induces matrix metalloproteinase expression *via* a β1 integrin:EGFR:ERK1/2 pathway. *(Submitted).*

Lee, Y-H. and **Schiemann, W.P.** (2012) Hypoxia-induced fibulin-5 (FBLN5) and EMT confer resistance to hypoxia-induced cell death. *(Submitted).*

Invited Seminars Presented by Dr. Schiemann Acknowledging Support of BC084651:

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **UC-Davis Cancer Center,** Sacramento, CA. (May 14, 2009).

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **Case Comprehensive Cancer Center,** Cleveland, OH. (July 16, 2009).

Schiemann, W.P. (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling, EMT, and tumorigenesis in mammary tumors. **The EMT International Association's 4th International Meeting on "Epithelial-Mesenchymal Transition,"** Tucson, AZ. (September 23, 2009).

Schiemann, W.P. (2009) The Abl and Cain of TGF-β signaling. **Department of Pharmacology,** Case Western Reserve University, Cleveland, OH. (October 5, 2009).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in mammary epithelial cells. *AACR Special Conference on "EMT and Cancer Progression and Treatment,"* Arlington, VA. (March 1, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β signaling in breast cancer. *University of Tennessee, Comparative and Experimental Medicine Research Seminar Series at the UT College of Veterinary Medicine*, Knoxville, TN (October 11, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β signaling in breast cancer. *Eppley Cancer Institute, University of Nebraska Medical Center,* Omaha, NE (October 28, 2010).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in Breast Cancer. *Translational Genomics Research Institute (TGen),* Phoenix, AZ (November 8, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β Signaling in breast cancer. *Lerner Research Institute, Cleveland Clinic Foundation,* Cleveland, OH (December 7, 2010).

Schiemann, W.P. (2011) Oncogenic TGF-β signaling in breast cancer. *Department of Biological Chemistry, Johns Hopkins University,* Baltimore, MD (January 25, 2011).

Schiemann, W.P. (2011) Oncogenic TGF-β signaling in breast cancer. *Department of Human and Molecular Genetics and the VCU Institute of Molecular Medicine, Virginia Commonwealth University,* Richmond, VA (April 12, 2011).

Schiemann, W.P. (2011) Mechanisms of EMT and metastasis stimulated by TGF-β. *FASEB Summer Research Conference on "The TGF-β Superfamily: Signaling In Development and Disease,"* Barga, Italy (August 23, 2011).

Schiemann, W.P. (2011) Mechanisms of EMT and metastasis stimulated by TGF-β. *Holden Comprehensive Cancer Center, University of Iowa,* Iowa City, IA (November 29, 2011).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling in breast cancer. *Rammelkamp Research Conferences, Rammelkamp Center for Education and Research, MetroHealth,* Cleveland, OH (February 14, 2012).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling during metastatic progression of triple-negative breast cancers. *Department of Chemistry, Purdue University,* West Lafayette, IN (February 20, 2012).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling in breast cancer metastasis. *College of Veterinary Medicine, Kansas State University,* Manhattan, KS (September 24, 2012).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling in metastatic breast cancer. *Medical University of South Carolina,* Charleston, SC (November 7, 2012).

CONCLUSION:

Our findings have clearly established new biological and pathological paradigms for FBLN5 and TGF-β. Importantly, we continue to *(i)* elucidate the mechanisms whereby FBLN5 induces oncogenic TGF-β signaling in normal and malignant MECs, and *(ii)* identify the FBLN5 effectors that contribute to the invasive and metastatic properties of TGF-β. Equally importantly, our findings have provided the first FBLN5 gene signature that underlies its biological activities, and this dataset has already uncovered fibromodulin as a novel FBLN5 gene target that regulates fibroblast survival. Our findings that FBLN5-deficient MEFs are largely unresponsive to TGF-β is exciting and may in fact be explained by our demonstration that FBLN5 binds directly to TGF-β, leading to its presentation to TGF-β receptors and the enhanced activation of autocrine TGF-β signaling in normal and malignant MECs. Our findings have also identified several novel FBLN5 effectors whose activity contributes to oncogenic TGF-β signaling. Given our recent finding that developing and progressing mammary tumors significantly upregulate their expression of FBLN5 at the point at which these tumors become metastatic, our results clearly establish FBLN5 as a new and potentially important biomarker to detect and track metastatic disease in patients with breast cancer. Moreover, the ability of FBLN5-deficiency to significantly attenuate cellular responses to TGF-β suggest that measures capable of antagonizing FBLN5 function may alleviate the initiation of oncogenic TGF-β signaling. Indeed, successful identification and implementation of FBLN5 molecules that are unable to bind and present TGF-β to its receptors on metastatic breast cancer cells holds tremendous potential to alleviate metastatic disease in breast cancer patients. Thus, translation of our findings will provide a novel set of biomarkers comprised of FBLN5 and its effectors that will be capable of predicting whether or not malignant MECs possess metastatic phenotypes. In addition, our findings will offer new inroads to target these metastatic lesions via employment of FBLN5 mutants that will suppress oncogenic TGF-β signaling in breast cancer cells. Collectively, we envision that further developing these reagents and clinical protocols will play a significant role in developing a "personalized medicine" approach tailored to treat individuals with metastatic breast cancer.

REFERENCES:

[1] Lee YH, Albig AR, Regner M, Schiemann BJ and Schiemann WP (2008). Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-β in mammary epithelial cells *via* a MMP-dependent mechanism. *Carcinogenesis*, **29**, 2243-2251.

[2] Bhowmick NA and Moses HL (2005). Tumor-stroma interactions. *Curr Opin Genet Dev*, **15**, 97-101.

[3] Bhowmick NA, Neilson EG and Moses HL (2004). Stromal fibroblasts in cancer initiation and progression. *Nature*, **432**, 332-337.

[4] Bierie B and Moses HL (2006). Tumour microenvironment: TGFβ: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer*, **6**, 506-520.

[5] Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, Arteaga CL, Neilson EG, Hayward SW and Moses HL (2005). Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks. *Oncogene*, **24**, 5053-5068.

[6] Albig AR, Neil JR and Schiemann WP (2006). Fibulins 3 and 5 antagonize tumor angiogenesis *in vivo*. *Cancer Res*, **66**, 2621-2629.

[7] Albig AR and Schiemann WP (2004). Fibulin-5 antagonizes vascular endothelial growth factor (VEGF) signaling and angiogenic sprouting by endothelial cells. *DNA Cell Biol*, **23**, 367-379.

[8] Albig AR and Schiemann WP (2005). Fibulin-5 function during tumorigenesis. *Future Oncol*, **1**, 23-35.

[9] Schiemann WP, Blobe GC, Kalume DE, Pandey A and Lodish HF (2002). Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-β and affects protein kinase cascades. *J Biol Chem*, **277**, 27367-27377.

[10] Galliher AJ and Schiemann WP (2006). β3 integrin and Src facilitate transforming growth factor-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res*, **8**, R42.
[11] Galliher AJ and Schiemann WP (2007). Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer Res*, **67**, 3752-3758.
[12] Galliher-Beckley AJ and Schiemann WP (2008). Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. *Carcinogenesis*, **29**, 244-251.
[13] Neil JR, Johnson KM, Nemenoff RA and Schiemann WP (2008). Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. *Carcinogenesis*, **29**, 2227-2235.
[14] Neil JR and Schiemann WP (2008). Altered TAB1:IκB kinase interaction promotes transforming growth factor β-mediated nuclear factor-κB activation during breast cancer progression. *Cancer Res*, **68**, 1462-1470.
[15] Neil JR, Tian M and Schiemann WP (2009). x-linked inhibitor of apoptosis protein and its E3 ligase activity promote transforming growth factor-β-mediated nuclear factor-κB activation during breast cancer progression. *J Biol Chem*, **284**, 21209-21217.
[16] Taylor MA, Amin J, Kirschmann DA and Schiemann WP (2011). Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor-β signaling in breast cancer cells. *Neoplasia*, **13**, 406-418.
[17] Tian M and Schiemann WP (2009). PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. *FASEB J*, **24**, 1105-1116.
[18] Wendt MK and Schiemann WP (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. *Breast Cancer Res*, **11**, R68.
[19] Wendt MK, Smith JA and Schiemann WP (2009). p130Cas is required for mammary tumor growth and transforming growth factor-β-mediated metastasis through regulation of Smad2/3 activity. *J Biol Chem*, **284**, 34145-34156.
[20] Wendt MK, Smith JA and Schiemann WP (2010). Transforming growth factor-β-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. *Oncogene*, **29**, 6485-6498.
[21] Wendt MK, Taylor MA, Schiemann BJ and Schiemann WP (2011). Downregulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. *Molecular biology of the cell*, **22**, 2423-2435.
[22] Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG and Moses HL (2004). TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*, **303**, 848-851.
[23] Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC and Moses HL (2008). Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*, **13**, 23-35.
[24] Allington TM, Galliher-Beckley AJ and Schiemann WP (2009). Activated Abl kinase inhibits oncogenic transforming growth factor-β signaling and tumorigenesis in mammary tumors. *FASEB J*, **23**, 4231-4243.
[25] Schiemann WP (2007). Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? *Expert Rev Anticancer Ther*, **7**, 609-611.
[26] Taylor MA, Parvani JG and Schiemann WP (2010). The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. *J Mammary Gland Biol Neoplasia*, **15**, 169-190.
[27] Tian M and Schiemann WP (2009). The TGF-β paradox in human cancer: an update. *Future Oncol*, **5**, 259-271.
[28] Wendt MK, Allington TM and Schiemann WP (2009). Mechanisms of the epithelial-mesenchymal transition by TGF-β. *Future Oncol*, **5**, 1145-1168.
[29] Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT and Giaccia AJ (2009). Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*, **15**, 35-44.

[30] Singh A and Settleman J (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*, **29**, 4741-4751.
[31] Creighton CJ, Chang JC and Rosen JM (2010). Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. *J Mammary Gland Biol Neoplasia*, **15**, 253-260.
[32] Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, Fan C, Zhang X, He X, Pavlick A, Gutierrez MC, Renshaw L, Larionov AA, Faratian D, Hilsenbeck SG, Perou CM, Lewis MT, Rosen JM and Chang JC (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A*, **106**, 13820-13825.
[33] Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong KK, Brandstetter K, Wittner B, Ramaswamy S, Classon M and Settleman J (2010). A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*, **141**, 69-80.
[34] Tischkowitz M, Brunet J-S, Begin L, Huntsman D, Cheang M, Akslen L, Nielsen T and Foulkes W (2007). Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC Cancer*, **7**, 134.
[35] Charpin C, Secq V, Giusiano S, Carpentier S, Andrac L, Lavaut MN, Allasia C, Bonnier P and Garcia S (2009). A signature predictive of disease outcome in breast carcinomas, identified by quantitative immunocytochemical assays. *Int J Cancer*, **124**, 2124-2134.
[36] Chaudhury A, Hussey GS, Ray PS, Jin G, Fox PL and Howe PH (2010). TGF-β-mediated phosphorylation of hnRNP E1 induces EMT *via* transcript-selective translational induction of Dab2 and ILEI. *Nature cell biology*, **12**, 286-293.
[37] Hussey GS, Chaudhury A, Dawson AE, Lindner DJ, Knudsen CR, Wilce MC, Merrick WC and Howe PH (2011). Identification of an mRNP complex regulating tumorigenesis at the translational elongation step. *Molecular cell*, **41**, 419-431.
[38] Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW, Hollier BG, Ram PT, Lander ES, Rosen JM, Weinberg RA and Mani SA (2010). Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A*, **107**, 15449-15454.

APPENDICES:

- Supporting Figures 1-24
- PDFs of Schiemann Laboratory Papers acknowledging DoD Support.

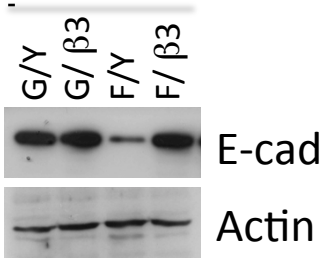
Figure 1

FBLN5-expressing MECs Exhibit EMT and Invasive Phenotypes:

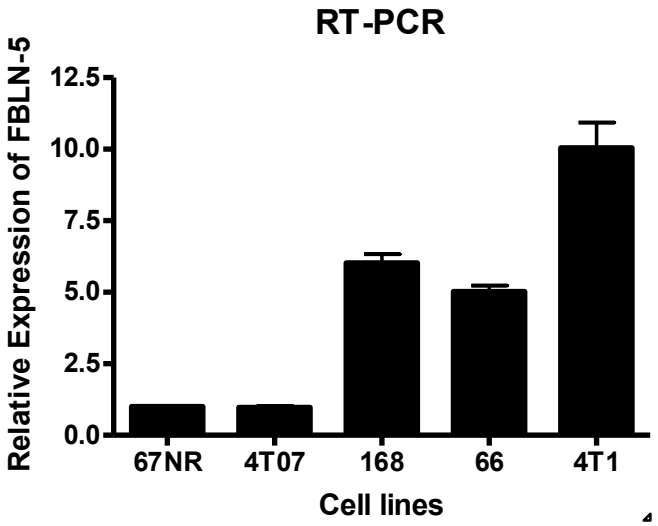
Role of FBLN5 in Breast Cancer Progression?

A

EMT Program

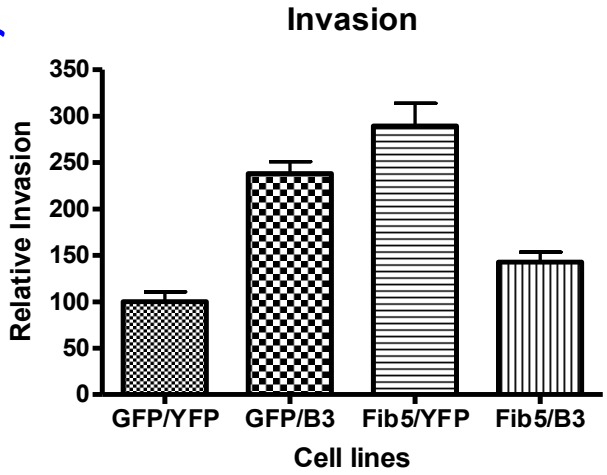


B



Tumorigenicity

C

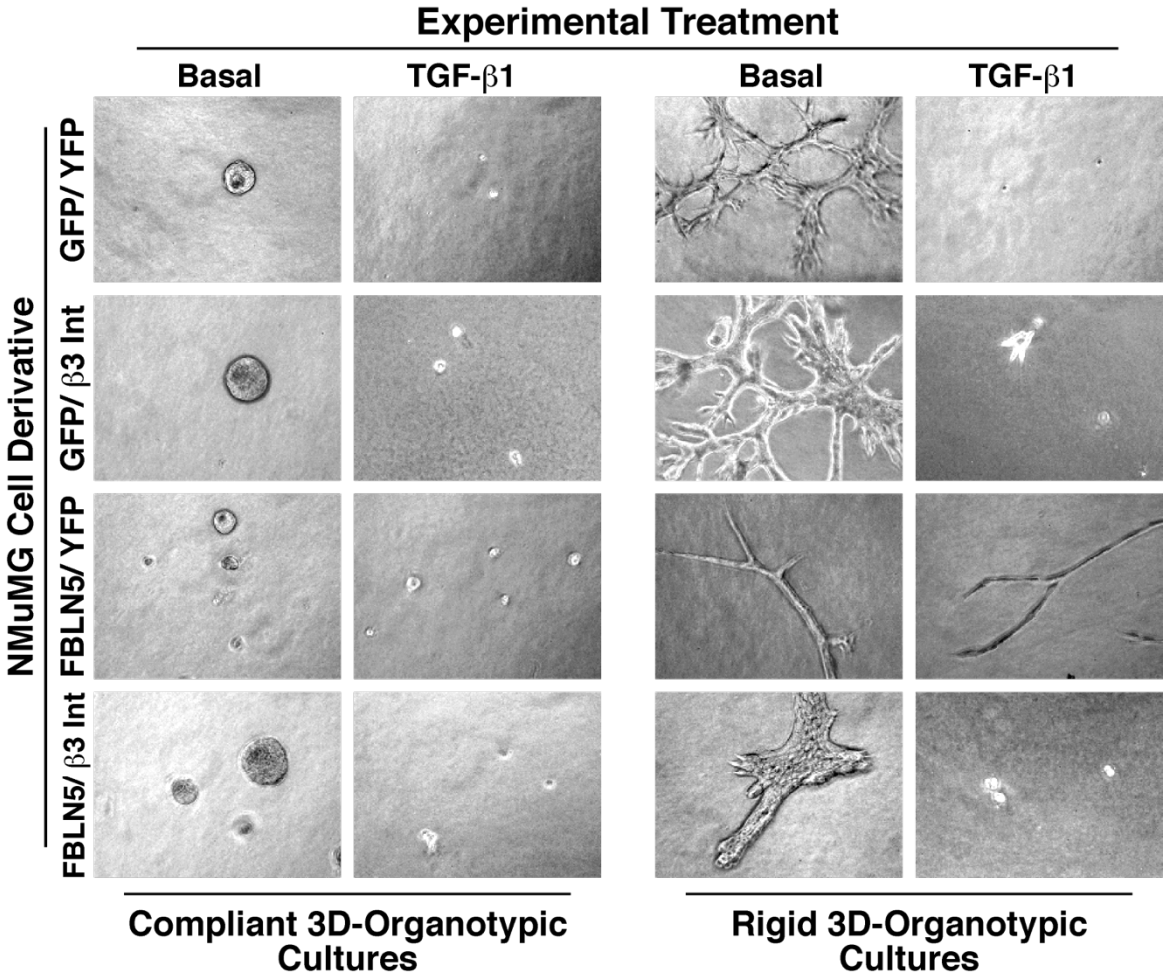


Legend

G = GFP
Y = YFP
F = FBLN5
B = β3 integrin

Figure 2

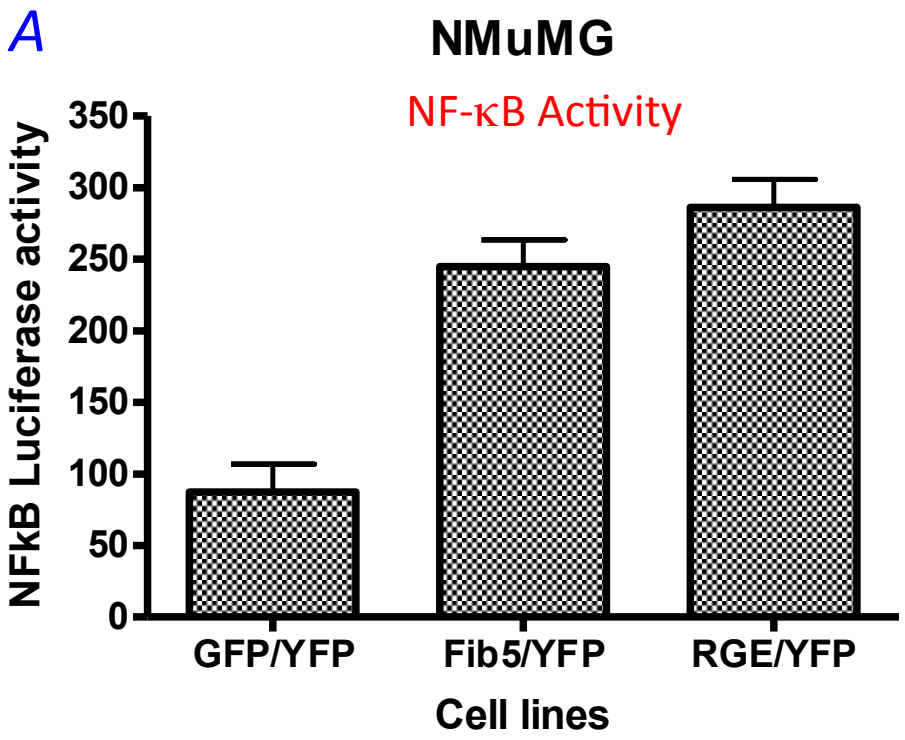
FBLN5 Induces Invasive Morphologies and Survival of MECs in Rigid Environments



NMuMG derivatives were cultured in the absence or presence of TGF-β1 (5 ng/ml) for 8 days in 3D-organotypic Cultrex matrices supplemented without (*i.e.,* compliant) or with type I collagen (3 mg/ml; *i.e.,* rigid). Bright-field images were captured and representative images from 3 independent experiments are shown.

Figure 3

FBLN5 Stimulates NF-κB Activity Independent of Integrin Binding in NMuMG Cells: *Targeting IκBα Destruction*



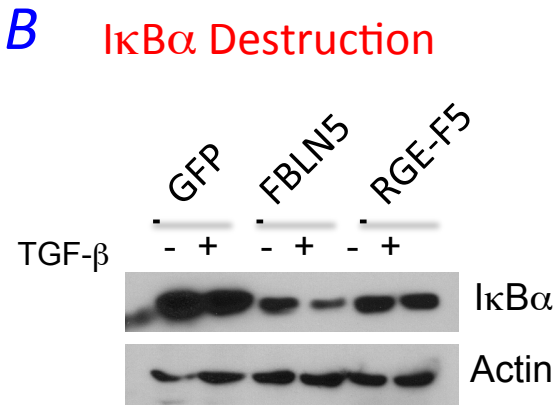


Figure 4

FBLN5 Suppresses the Coupling of TNF-α to Caspases 3 & 7 in NMuMG Cells

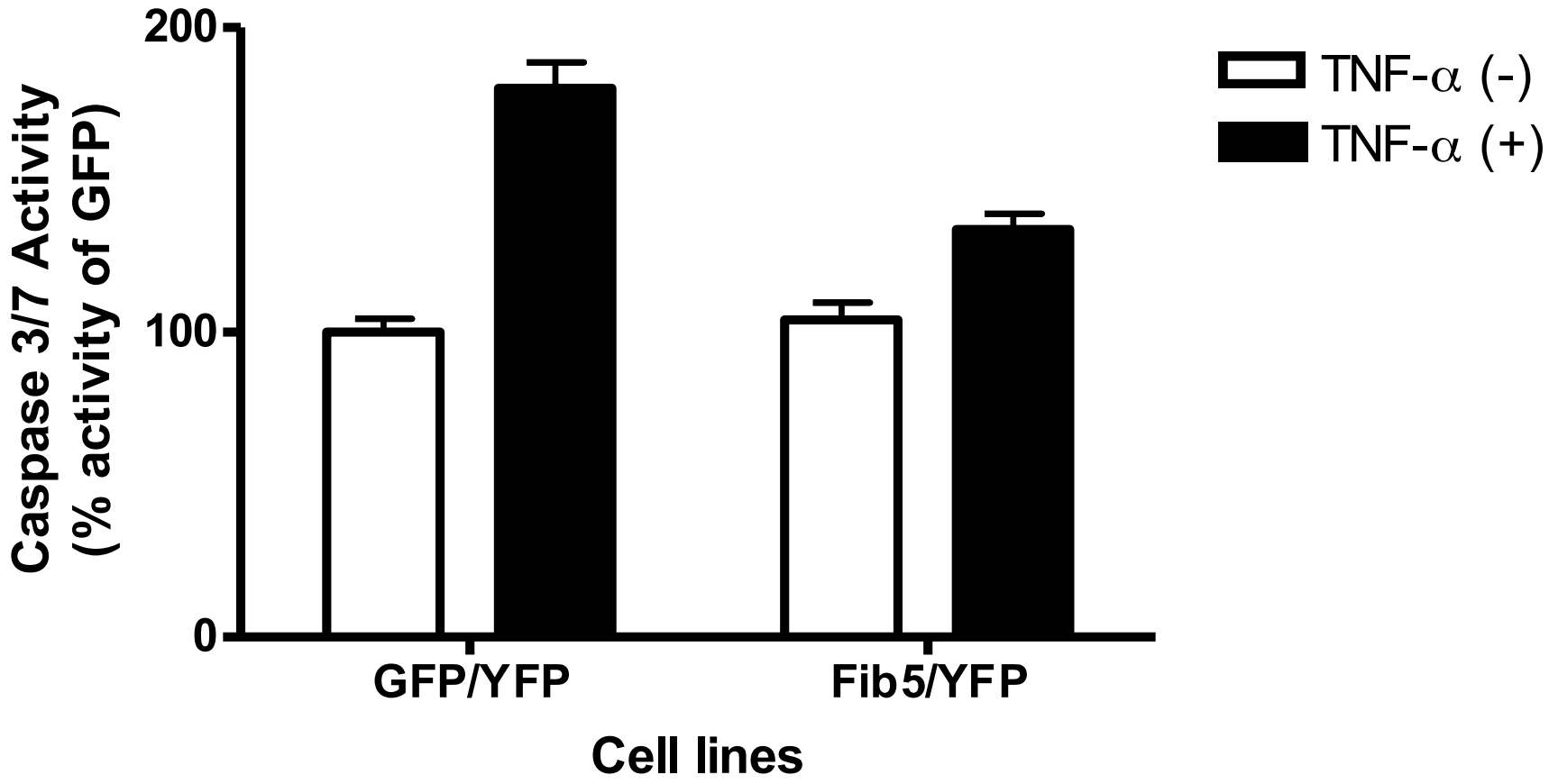


Figure 5

Hypoxia Inactivates TGF-β Signaling in Traditional 2D- and 3D-Organotypic Cultures

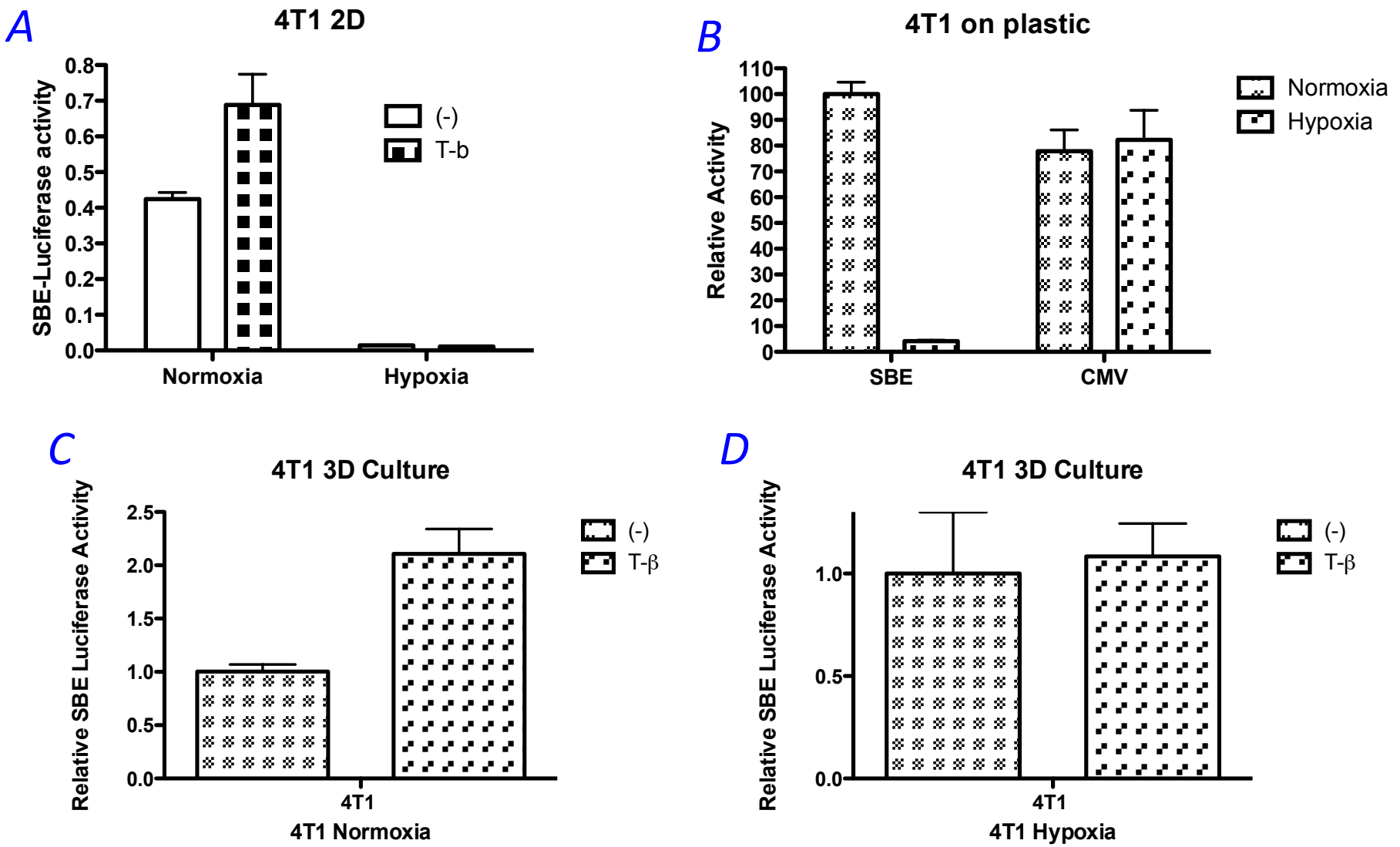


Figure 6

Hypoxia Induces FBLN5 Expression

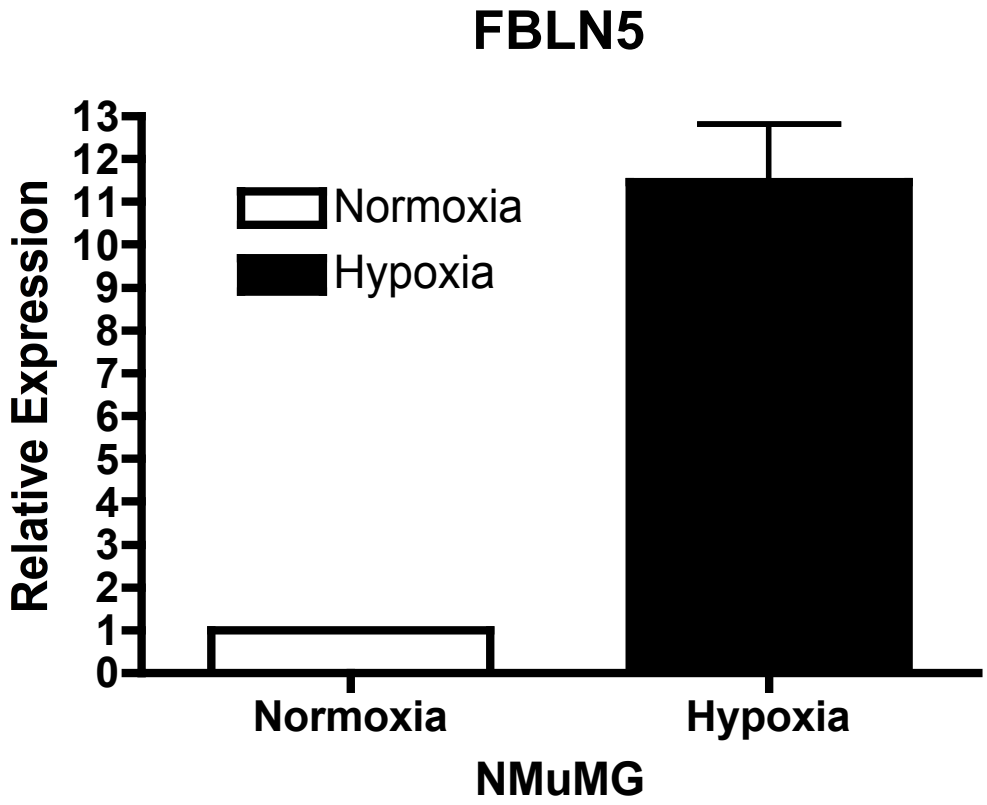
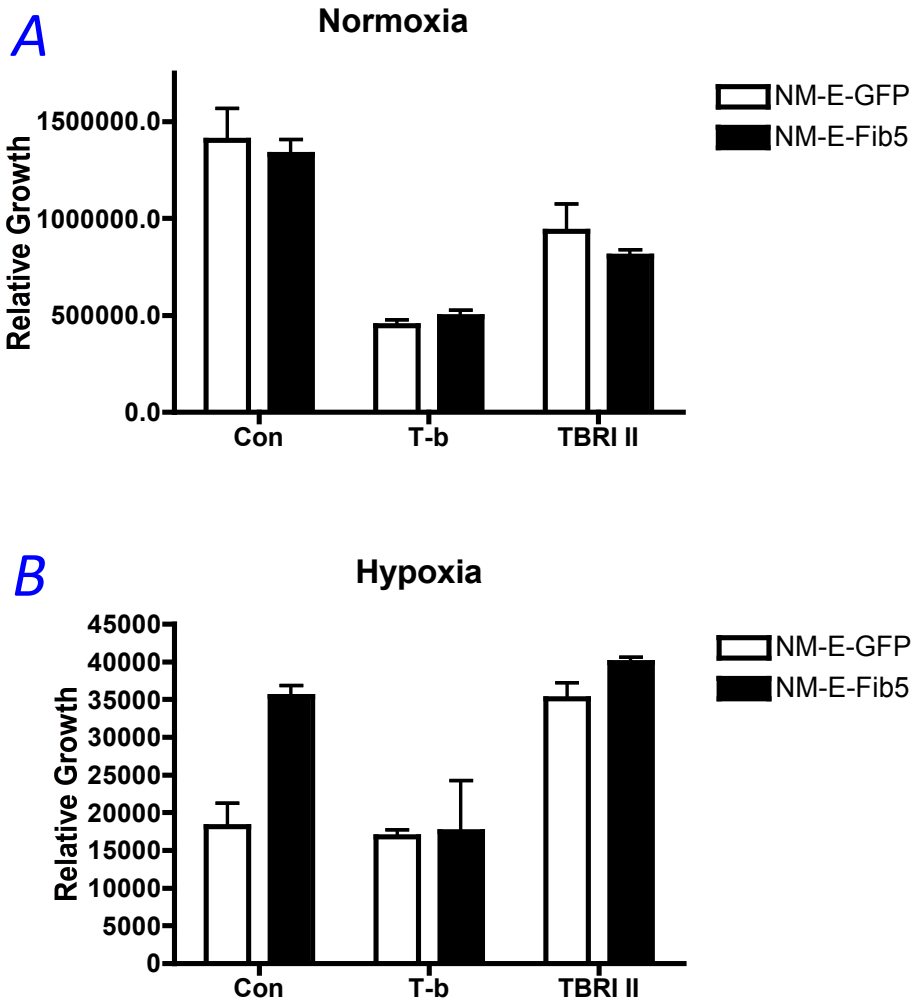


Figure 7

FBLN5 Stimulates the Growth of Malignant NM-E Cells Under Hypoxic Conditions



Legend

Con = Control
T-b = TGF-β
TBRI II = TβRI Inhibitor

NM-E-GFP = GFP-expressing NM-E Cells
NM-E-Fib5 = FBLN5-expressing NM-E Cells

Figure 8

FBLN5 Protects NMuMG Cells from Hypoxia and TGF-β-induced Cell Death

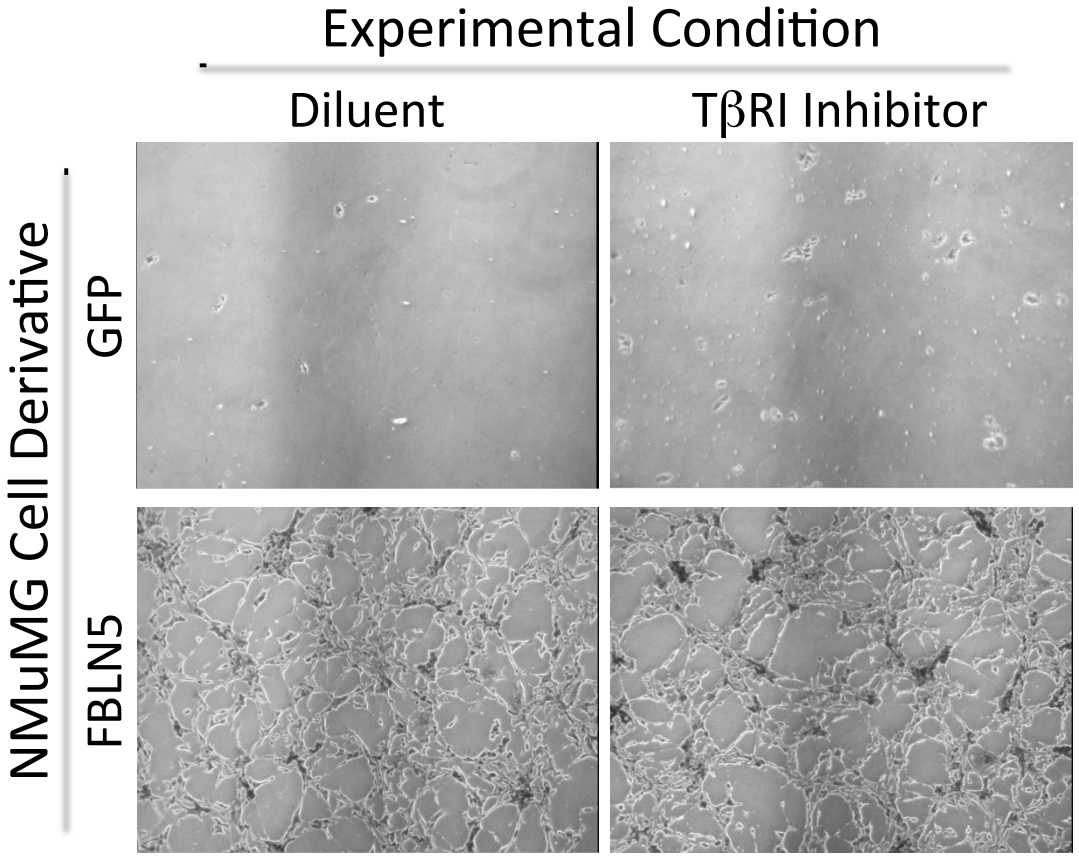


Figure 9

FBLN5 Inhibits Caspase-3 Cleavage and Activation in NMuMG Cells Induced to Undergo Apoptosis in Response to Hypoxia

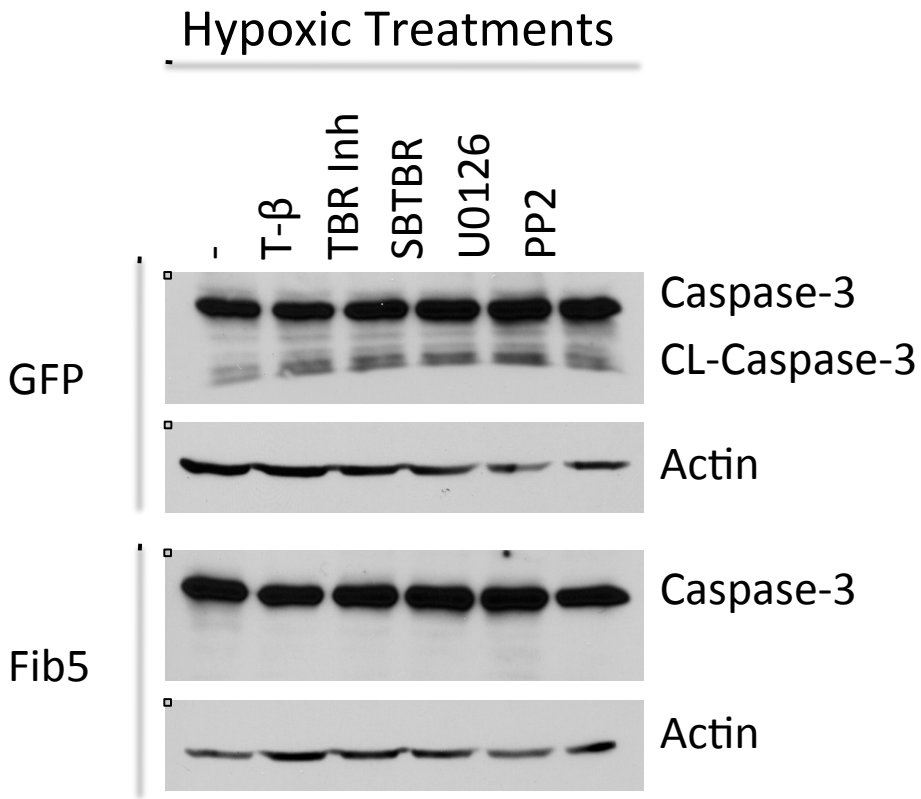
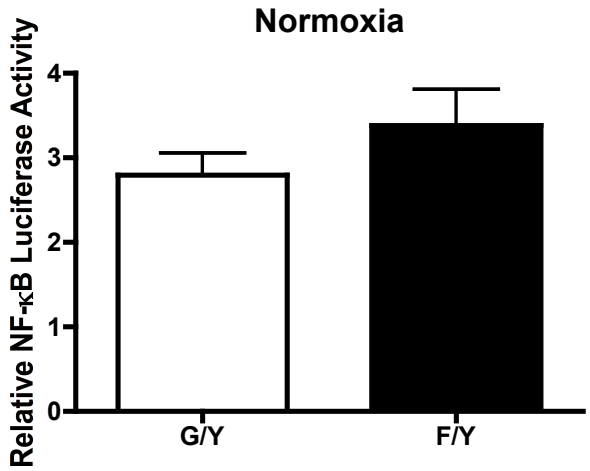


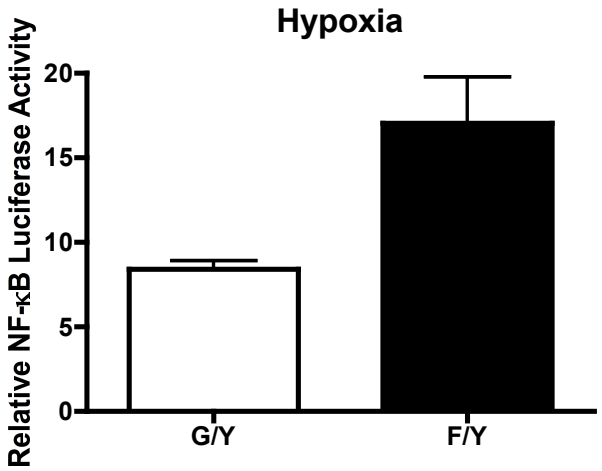
Figure 10

Hypoxia and FBLN5 Cooperate in Activating NF-kB to Suppress Apoptosis of Normal Mammary Epithelial Cells

A



B



Legend

G = GFP
Y = YFP
F = FBLN5

Figure 11

FBLN5 is a Potent Inducer of MMP Expression in Normal NMuMG Cells

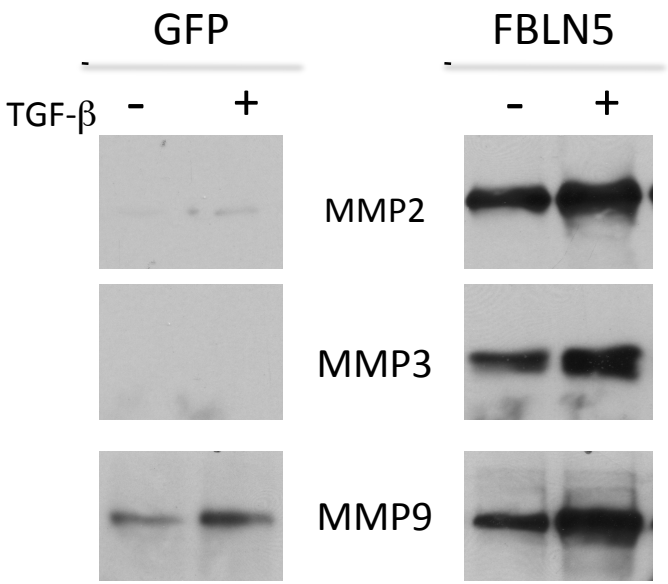


Figure 12

FBLN5 is a Potent Inducer of MMP Expression in Metastatic MECs

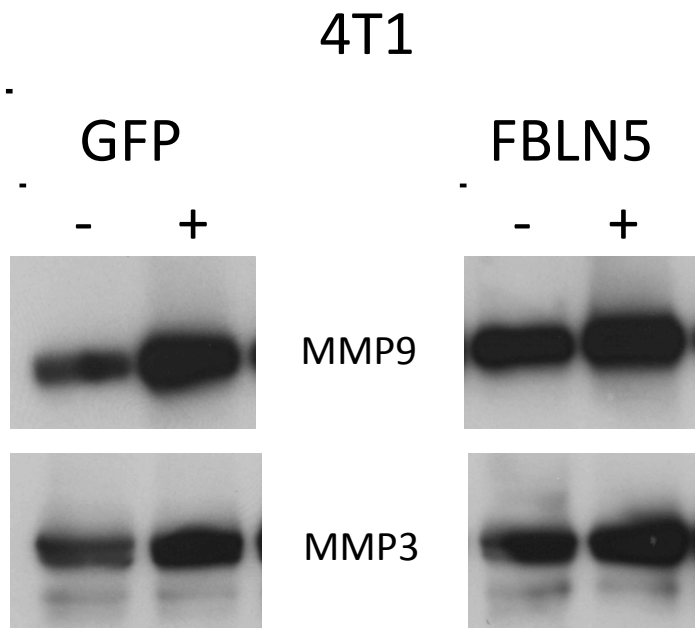
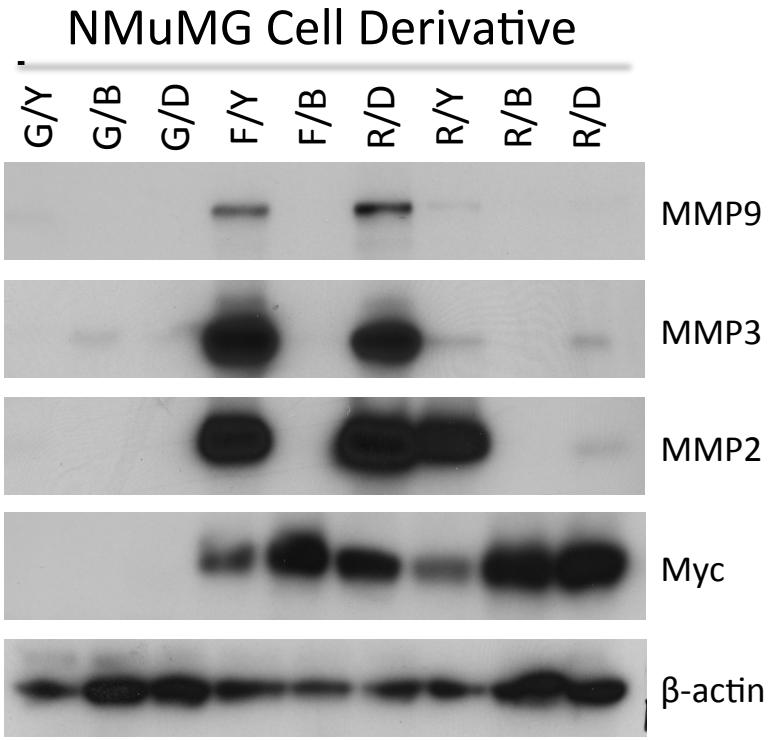


Figure 13

β3 Integrin Masks FBLN5 Stimulation of MMP Expression in NMuMG Cells

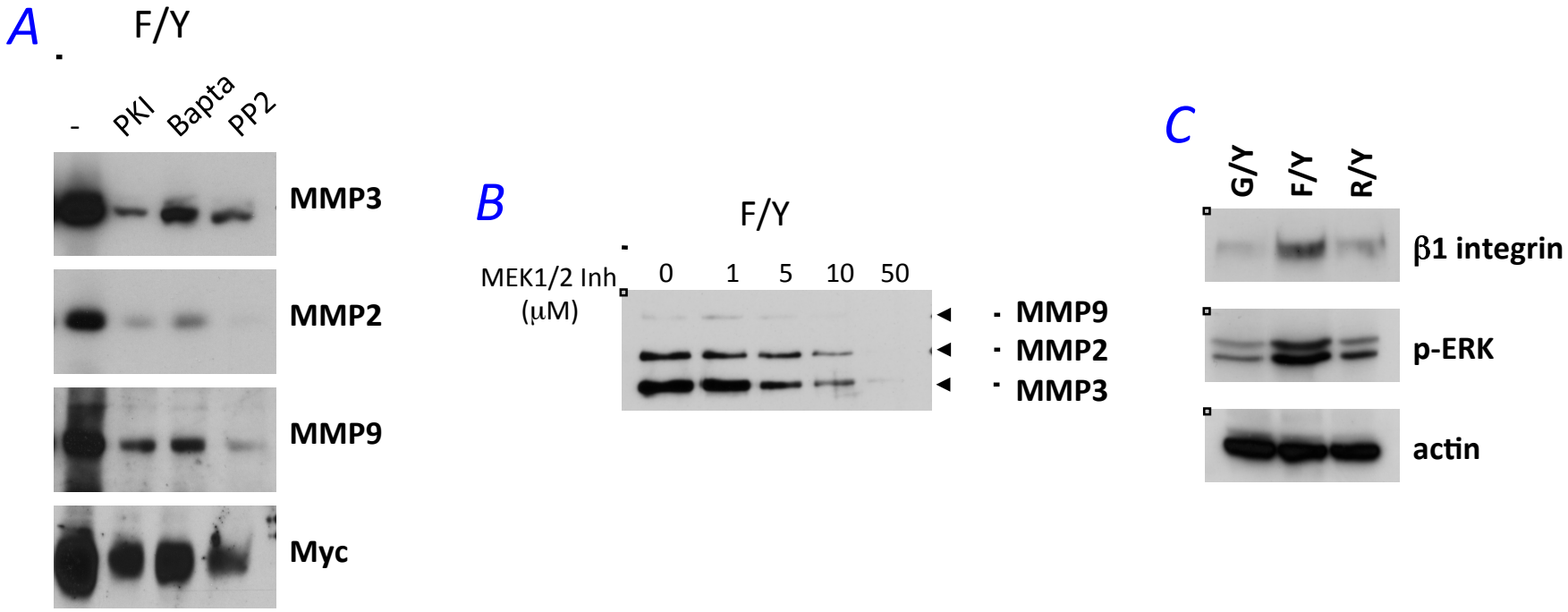


Legend

G = GFP
Y = YFP
F = FBLN5
R = RGE-FBLN5
B = β3 integrin
D = D119A-β3 integrin

Figure 14

FBLN5 Regulates MMP Expression in NMuMG Cells *via* MAP Kinase-, Calcium-, and Src-dependent Mechanisms

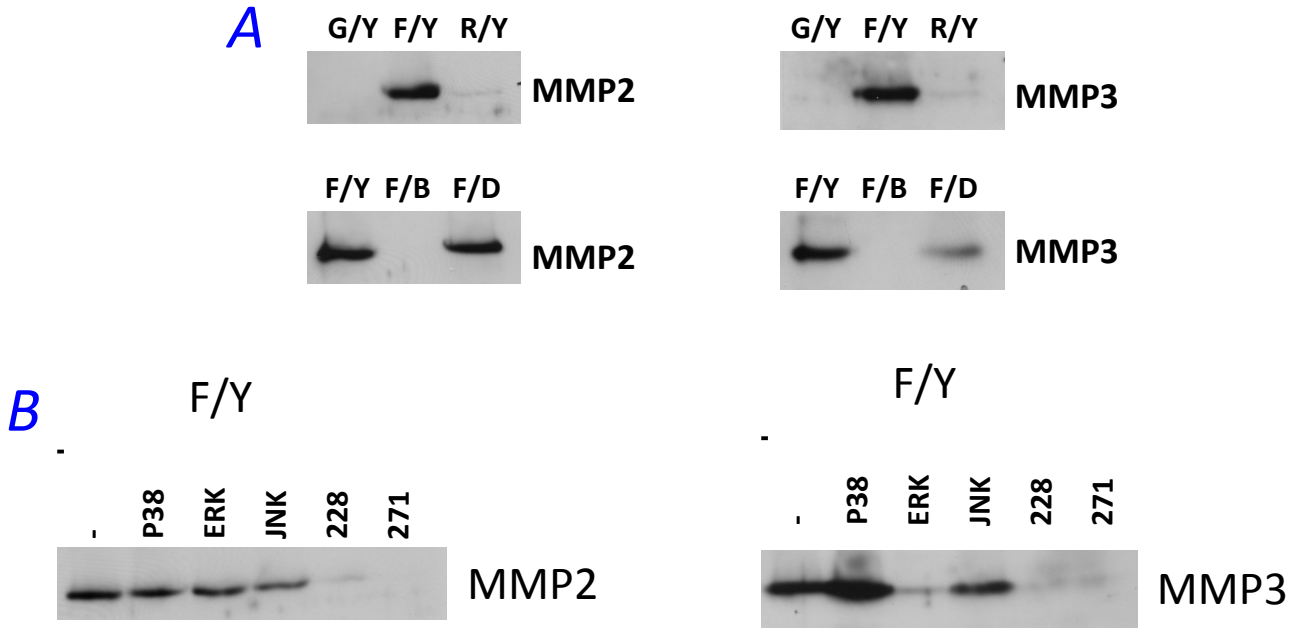


Legend

PKI = mixture of ERK1/2, p38 MAPK, and JNK inhibitors
Bapta = Calcium chealtor
PP2 = Src inhibitor

Figure 15

FBLN5 Induction of MMP Expression is RGD-dependent and Coupled to Activation of ERK and FAK/Pyk2: *Role of β3 Integrin in Suppressing FBLN5 Coupling to MMP Expression*



Legend

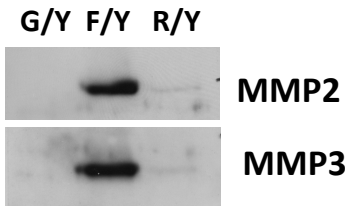
P38 = p38 MAPK inhibitor
ERK = ERK1/2 inhibitor
JNK = JNK inhibitor
228 = FAK inhibitor
271 = FAK & Pyk2 inhibitor

G = GFP
Y = YFP
F = FBLN5
R = RGE-FBLN5
B = β3 integrin
D = D119A-β3 integrin

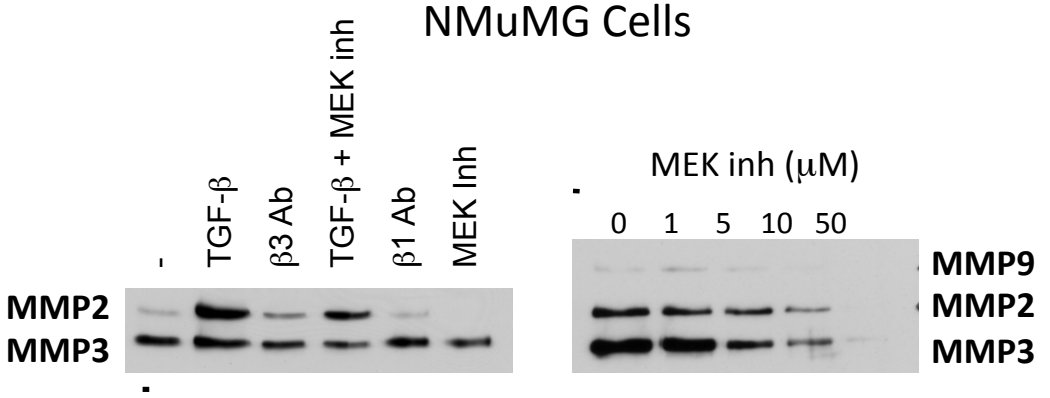
Figure 16

FBLN5 Induces MMP Expression *via* a β1 Integrin-Dependent Pathway in Normal NMuMG Cells

A FBLN5 Requires Integrin Ligation to Induce MMP Expression

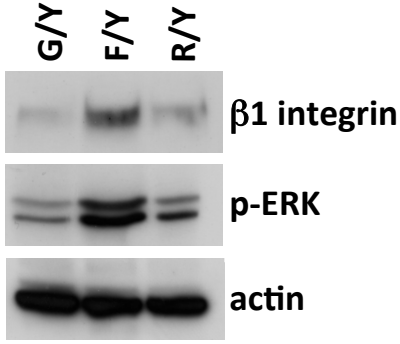


B FBLN5 Induces MMP Expression *via* a β1 Integrin & ERK1/2-dependent Pathway in F/Y-Expressing NMuMG Cells



F/Y NMuMG Cells

C β1 Integrin Couples FBLN5 to ERK1/2 Activation



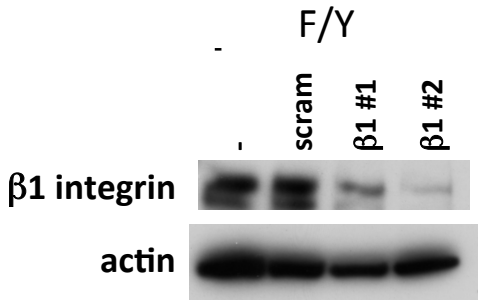
Legend

G = GFP
Y = YFP
F = FBLN5
β3 Ab = Neutralizing β3 integrin antibodies
β1 Ab = Neutralizing β1integrin antibodies
P-ERK = phosphorylated/activated ERK1/2

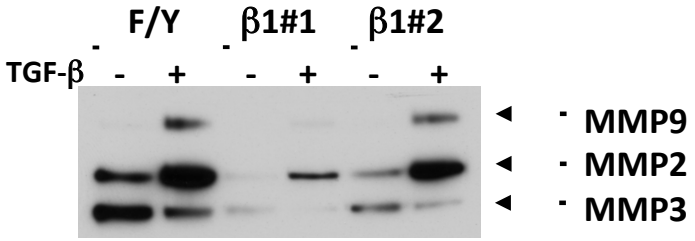
Figure 17

β1 Integrin-depletion Suppresses FBLN5- and TGF-β-mediated MMP Expression in NMuMG Cells

A Monitoring the Extent of β1 Integrin-deficiency in Using Two Independent shRNAs



B β1 Integrin-deficiency Uncouples FBLN5 and TGF-β From MMP Expression



Legend

Y = YFP
F = FBLN5
Scram = nonsilencing shRNA
β1 #1 = β1 integrin shRNA#1
β1 #2 = β1 integrin shRNA#2

Figure 18

FBLN5 Stimulates MMP Expression *via* a EGFR-dependent Pathway

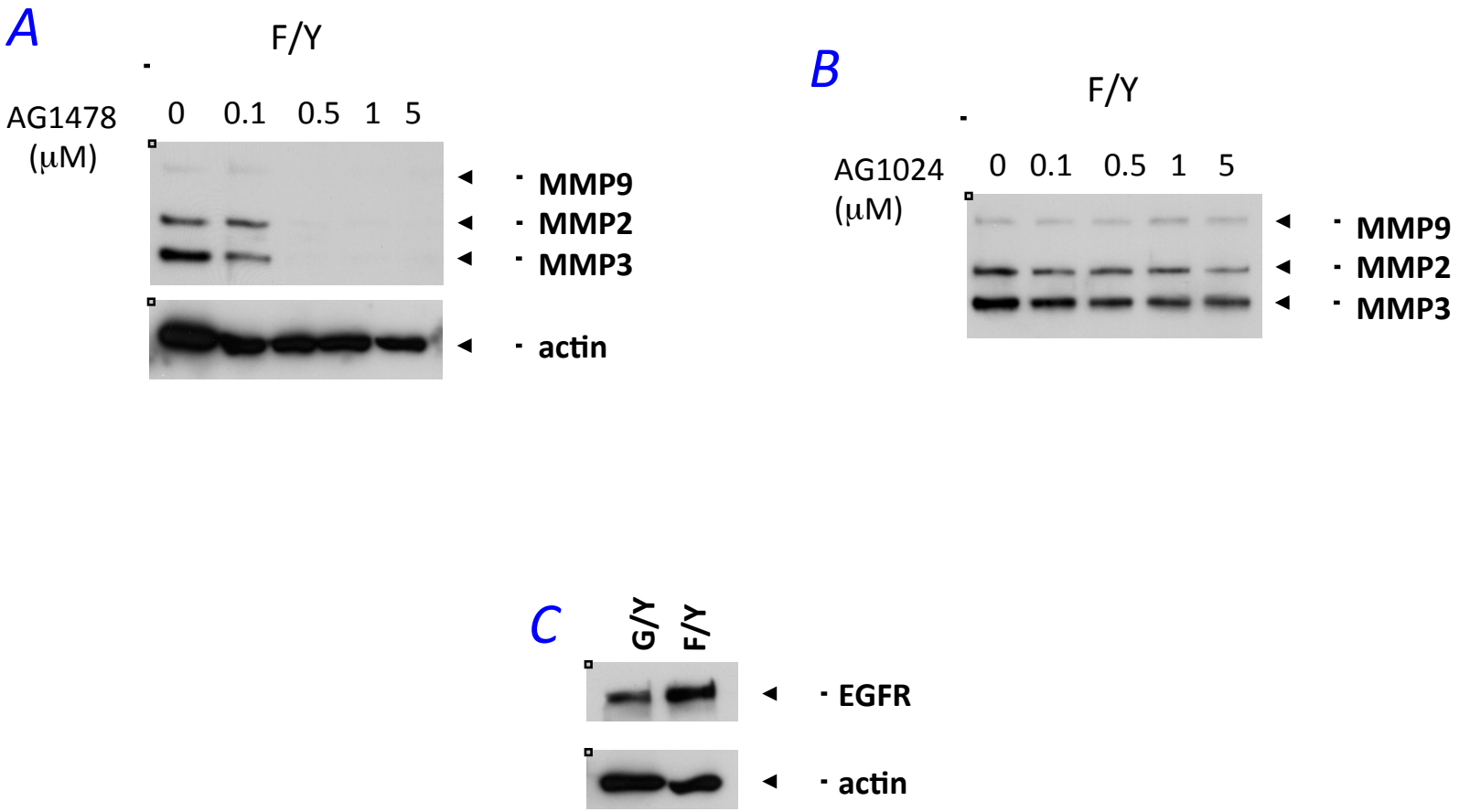


Figure 19

FBLN5 Potentiates EGF-mediated Activation of ERK1/2: *Role of EGFR Stabilization?*

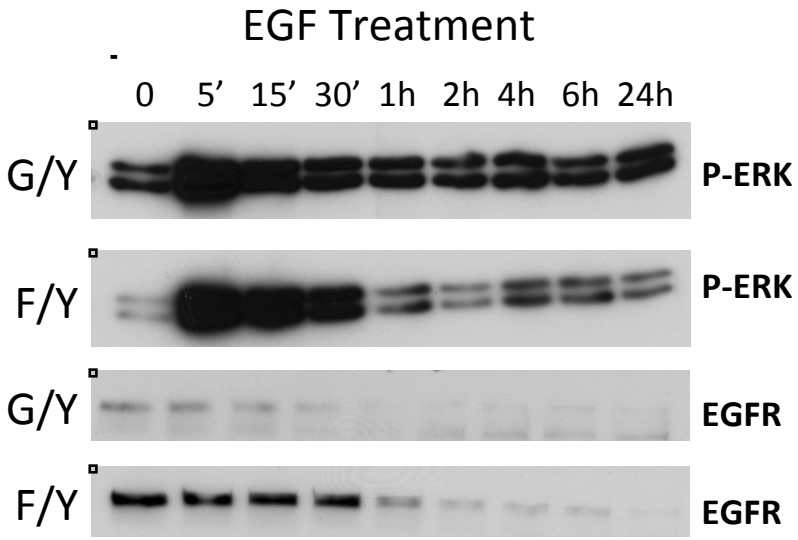


Figure 20

FBLN5 Potentiates MMP Expression and MAP Kinase Mediated Phosphorylation *via* a β1 Integrin- and EGF-dependent Pathway

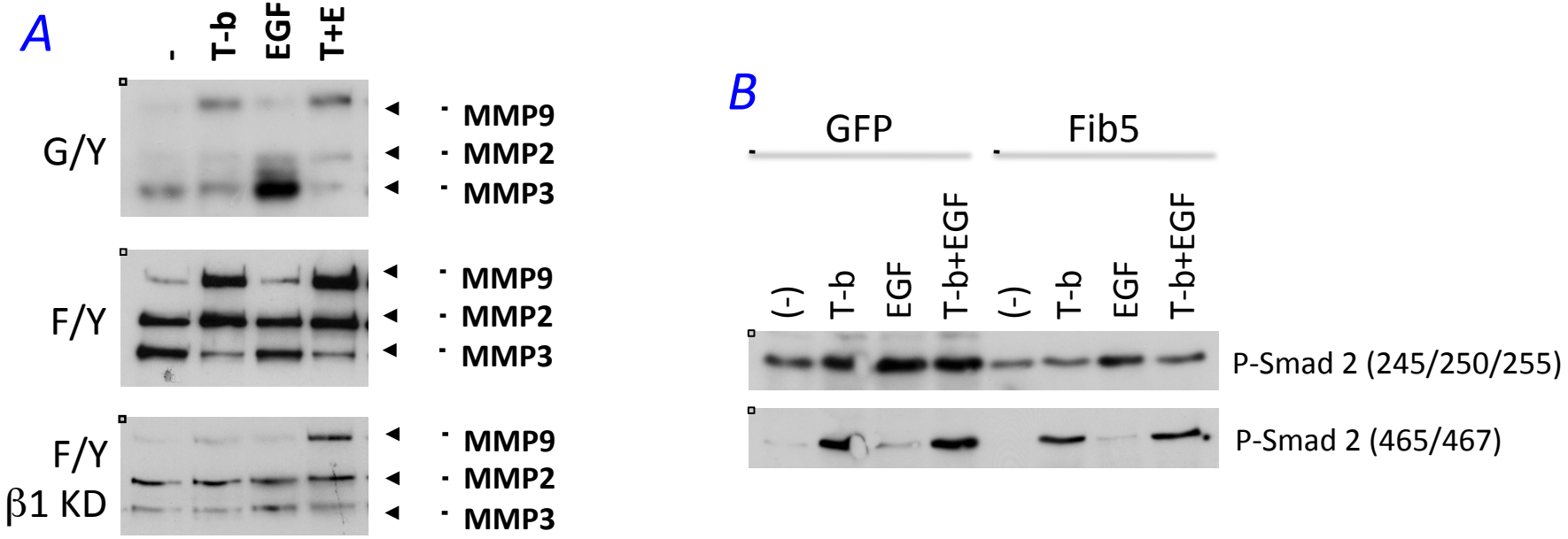
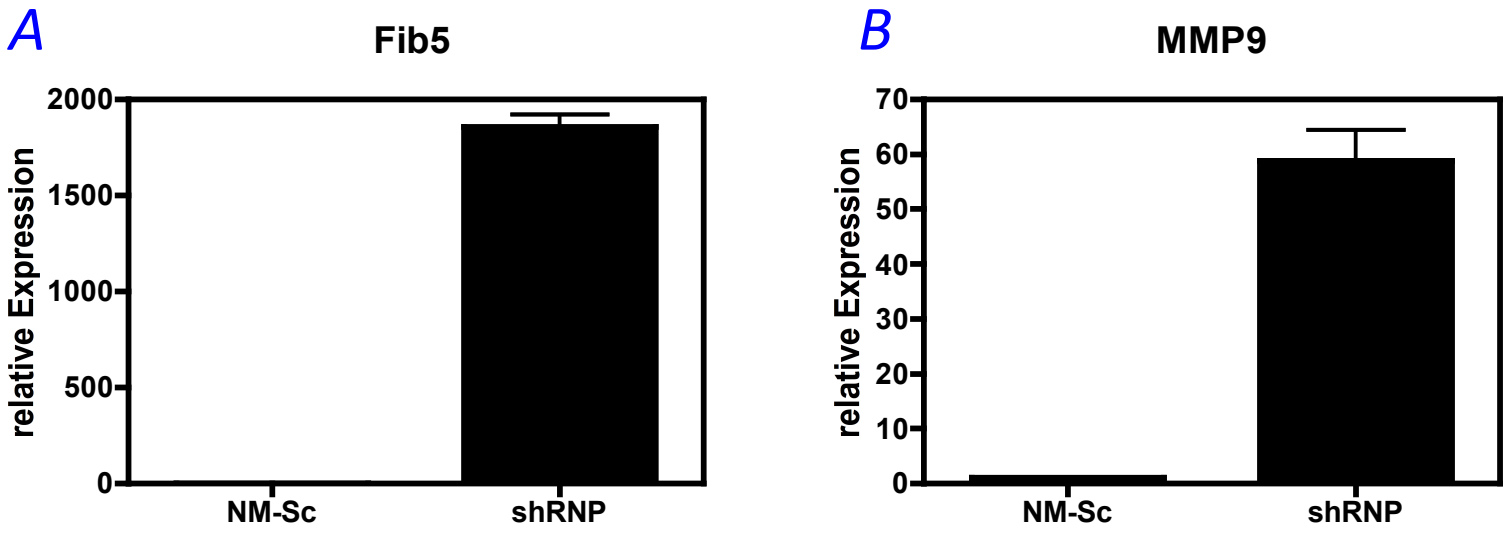


Figure 21

hnRNP E1-Deficiency Dramatically Induces the Expression of FBLN5 and MMP-9 in NMuMG Cells



Legend

NM-Sc = scrambled shRNA
shRNP = shRNA against hnRNP E1

Figure 22

FBLN5 Induces Dab2 Expression *via* a β1 Integrin-dependent Pathway

Legend

- G = GFP
- Y = YFP
- F = FBLN5
- shRPE = hnRNP E1 shRNA
- sc = nonsilencing shRNA
- B1kd = β1 integrin shRNA

Figure 23

FBLN5 Induces EMT *via* a β1 Integrin-dependent Pathway

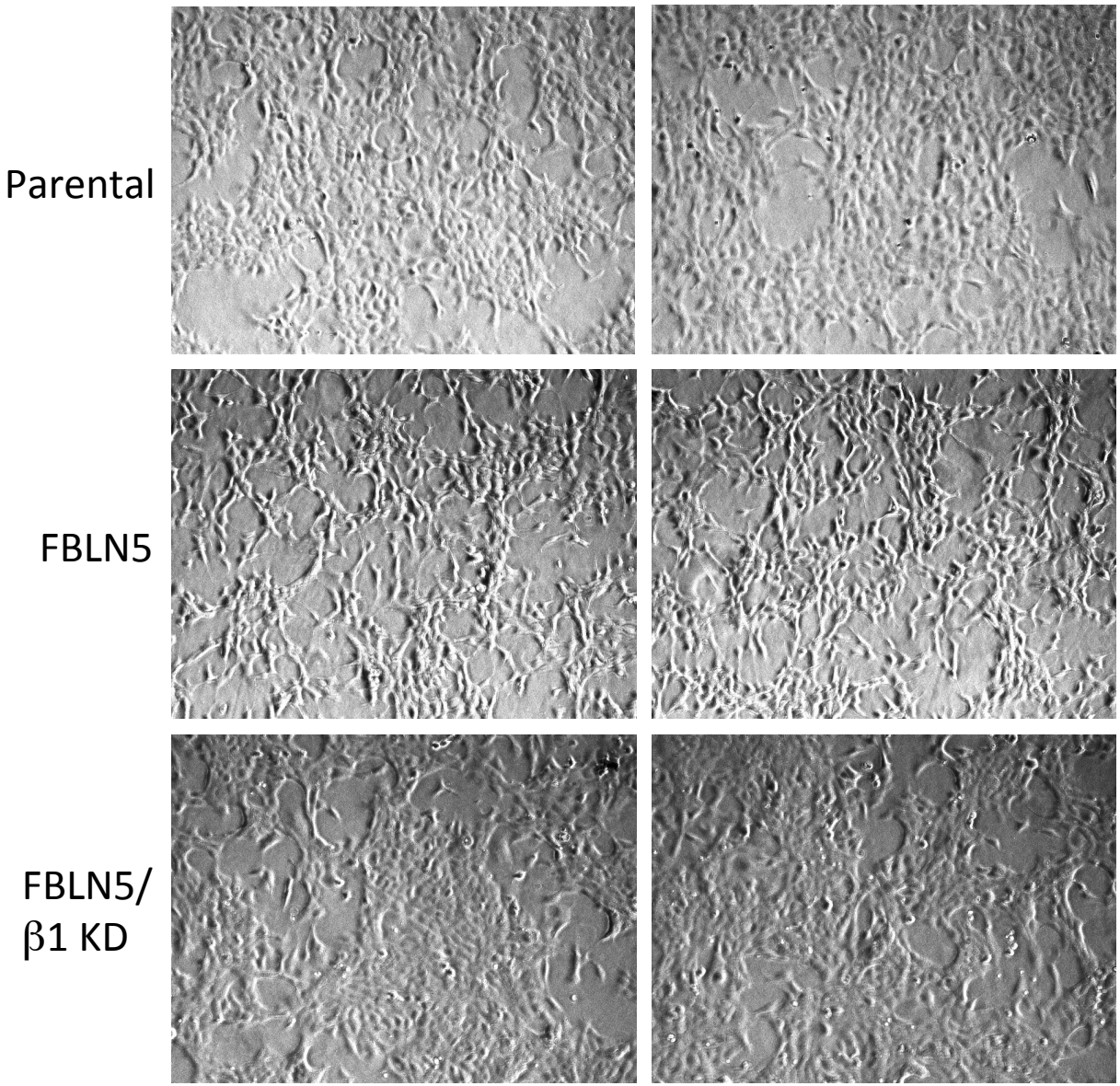


Figure 24

FBLN5 Significantly Enhances EGFR-driven Mammary Tumor Development and Metastatic Progression in Mice

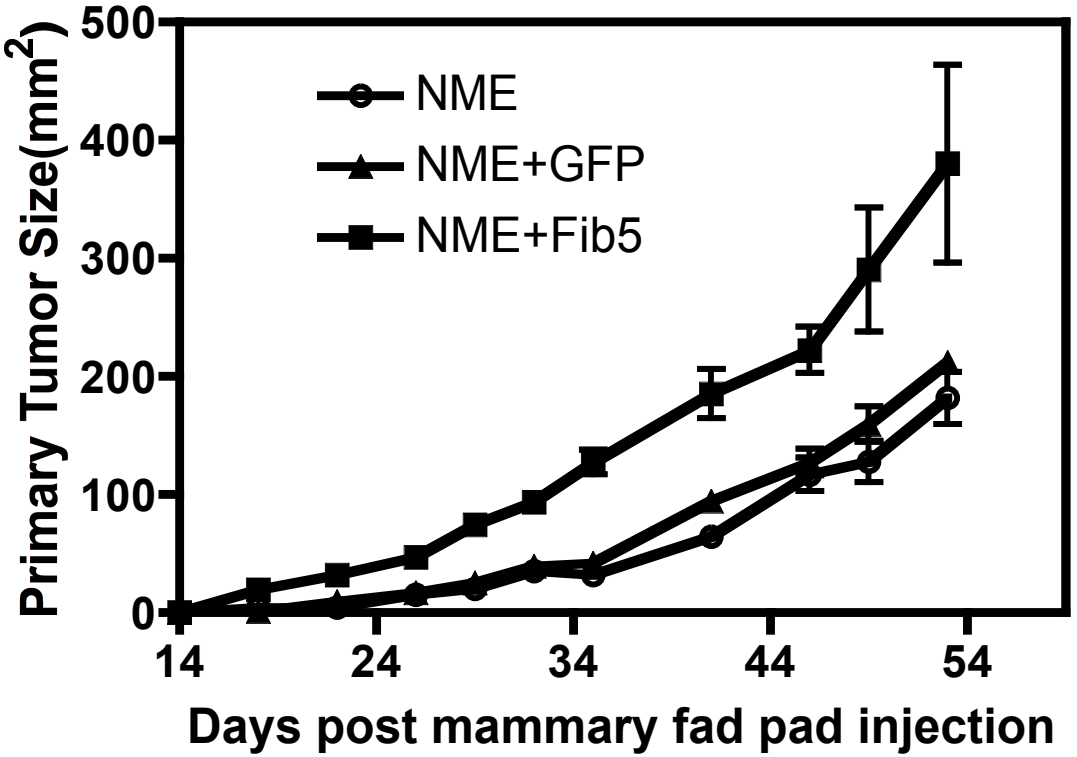
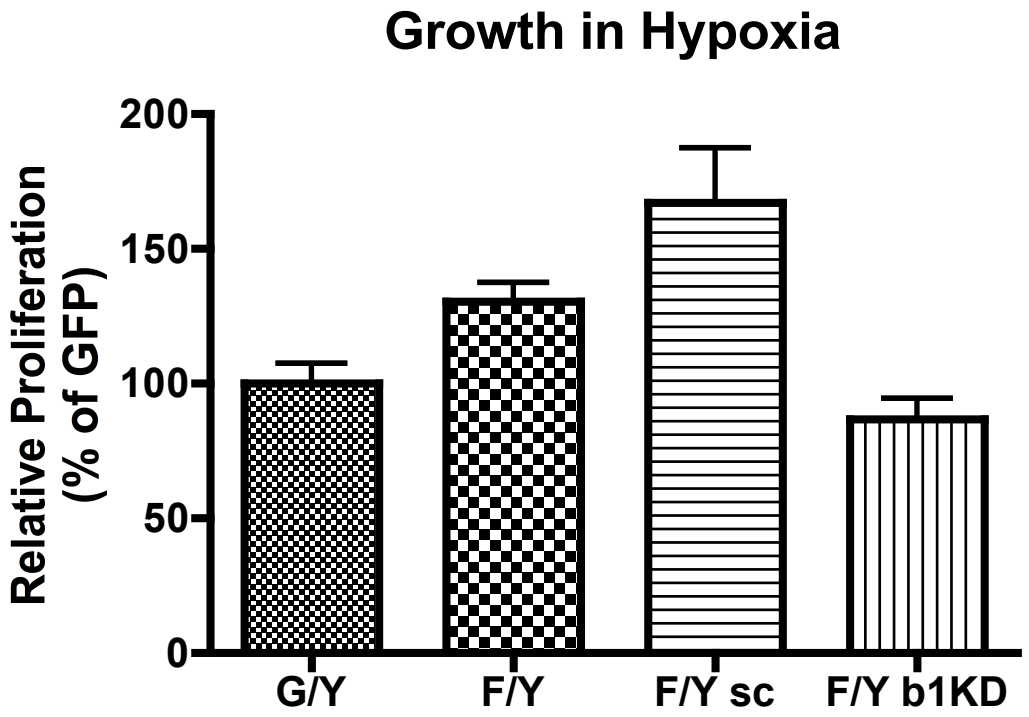


Figure 25

FBLN5 Stimulates the Proliferation of NMuMG Cells Under Hypoxic Conditions *via a* β1 integrin-Dependent Manner

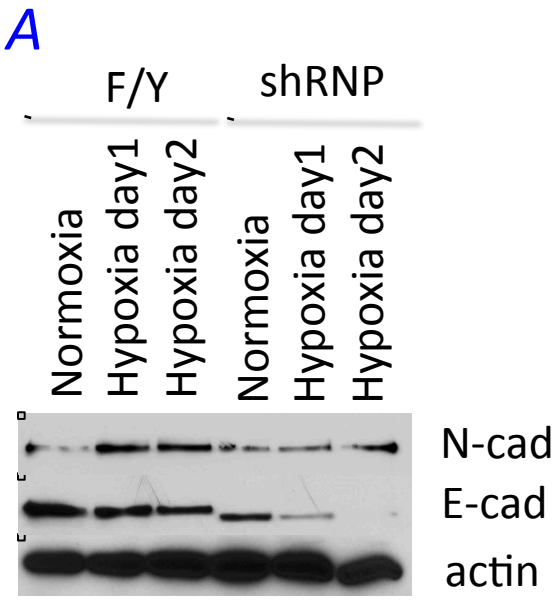


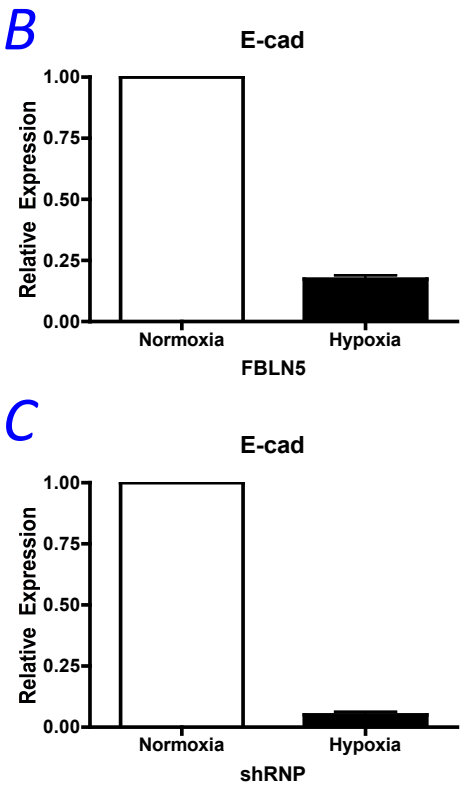
Legend

G = GFP
Y = YFP
F = FBLN
sc = nonsilencing shRNA
B1kd = β1 integrin shRNA

Figure 26

Hypoxia Induces EMT Programs in NMuMG Cells





Legend

Y = YFP
F = FBLN
shRNP = hnRNP E1 knockdown

Figure 27

Hypoxia and FBLN5 Enhance MEC Motility in Normal and Malignant MECs

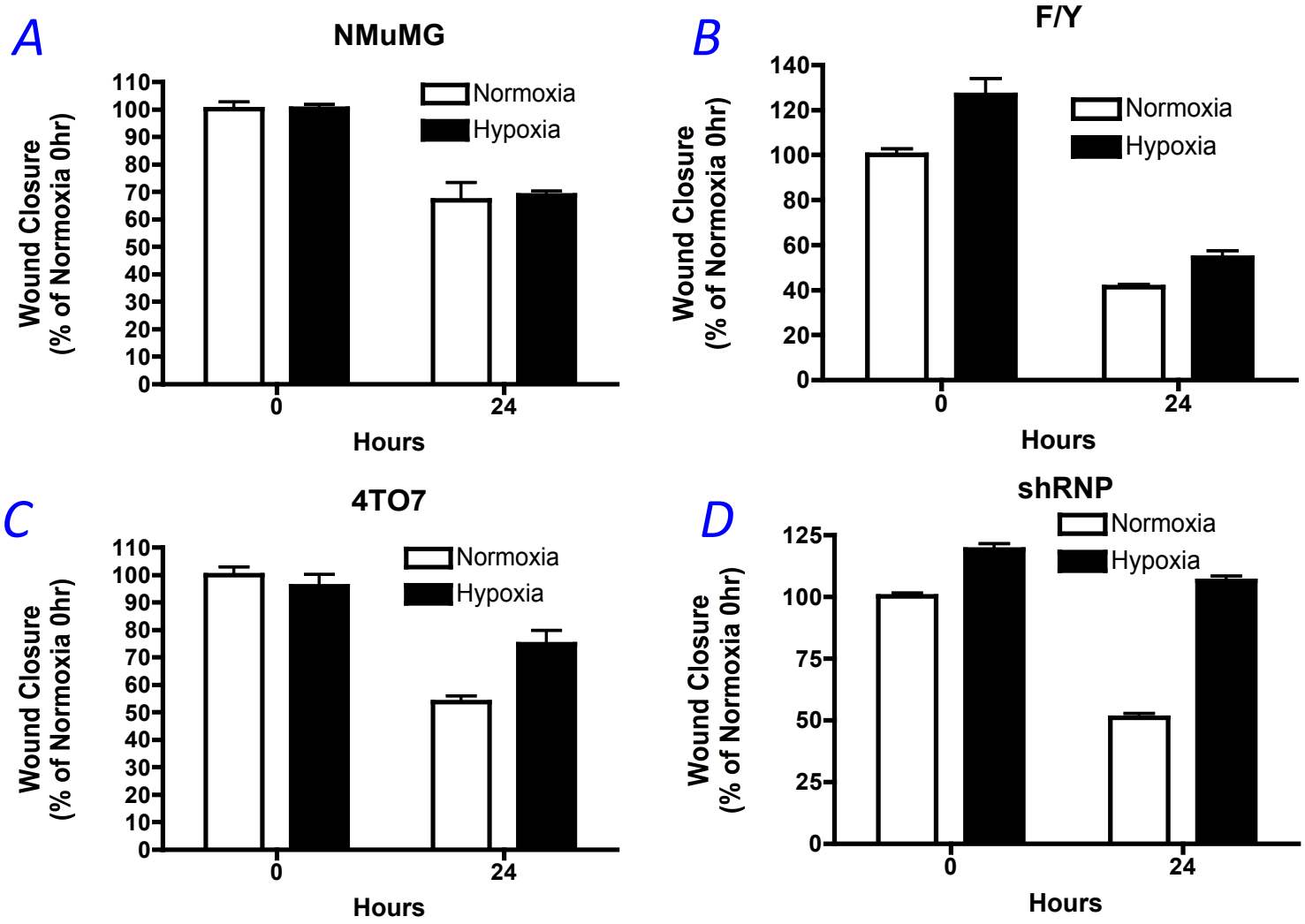


Figure 28

EMT Programs Induced by Hypoxia are Reversible

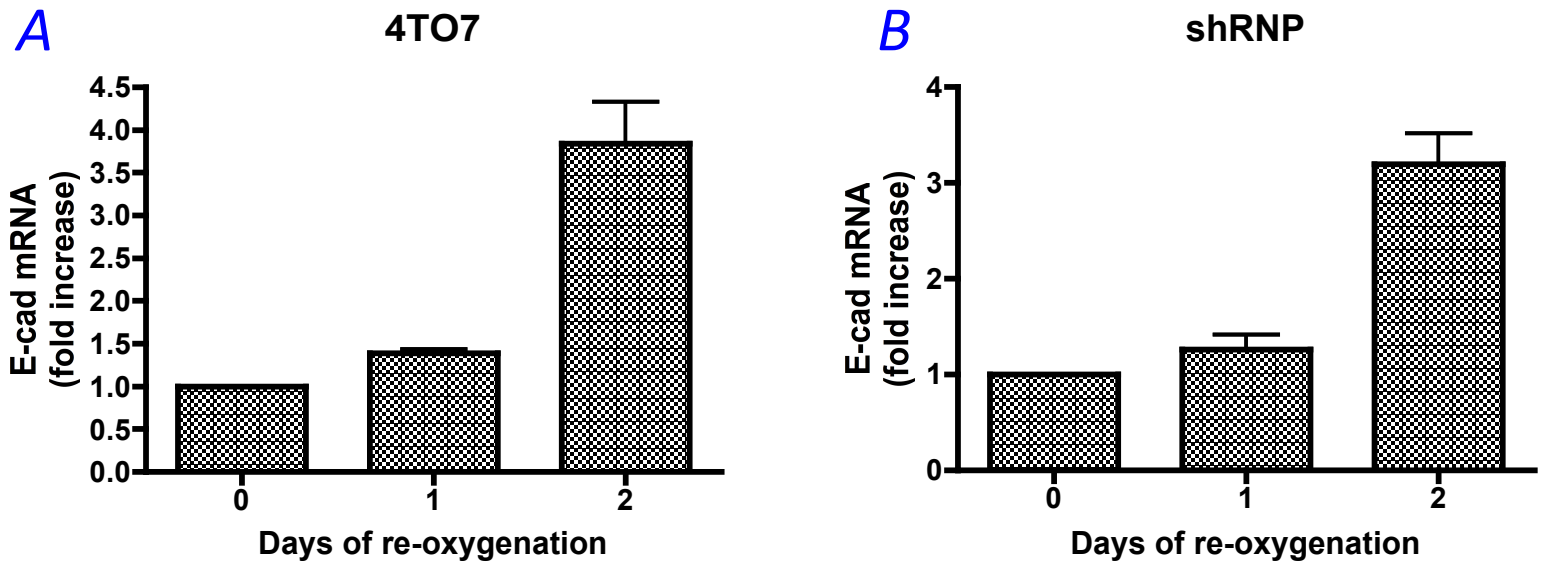


Figure 29

Malignant MECs are More Resistant to Hypoxia-Induced Cell Death as Compared to Their Normal Counterparts

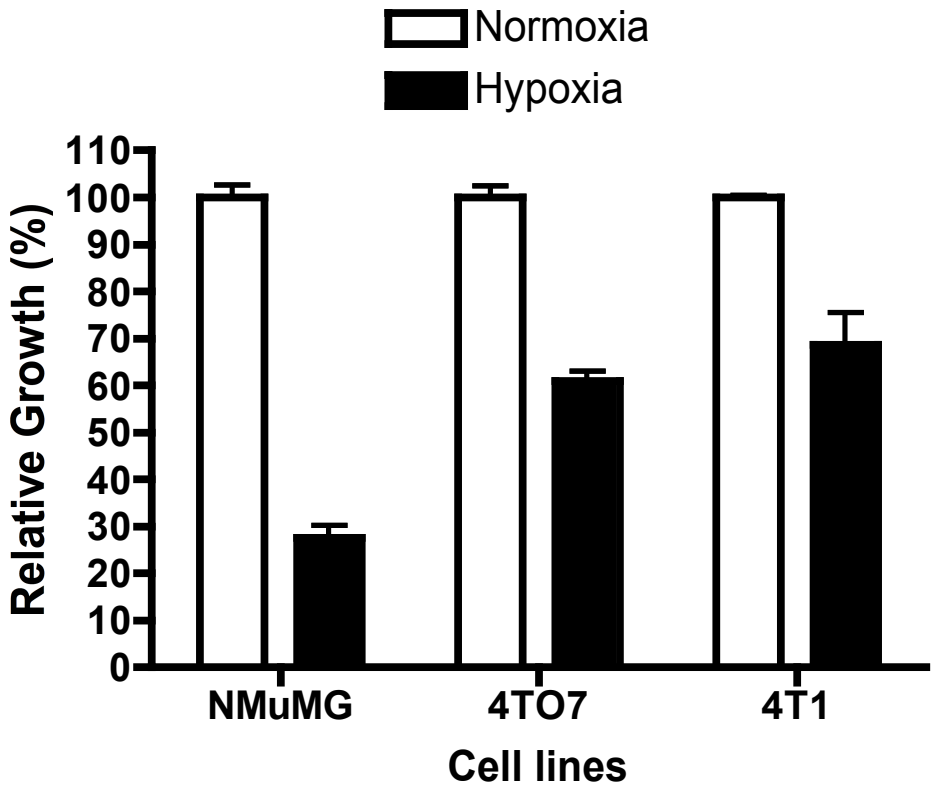


Figure 30

Malignant MECs are More Resistant to Hypoxia and Anoikis in Suspension as Compared to Their Normal Counterparts

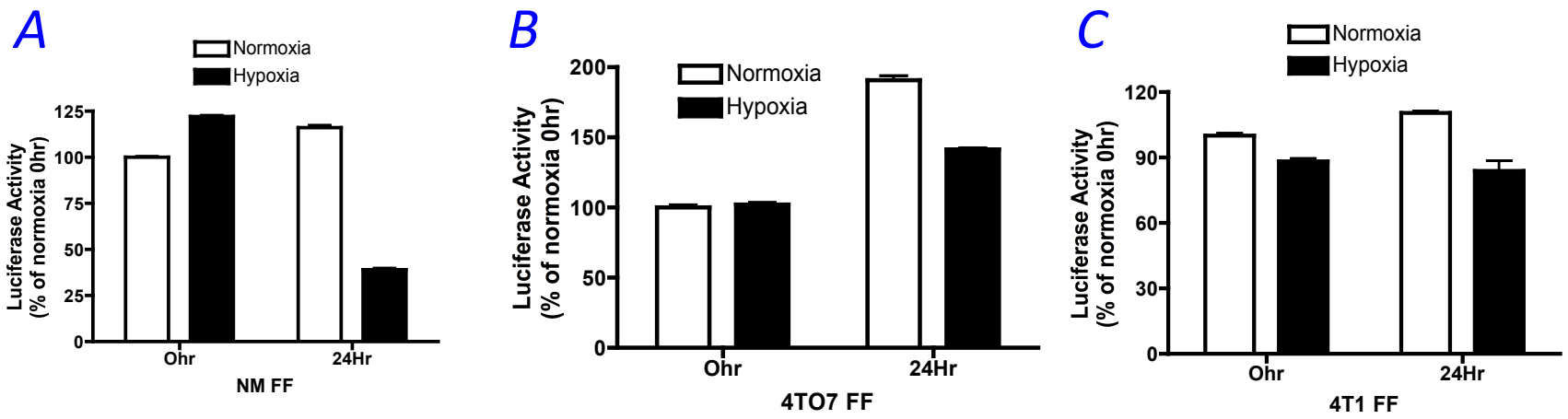


Figure 31

Malignant MECs Progressively Downregulate Their Expression of p27

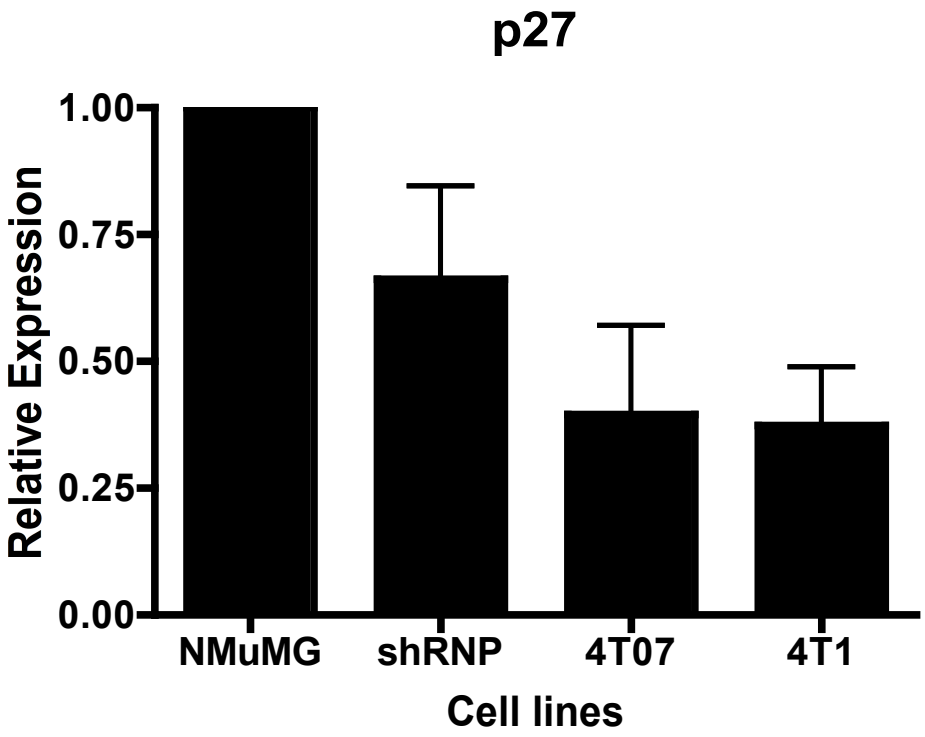
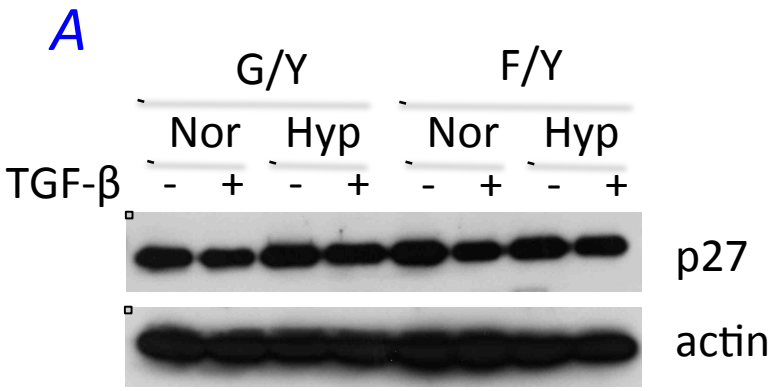
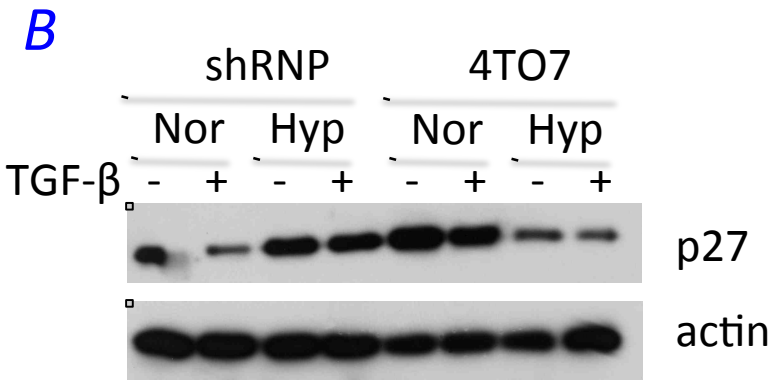


Figure 32

p27 Expression is Sustained in Normal MECs Under Hypoxic Conditions, But is Dramatically Downregulated in Their Malignant Counterparts





Legend

G = GFP
Y = YFP
F = FBLN
Nor = Normoxia
Hyp = Hypoxia

Figure 33

p27 Expression is Stabilized in Malignant MECs as Compared to Their Normal Counterparts

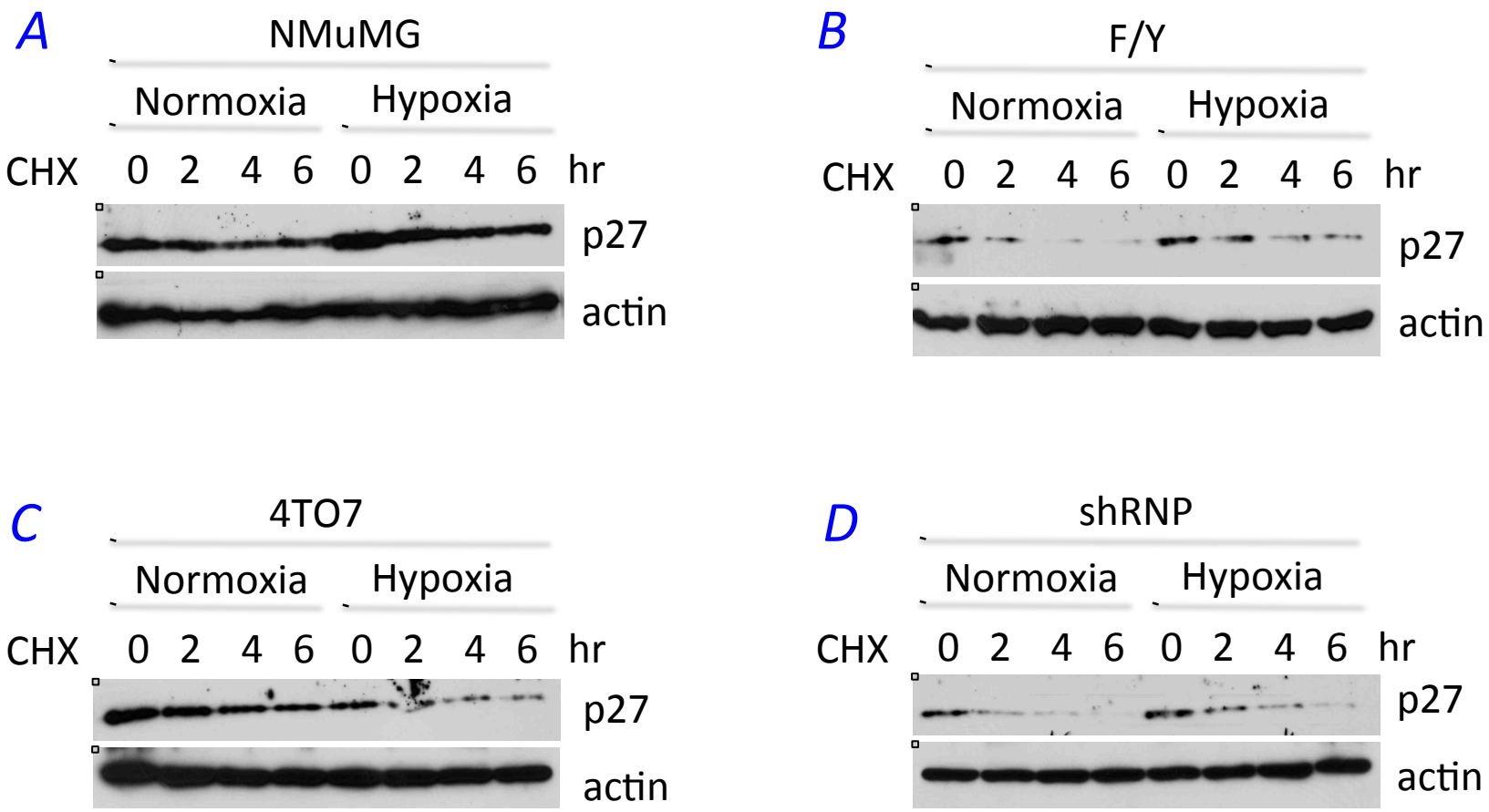


Figure 34

p27 Expression is Stabilized in Malignant MECs as Compared to Their Normal Counterparts

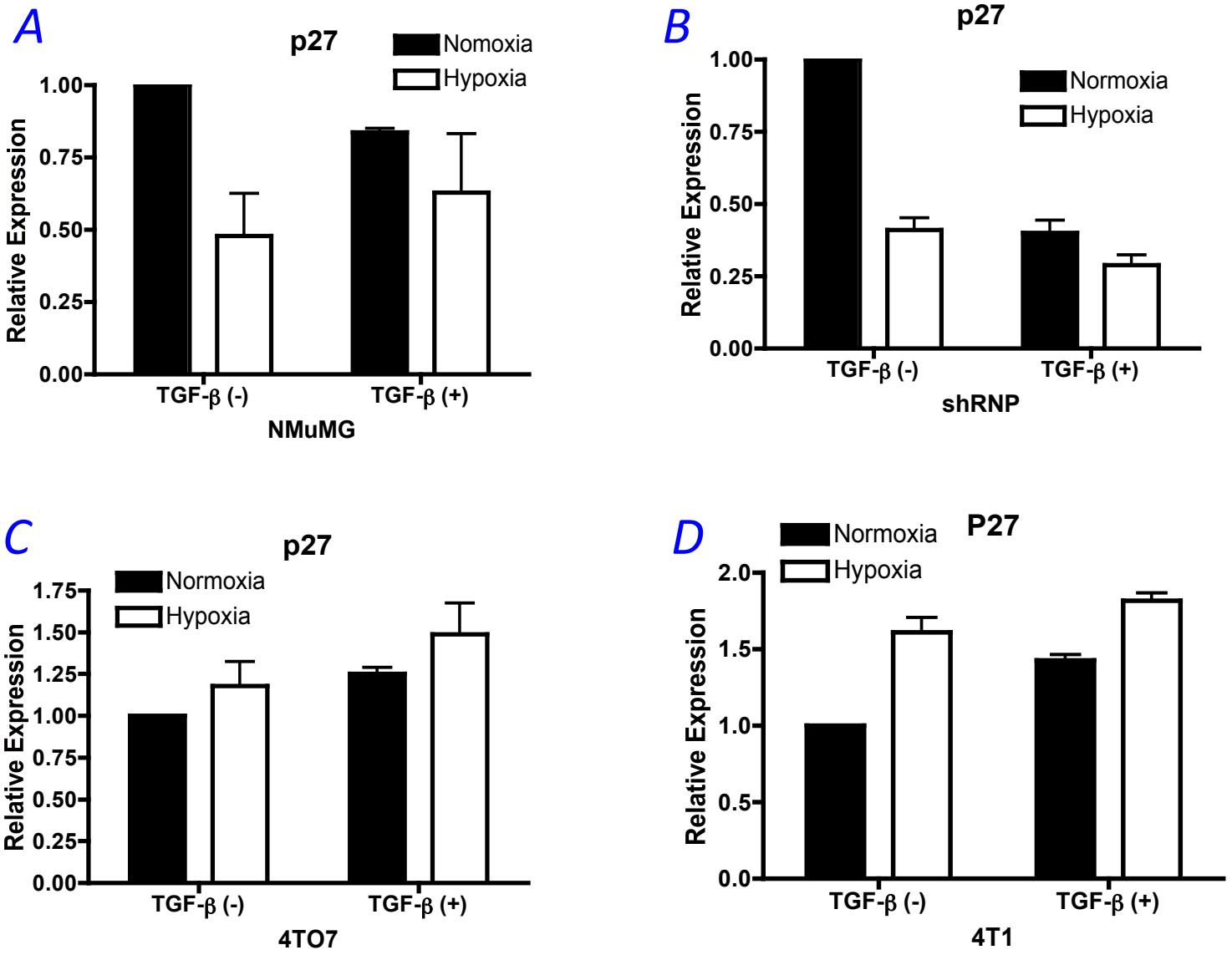
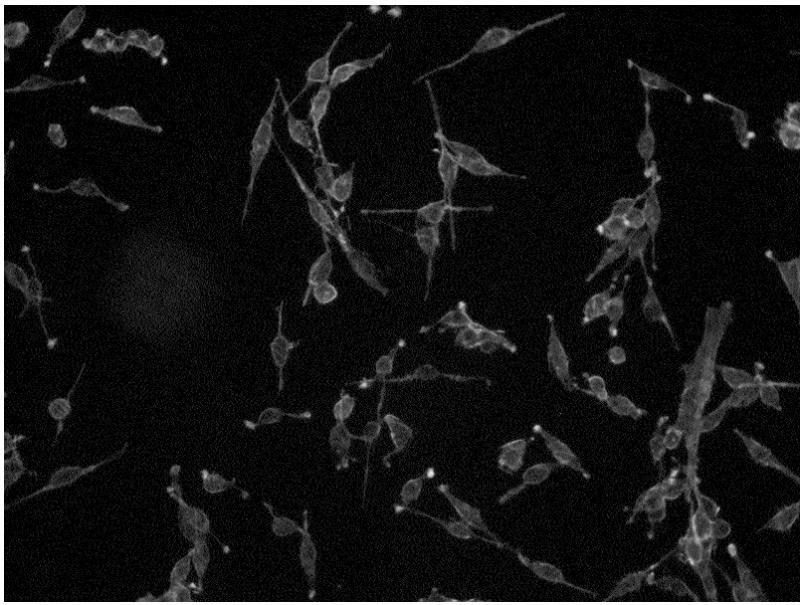


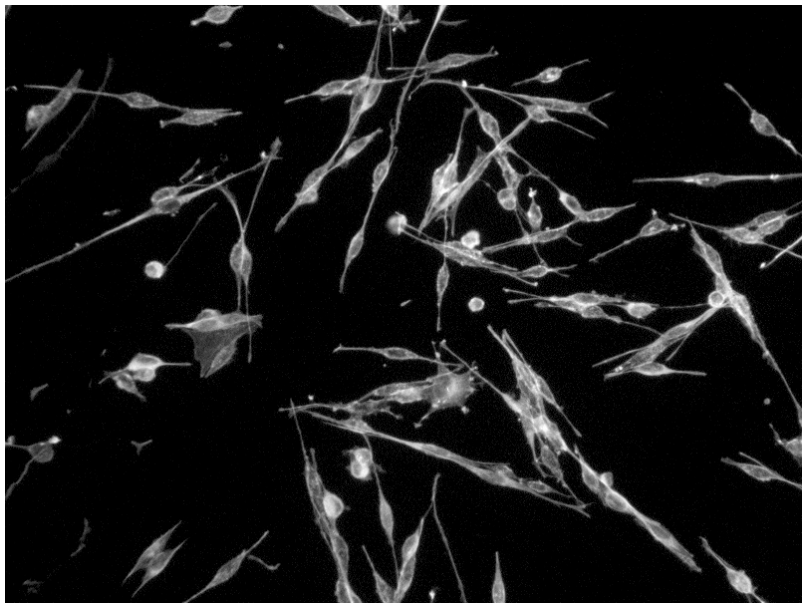
Figure 35

Hypoxia Induces EMT Programs and Mesenchymal Phenotypes

Normoxia



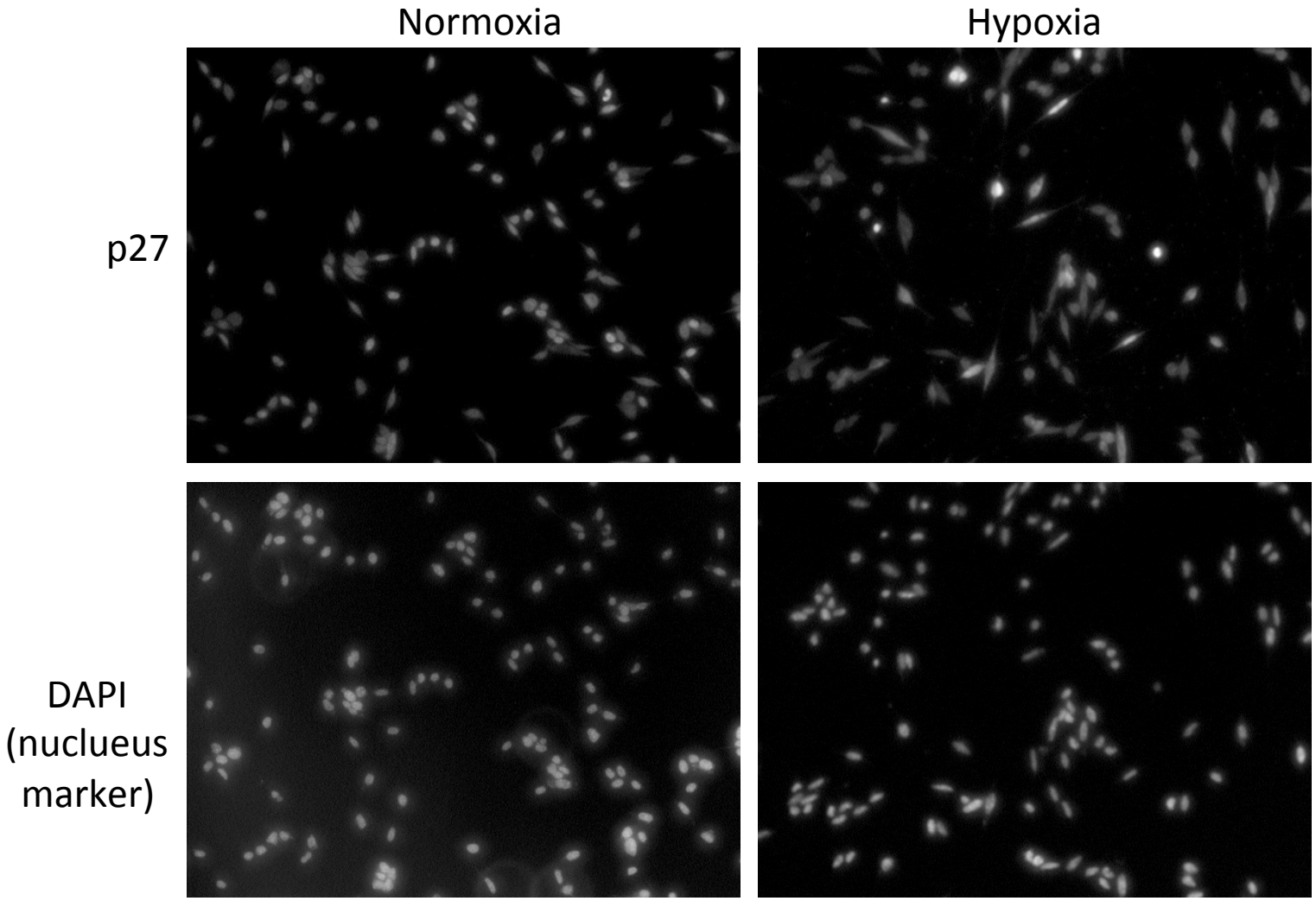
Hypoxia



- Actin Cytoskeleton Visualized with Fluorescently-labeled Phalloidin

Figure 36

Hypoxia Induces Nuclear to Cytoplasmic Redistribution of p27 in Malignant MECs



NIH Public Access
Author Manuscript
Future Oncol. Author manuscript; available in PMC 2010 April 22.

Published in final edited form as:
Future Oncol. 2009 October ; 5(8): 1109–1111. doi:10.2217/fon.09.87.

EMT in Tumor Metastasis: A Method to the Madness

Venkateshwar G. Keshamouni[1] and **William P. Schiemann**[2]

[1] Division of Pulmonary and Critical Care, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI-48109

[2] Department of Pharmacology, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO-80045

Keywords

Epithelial-mesenchymal transitions; metastasis; invasion; tumor microenvironment

Were it not for the ability of carcinoma cells to metastasize and colonize distant organs, all solid tumors would present medically as a group of chronic but manageable diseases. There has been significant progress in the understanding of how cancer cells acquire five of the six essential hallmarks proposed for their transformation (1). Unfortunately, it still remains unclear as to how and when cancer cell acquire the ability to metastasize – *i.e.*, the sixth and final hallmark which is responsible for more than 90 percent of the cancer related mortality (1). However, it has long been recognized that the dissemination of cancer is not simply a random dispersion of cells, but instead represents an ordered and systematic method to this madness. Indeed, epithelial-mesenchymal transition (EMT) is one such method that has been proposed to initiate the metastasis of carcinoma cells (2).

EMT was first recognized as a conserved embryonic and developmental process that facilitates the dispersion cells that ultimately lead to the generation of distinct tissue types (3). In undergoing EMT, cells lose their epithelial properties, while acquiring mesenchymal properties that enable transitioned cells to migrate to predetermined destinations (4). The idea that a similar process is reactivated during tumor progression and other pathologies, including wound healing, tissue regeneration and organ fibrosis, has gained significant ground and acceptance in recent years. Indeed, this fact is readily apparent in the sheer number of publications on this topic, and in the number of EMT-focused sessions and dedicated meetings that have grown exponentially in last few years. It is now widely accepted that EMT plays an important role during tumor progression and confers certain fundamental abilities to cancer cells that are essential for tumor metastasis. These include the ability to migrate, invade and resist anoikis (5,6).

The precise contribution of EMT to tumor metastasis is still a subject of considerable debate in the scientific literature (7). Recent reports of EMT in in-vivo animal models and human studies (8–11), to a certain degree softened the arguments for lack of concrete in-vivo evidence. However, convincing demonstration of a true phenotypic switch is still yet to come. The other dismissive argument that EMT is simply reflective of genomic instability in

*Address for Correspondence: Venkateshwar Keshamouni, Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical Center, 4062 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109, Phone: 734-936-7576, Fax: 734-615-2331, vkeshamo@med.umich.edu And William P. Schiemann, Department of Pharmacology, MS 8303, University of Colorado Denver, RC1 South Tower, Room L18-6110, 12801 East 17th Avenue, PO Box 6511, Aurora, CO 80045, Tel: 303-724-1541, Fax: 303-724-3663, bill.schiemann@ucdenver.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cancer cells is also fading in light of more and more studies reporting EMT that occurs in normal epithelial cells from various organs in response to injury (8,10,12).

Reports of EMT conferring resistance to certain class of drugs and therapeutic modalities, and correlation of EMT gene signatures with poor outcomes have been described (13–15). These observations, together with the recent finding that EMT may confer stem cell like properties to resulting mesenchymal cells (16) have highlighted the clinical relevance of this process. Consequently, several groups both in the industry and academia are actively pursuing the discovery of novel molecules to target EMT (17).

Recently, Kalluri and Weinberg proposed to classify EMT into three distinct subtypes based on the biological context in which they occur (4). This new terminology was not available at the time the reviews for this special supplement were accepted for publication, and as such, this classification is not used herein. With the exception of the review by Micalizzi et al., the other articles have predominantly discussed what now could be referred to as type III EMT in the new classification system – *i.e.*, EMT in the context of tumor progression. In contrast, the article by Micalizzi et al., describes the regulators of developmental EMT, which now is called type I EMT in the new classification scheme, and discusses the transcriptional reactivation of type I EMT in the context of type III EMT. Particularly interesting is the discussion of their own work investigating the role of two new players, Six 1 and Six 4, in the EMT of mouse mammary tumors. Radaelli et al., provide a very elegant historical perspective by discussing some of the early descriptions of EMT in mouse tumors, some of which date as far back as the year 1854. They also presented an interesting comparison of EMT in mouse and human pathologies. A very comprehensive review of the regulatory pathways implicated in TGF-β-induced EMT in normal and malignant cells of breast is provided in the article by Wendt et al. lastly, van Zijl et al review the evidence for EMT in hepatocellular carcinoma and discussed its implications for the treatment of these tumors.

Pathways and molecules that distinguish EMT in tumor progression from other two biological contexts are far from clear. However, any effort to identify context-specific signals should consider the physiological state of the epithelium in which EMT is taking place – *i.e.*, whether it transpires in normal, transformed, or injured epithelium, and how these unique epithelial states impact the functional consequences of the resulting EMT. Indeed, the vast majority of EMT studies to date have focused on assessing the functional consequences of EMT in solely altering the behaviors and functions of tumor cells, not their accompanying stromal components. Given the dramatic changes that take place during EMT, it is wholly reasonable to expect EMT to also elicit powerful alterations within tumor microenvironments, as well as to target the activities and behaviors of various stromal supporting cells. Therefore, the implications of EMT on the interactions of tumor cells with their accompanying stromal and microenvironmental components clearly need to be explored in the future studies.

Acknowledgments

The research work in Keshamoun's lab is supported by grants from Flight Attendant Medical Research Institute, NIH (CA132571), and American Cancer Society (CSM-116801). Dr. Schiemann is supported in part by grants from the NIH (CA114039 and CA129359), the DOD (BC084651), and the Komen Foundation (BCTR0706967).

Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70. ** Seminal review describing six hallmarks of a cancer cell. [PubMed: 10647931]
2. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–454. [PubMed: 12189386]
3. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: The importance of changing cell state in development and disease. J Clin Invest 2009;119:1438–1449. [PubMed: 19487820]
4. Kalluri R, Weinberg RA. J Clin Invest. The basics of epithelial-mesenchymal transition 2009;119:1420–1428. * Proposed classification of EMT into three subtypes.
5. Yang J, Weinberg RA. Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. Developmental cell 2008;14:818–829. [PubMed: 18539112]
6. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006;7:131–142. [PubMed: 16493418]
7. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. Cancer Res 2005;65:5996–6000. discussion 6000-5991. [PubMed: 16024596]
8. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A 2006;103:13180–13185. * First paper to demonstrate in vivo EMT in the context of tissue fibrosis by tracking cell fate. [PubMed: 16924102]
9. Trimboli AJ, Fukino K, de Bruin A, Wei G, Shen L, Tanner SM, Creasap N, Rosol TJ, Robinson ML, Eng C, et al. Direct evidence for epithelial-mesenchymal transitions in breast cancer. Cancer Res 2008;68:937–945. * First in-vivo evidence of EMT in the context of tumor progresssion in a mouse model of breast cancer. [PubMed: 18245497]
10. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007;282:23337–23347. [PubMed: 17562716]
11. Reckamp KL, Gardner BK, Figlin RA, Elashoff D, Krysan K, Dohadwala M, Mao J, Sharma S, Inge L, Rajasekaran A, et al. Tumor response to combination celecoxib and erlotinib therapy in non-small cell lung cancer is associated with a low baseline matrix metalloproteinase-9 and a decline in serum-soluble e-cadherin. J Thorac Oncol 2008;3:117–124. * First correlation of EMT markers to therapeutic response in patients with lung cancer. [PubMed: 18303430]
12. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002;110:341–350. * First paper to demonstate contribution of EMT to tissue fibrosis. [PubMed: 12163453]
13. Dimeo TA, Anderson K, Phadke P, Feng C, Perou CM, Naber S, Kuperwasser C. A novel lung metastasis signature links wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. Cancer Res. 2009
14. Singh A, Greninger P, Rhodes D, Koopman L, Violette S, Bardeesy N, Settleman J. A gene expression signature associated with “K-ras addiction” Reveals regulators of emt and tumor cell survival. Cancer Cell 2009;15:489–500. [PubMed: 19477428]
15. Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N, Iwata KK, Gibson N, Haley JD. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer Res 2005;65:9455–9462. [PubMed: 16230409]
16. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–715. ** First paper to demonstrate a link between EMT and stemness. [PubMed: 18485877]
17. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: New insights in signaling, development, and disease. J Cell Biol 2006;172:973–981. [PubMed: 16567498]

NIH Public Access
Author Manuscript
Future Oncol. Author manuscript; available in PMC 2010 April 21.

Published in final edited form as:
Future Oncol. 2009 October ; 5(8): 1145–1168. doi:10.2217/fon.09.90.

Mechanisms of Epithelial-Mesenchymal Transition by TGF-β

Michael K. Wendt[*], **Tressa M. Allington**[*], and **William P. Schiemann**
Department of Pharmacology, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045.

Summary

The formation of epithelial cell barriers results from the defined spatiotemporal differentiation of stem cells into a specialized and polarized epithelium, a process termed mesenchymal-epithelial transition. The reverse process, epithelial-mesenchymal transition (EMT), is a metastable process that enables polarized epithelial cells acquire a motile fibroblastoid phenotype. Physiological EMT also plays an essential role in promoting tissue healing, remodeling, or repair in response to a variety of pathological insults. On the other hand, pathophysiological EMT is a critical step in mediating the acquisition of metastatic phenotypes by localized carcinomas. Although metastasis clearly is the most lethal aspect of cancer, our knowledge of the molecular events that govern its development, including those underlying EMT, remain relatively undefined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that oversees and directs all aspects of cell development, differentiation, and homeostasis, as well as suppresses their uncontrolled proliferation and transformation. Quite dichotomously, tumorigenesis subverts the tumor suppressing function of TGF-β, and in doing so, converts TGF-β to a tumor promoter that stimulates pathophysiological EMT and metastasis. It therefore stands to reason that determining how TGF-β induces EMT in developing neoplasms will enable science and medicine to produce novel pharmacological agents capable of preventing its ability to do so, thereby improving the clinical course of cancer patients. Here we review the cellular, molecular, and microenvironmental mechanisms used by TGF-β to mediate its stimulation of EMT in normal and malignant cells.

Keywords

Epithelial-mesenchymal Transition; Metastasis; Signal Transduction; Transforming growth factor-β; Tumor Microenvironment

1. INTRODUCTION

The epithelium is comprised of highly specialized and diverse cells that play critical roles in nearly all biological processes [1,2]. Indeed, epithelial cells serve as protective barriers that line both the outer (*i.e.*, skin) and inner (*i.e.*, airways, gastrointestinal tract, etc.) body cavities, as well as behave as secretory and glandular tissues. In addition, epithelial cell function varies widely between tissues and ranges from nutrient absorption in the intestines to gaseous exchange in the lungs to lactogenesis in the mammary gland. Equally important is the role of the epithelium in providing the first line of defense against exterior insults and infections, while simultaneously enabling the exchange of vital nutrients needed to maintain tissue homeostasis. The fidelity and function of the epithelium is maintained through its

Correspondence: William P. Schiemann, Department of Pharmacology, MS-8303, University of Colorado Denver, Anschutz Medical Campus, RC1 South Tower, Room L18-6110, 12801 East 17th Avenue, PO Box 6511, Aurora, CO 80045. Phone: (303)724-1541. Fax: (303)724-3663. Bill.Schiemann@ucdenver.edu .
[*]M.K. Wendt and T.M. Allington were equal contributors to this work.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

continual renewal and repair, and as such, it perhaps is not surprising to learn that the majority (*i.e.*, ~90%; [3]) of cancers arise in cells derived from epithelial origins. Thus, it is imperative that science and medicine uncover the sequence of events that enable specialized and polarized epithelial cells to dedifferentiate along a tumorigenic pathway that terminates in their acquisition of metastatic phenotypes.

Recent evidence has linked the development of tissue fibrosis and cancer metastasis to the inappropriate reactivation of epithelial-mesenchymal transition (EMT), which is the process whereby immotile, polarized epithelial cells transition into highly motile, apolar fibroblastoid-like cells (Figure 1; [1,2,4-6]). Indeed, EMT is a normal physiological process essential for proper embryogenesis and tissue morphogenesis, particularly for the formation of the mesoderm, neural crest, cardiac valve, and secondary palate [1,2,7]. With respect to adult tissues, EMT also is engaged in wounded epithelia to facilitate their healing, remodeling, and repair in response to tissue damage. Thus, fully differentiated epithelial cells harbor a dormant embryonic transcriptional EMT program that can be reinitiated in response to a variety of specific environmental cues and signals, one of which is the pleiotropic cytokine, transforming growth factor-β (TGF-β). Interestingly, these same cellular and morphological features are observed in cells undergoing pathophysiological EMT, which underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders of the lung, liver, and kidney [1,2,4-6,8,9]. Along these lines, aberrant reinitiation of EMT also engenders the acquisition of invasive and metastatic phenotypes in developing and progressing carcinomas, leading to their dissemination and colonization of distant organ sites suitable to support their metastatic growth. A commonality of physiological and pathophysiological EMT is their ability to be induced by TGF-β, which now is recognized as a master regulator of this transdifferentiation process.

TGF-β is a ubiquitously expressed and multifunctional cytokine that not only regulates EMT, but also oversees the development, differentiation, and survival of essentially all cell types and tissues [10-13]. TGF-β also is a powerful suppressor of cell growth and proliferation, particularly in cells of epithelial, endothelial, and hematopoietic origins [10-13]. Quite dichotomously, aberrations in the TGF-β signaling system regularly take place during tumorigenesis and elicit resistance to its anti-proliferative activities, contributing to the formation of human neoplasms. Upon being liberated from the cytostatic activities of TGF-β, cancer cells proliferate, invade, and metastasize beyond their tissue of origin when stimulated by TGF-β. How TGF-β suppresses these processes in normal epithelial cells is unclear, as is how TGF-β promotes these processes in their malignant counterparts. Despite the continued uncertainty of the molecular events associated with the diametric activities of TGF-β, it is absolutely clear that this cytokine stimulates the two deadliest aspects of cancer, namely cell invasion and metastasis. Moreover, recent studies indicate that acquisition of metastatic phenotypes by carcinoma cells is critically dependent upon their ability to undergo EMT [4-6,8,14]. Indeed, TGF-β stimulation of EMT was demonstrated originally by Miettinen *et al* [15] who observed normal mammary epithelial cells (MECs) to acquire fibroblastoid phenotypes in response to TGF-β. In addition, TGF-β3-deficient mice develop cleft palate due to defective palatogenesis associated with aberrant EMT [16]. Similar inactivation of TGF-β2 function impairs endocardial cushion development in chick hearts due to their absence of Slug expression and its ability to activate EMT [17]. Finally, Smad3-deficiency affords protection against EMT-driven retinal [18,19] and renal [20] fibrosis in mice. Thus, these and other seminal studies have clearly established TGF-β as a master regulator of EMT. This review focuses on the myriad of evidence supporting this designation for TGF-β, particularly the cellular, molecular, and microenvironmental mechanisms that underlie the ability of TGF-β to induce EMT in normal and malignant cells.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2. TGF-β SIGNALING & EMT

The general mechanisms whereby TGF-β activates responsive cells and regulates their behavior is depicted in Figures 2 and 3. As shown, transmembrane signaling by TGF-β commences *via* its binding to three high-affinity receptors, namely the TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). When and where it is expressed, TβR-III clearly is the most abundant TGF-β receptor on the cell surface where it functions as an accessory receptor that binds and presents TGF-β to its signaling receptors, TβR-I and TβR-II, both of which possess intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains [11,12,21-23]. The binding of TGF-β to TβR-II enables the recruitment and activation of TβR-I, leading to its induction of canonical Smad2/3-dependent signaling. Once activated, Smad2/3 form heterocomplexes with Smad4 and translocate into the nucleus where they regulate the cell type-specific expression of TGF-β-responsive genes [11,12,21-23]. It is interesting to note that the variety of cell responses exhibited in response to TGF-β are governed primarily by the cell type-specific expression of various Smad2/3-interacting transcription factors (*e.g.*, AP-1 and Forkhead family members, Stats, etc. [11,22]), as well as their association with additional transcriptional activators or repressors [11,12,21-23]. Moreover, the amplitude and duration of Smad2/3 signaling is modulated by several mechanisms, including the expression of *(i)* adapter and/or anchoring proteins SARA [24], Hgs [25], and Dab2 [26] that enable Smad2/3 phosphorylation by TβR-I, and *(ii)* the inhibitory Smad, Smad7, which prevents the phosphorylation of Smad2/3 [27-29] and induces the degradation of TGF-β receptors [30,31]. In addition, the inhibitory functions of Smad7 are regulated by its interaction with STRAP [32], which potentiates the anti-TGF-β activity of Smad7, and by its association either with AMSH2 [33] or Arkadia [34-36], both of which negate the anti-TGF-β activity of Smad7. As alluded to above, the activation of Smad2/3 by TGF-β represents the canonical TGF-β signaling system, which is shown diagrammatically in Figure 3.

Also depicted in Figure 3 is the coupling of TGF-β to a variety of noncanonical signaling systems, including *(i)* the MAP kinases ERK1/ERK2, p38 MAPK, and JNK; *(ii)* the growth and survival kinases PI3K, AKT/PKB, and mTOR; and *(iii)* the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 [37-45]. In addition, TGF-β typically represses NF-κB activity in normal epithelial cells [46,47], but readily activates this transcription factor in their malignant counterparts [47-51]. More recently, TGF-β has been shown to activate a number of protein tyrosine kinases (PTKs), including FAK [52,53], Src [43-45,54], and Abl [55,56], which results in the inappropriate amplification of noncanonical TGF-β signaling in mesenchymal or dedifferentiated epithelial cells. Moreover, imbalances in the activation status of canonical and noncanonical TGF-β signaling systems may very well underlie the ability of TGF-β to induce EMT in normal and malignant cells. The importance of canonical and noncanonical TGF-β signaling systems to promote physiological and pathophysiological EMT is presented in greater detail below.

3. DEFINING EMT

The phenomenon of EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells. An inherent characteristic or hallmark of EMT, including that stimulated by TGF-β, is the dramatic phenotypic change in epithelial cell morphology [4-6,8,14]. Typically, fully differentiated epithelium manifests as a single layer of polarized epithelial cells comprised of well-defined apical and basolateral surfaces, as well as a clearly demarcated actin cytoskeleton arranged into discrete “cobblestones” that reflect regions of concentrated actin fibers at cell-cell junctions. In response to the initiation of EMT, cell-cell junctions disassemble and filamentous actin undergoes a dramatic redistribution to form prominent stress fibers, which is tracked experimentally *via* the use of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

a fluorescently-labeled mushroom toxin, phalloidin. The combined effect of these various cell biological activities is a loss of epithelial cell polarity (Figure 1).

Examining the biochemical and molecular alterations in cell-cell junction formation and dissolution has enabled science and medicine to garner a more complete assessment of the events underlying EMT. Indeed, a number of recent examinations have elucidated a variety of molecular complexes and scaffolds that govern the development of cell-cell junctions, including tight junctions, adherens junctions, and desmosomes [5]. Not surprisingly, a series of coordinated and dynamic processes underlie formation of these macromolecular complexes during the development and maintenance of the epithelium, while changes in the expression and localization of junctional proteins constitute useful measures to track the progression of EMT. For instance, tight junctions are formed by the actions of the transmembrane proteins, claudins, occludins, and JAMs (**J**unctional **A**dhesion **M**olecules), which are linked to the actin cytoskeleton *via* the scaffold proteins ZO-1, −2, −3 [57,58]. Moreover, following their formation, tight junctions and their constituents play essential roles in regulating the biology, homeostasis, and architecture of epithelial cells, and in preventing the initiation of EMT and tumorigenesis [59]. In contrast, the initiation of EMT induces a drastic modulation of tight junction localization in epithelial cells [15,38]. For instance, the function of Par6 (partitioning-defective 6), which governs the formation of tight junctions, the establishment of apical-basolateral polarity, and the initiation of polarized cell migration [60], is compromised by its physical interaction with TβR-I and subsequent phosphorylation by TβR-II in epithelial cells stimulated with TGF-β [61]. Once phosphorylated, Par6 recruits and interacts with E3 ubiquitin ligase, Smurf1, which ubiquitinates the small GTPase, RhoA, leading to its degradation and subsequent dissolution of tight junctions during EMT stimulated by TGF-β [62]. The importance of Par6 to EMT induced by TGF-β is highlighted by the ability of TβR-II-resistant Par6 mutants (*i.e.*, S345A-Par6) to prevent MECs from undergoing EMT in response to TGF-β [61].

Unlike tight junctions, adherens junctions consist of transmembrane E-cadherin (**E**pithelial-cadherin) proteins that are linked to the actin cytoskeleton by α- and β-catenins [63]. TGF-β stimulation of EMT represses E-cadherin transcription (*see below*), as well as disrupts its localization at the plasma membrane in part *via* diminished activation of the small GTPase, Rac1 [62]. The net effect of altered E-cadherin function during EMT is the dissolution of adherens junctions. In addition, the loss of cell-cell contacts parallels the development of prominent actin filaments and the appearance of fibroblastoid-like phenotypes in transitioning epithelial cells, processes requiring the activation of RhoA by TGF-β [64,65]. The mechanisms underlying TGF-β regulation of adherens junction expression and function are discussed below.

4. EMT, TGF-β, & CELL MICROENVIRONMENTS

Maintaining homeostasis within cell microenvironments is essential to alleviating disease development in humans, particularly cancer. Tumor development has been likened to that of dysfunctional miniature organs that house a mixture of malignant and normal cells, including fibroblasts, endothelial, and immune cells [66]. It also is important to remember that the growth and progression of tumors are not inherent properties of the cancer cells themselves, but instead are dictated in large part by a delicate balance between positive and negative proliferative signals produced by diverse cell types within tumor microenvironments. Indeed, alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β in developing neoplasms. Biologically, TGF-β is a master inhibitor of cell cycle progression; however, this cytokine also functions as a master regulator of ECM production, deposition, and remodeling, all of which are essential processes during EMT.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Along these lines, recent evidence has shown that TGF-β stimulation of cancer progression proceeds in part *via* its reprogramming of cell microenvironments, particularly by its ability to target the behaviors of neighboring endothelial cells (ECs) and fibroblasts. Moreover, ECs and fibroblasts typically respond to TGF-β by synthesizing and secreting numerous cytokines, growth factors, and ECM components capable of driving the progression of tumors from indolent to aggressive states [67,68]. A vital component of normal and malignant cell microenvironments is the ECM, which functions as *(i)* a gel-like structural scaffold for cells comprised of polysaccharides and fibrous proteins, including collagen, fibronectin, and elastin; and *(ii)* a molecular sensor that monitors, detects, and responds rapidly to physiological and pathophysiological changes within cell microenvironments. Indeed, under physiological conditions, the ECM serves as a storage reservoir that sequesters numerous growth factors and cytokines that can be rapidly released in response to ECM perturbations or insults, thereby circumventing the need for *de novo* protein synthesis to elicit biological behaviors [69]. Thus, the microenvironment of epithelial cells plays a critical role in maintaining their polarization and differentiation, processes that are disrupted temporarily during physiological EMT and its modification of epithelial cell microenvironments. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis. Table 1 identifies numerous EMT-associated genes whose expression is regulated by TGF-β, and readers desiring more in depth discussions of the activities and functions of these genes in governing EMT and epithelial cell biology are directed to several recent reviews [1,2,4-6]. In the following sections, we highlight many of the mechanisms that underlie the ability of TGF-β to induce EMT and its associated alterations within the microenvironments of transdifferentiating cells.

4.1. Matrix Metalloproteinases

Matrix **m**etallo**p**roteinases (MMPs) comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, and of wound healing and cell growth. MMPs also possess the ability to degrade nearly all ECM and basement membrane components, as well as the ability to promote the development and progression human malignancies [70,71]. Along these lines, TGF-β enhances the tumorigenicity and invasiveness of breast cancer cells by inducing their expression of MMPs 2 and 9 [72,73], which is consistent with the general importance of upregulated MMP expression in mediating the acquisition invasive phenotypes in several cancers [74]. Indeed, aberrant MMP expression (*e.g.*, MMP-7 or matrilysin) facilitates the development of mammary fibrosis and desmoplasia, which increase tumor rigidity and the selection, expansion, and dissemination of metastatic cells [75,76]. Similarly, upregulated MMP-3 expression is sufficient to induce lung and mammary fibrosis [77,78], and to stimulate EMT in carcinomas [79]. Thus, elucidating the connections between aberrant MMP expression and the development of fibrosis and/or EMT will offer important clues as to how EMT promotes cancer progression. For instance, does pathophysiologic EMT solely mediate the acquisition of invasive phenotypes by developing carcinomas, or does this event simply reflect the transdifferentiation of a subset of carcinoma cells into tumor supporting stroma cells (*e.g.*, myofibroblasts) [80]? Indeed, tumor-associated myofibroblasts upregulate their production and secretion of TGF-β, which may serve in establishing a positive feedback loop that drives the selection and expansion of metastatic carcinoma cells [81-83]. Collectively, these findings point to the need for additional studies to fully address these questions, particularly since the expression and activity of MMPs alters the expression of E-cadherin, Snail, vimentin, and TGF-β in a manner consistent with the induction of EMT [79].

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

4.2. Neuronal Cell Adhesion Molecule

Neuronal **c**ell **a**dhesion **m**olecule (NCAM) is a member of the immunoglobulin superfamily and has been implicated as a mediator of tumor progression and metastasis [84]. Recently, TGF-β stimulation of EMT was observed to induce NCAM expression in a manner correlated with downregulated expression E-cadherin [85]. Functionally, upregulated expression of NCAM during EMT facilitates the formation of β1 integrin-containing focal adhesion complexes [85]. Interestingly, the extracellular domain of NCAM is cleaved proteolytically by MMP-28 (epilysin), which also induces EMT through its ability activate latent TGF-β complexes from inactive ECM depots [86]. In addition, MMP-28 expression also is upregulated in a EMT-dependent manner in wounded epithelial cells, and in metastatic breast cancer cells [87]. Thus, future studies need to determine the physiological and pathophysiological connections between NCAM, MMP-28, and TGF-β during the initiation of EMT in normal and malignant epithelial cells.

4.3. Urokinase Plasminogen Activator

Urokinase **p**lasminogen **a**ctivator (uPA) is a serine protease whose elevated expression in human cancer correlates with advanced disease sates and poor clinical outcomes, presumably through its ability to promote cancer cell invasion and metastasis [88,89]. Accordingly, uPA expression is essential for breast and ovarian cancer metastasis in mice [90,91], and for hypoxia-induced EMT in breast cancer cells *via* uPA receptor-mediated activation of AKT and Rac1 [92]. TGF-β is a potent inducer of uPA expression, yet the role of this event in mediating EMT and metastasis stimulated by TGF-β remains to be elucidated fully. Recently, the activation of JNK1/2 was shown to be essential for TGF-β stimulation of uPA expression and EMT [93], which is consistent with the notion that noncanonical TGF-β signaling promotes its oncogenic activities in epithelial cells.

4.4. Plasminogen Activator Inhibitor-1

Plasminogen **a**ctivator **i**nhibitor-1 (PAI-1) is an antagonist of tissue-type plasminogen activator (tPA) and uPA, as well as a physical interactor of the ECM ligand, vitronectin [94,95]. tPA and uPA both activate the serine protease activity of plasminogens (or plasmins), resulting in the degradation of blood plasma proteins, such as fibrin and von Willebrand factor, and of ECM proteins, such as fibronectin, thrombospondin, and laminin [95]. Through its ability to inhibit tPA and uPA, PAI-1 prevents the activation of intravascular and cell-associated plasminogen, and as such, impedes the breakdown of blood clots and ECM proteins necessary to enable carcinoma cells to undergo invasion and extravasation reactions during metastasis [95].

TGF-β is principal player involved in stimulating PAI-1 transcription in part *via* activation of p53, which binds and stabilizes PAI-1 transcripts [96,97]. Quite dichotomously, overexpression of PAI-1 has been observed to reduce the migration and invasion of breast and ovarian cancers [94,98]; however, PAI-1 polymorphisms or its aberrantly elevated expression also have been associated with a poor prognosis and the increased risk of metastasis in breast cancer patients [99]. Thus, the precise mechanisms underlying the dynamic relationship between PAI-1, plasminogen, and TGF-β regulatory loops, as well as their impact on cancer cell motility, remain an active and important topic of investigation.

4.5. Collagen

Collagen is an abundant ECM molecule that assembles into tensilely strong fibers that provide mechanical support to tissues. The major types of collagen, types I-IV, are distributed differentially in specific tissues of the body. For instance, collagen IV is a major component of the basal lamina, a specialized component of the basement membrane in the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

mammary gland. Invading breast cancer cells must degrade collagen IV to migrate into surrounding tissue. Interestingly, Endo180 is a cell surface receptor that promotes the uptake of collagen for its degradation intracellularly. Moreover, Endo180 expression is *(i)* elevated significantly in highly invasive breast cancer cells; *(ii)* induced transcriptionally by TGF-β stimulation in breast cancer cells; and *(iii)* reduced the collagen content and enhanced the growth of mammary tumors produced in mice [100]. In addition, TGF-β also governs collagen function by upregulating the expression of MMP-2 and other collagenases in normal and malignant MECs, leading to their enhanced migration and invasion [72,73,101].

4.6. Fibronectin

Fibronectin is a large and critical ECM glycoprotein whose elevated production by cancer cells classically is associated with the acquisition of EMT, and more recently, with the development of the metastatic niche [67]. TGF-β is a potent inducer of fibronectin production and deposition into the ECM [102], where it binds integrins and regulates cell adhesion and motility. The synthesis and secretion of fibronectin into the ECM is primarily mediated by fibroblasts, and by epithelial cells induced to undergo EMT (Table 1; [103]). With respect to the latter, nontumorigenic EpH4 MECs engineered to express oncogenic Ras (*i.e.*, EpRas cells) significantly upregulate their expression of fibronectin and its receptor, α5β1 integrin when stimulated with TGF-β [104]. More importantly, administration of neutralizing α5 integrin antibodies to TGF-β treated EpRas cells inhibited their migration and induced a significant apoptotic response [104]. Thus, the synthesis and deposition of fibronectin, coupled with changes in expression and activation of integrins (*see below*), clearly represent an important mechanism that enables TGF-β to stimulate invasive migration during EMT.

4.7. Cadherin Switching

A phenotypic hallmark of EMT is its ability to downregulate the expression and function of E-cadherin, which is critical in mediating epithelial cell integrity and cell-cell adhesion [105]. Reduced E-cadherin expression in developing and progressing carcinomas takes places through several mechanisms that function en masse to promote cancer cell invasion [5]. For instance, E-cadherin can be inactivated by genetic mutations, and humans harboring these E-cadherin mutations have significantly increased risk of developing cancer [106]. In addition, epigenetic silencing of the E-cadherin *(CDH1)* promoter *via* hypermethylation of its 5′ CpG island also enhances the development of carcinomas [107]. Along these lines, TGF-β stimulation of EMT also represses the synthesis of E-cadherin transcripts in large part *via* its ability to induce the expression of the Snail/ZEB family of basic helix-loop-helix transcription factors, including that of Snail1, ZEB1, Snail2/Slug, Twist, and ZEB2/SIP1 [105,108-110]. Although the relative contribution of canonical and noncanonical TGF-β signaling in mediating transcriptional activation of these E-cadherin repressors remains to be determined definitely, recent evidence suggests that these events do take place in a cell type-specific manner in response to TGF-β. For example, activation of Smad2/3 by TGF-β in MECs induces their expression of the nuclear high mobility group A2 (HMGA2), which promotes EMT by stimulating the expression of Snail1, Snail2/Slug, and Twist, and by inhibiting the expression of ID2 (inhibitor of differentiation 2) [111]. In addition, while the functional consequences of diminished E-cadherin expression on the behavior of transitioning epithelial cells is well established, recent studies have determined that these same cells also exhibit upregulated expression of N-cadherin (*i.e.*, **N**euronal-cadherin) [65], an event linked to elevated cell motility and poor clinical outcomes in cancer patients [112-114]. At present, the necessity of increased N-cadherin expression in mediating EMT, particularly that stimulated by TGF-β, remains to be clarified. Indeed, we [45] and others [115,116] recently established murine 4T1 breast cancer cells as a model of advanced stage breast cancer whose increased malignancy is governed by TGF-β. Interestingly, while 4T1

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cells undergo EMT and downregulate E-cadherin when stimulated by TGF-β [44,45], these cells fail to express and/or elevate their expression of N-cadherin during EMT initiated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Thus, future studies aimed at determining the exact nature of N-cadherin in promoting the acquisition of EMT and metastatic phenotypes clearly are warranted.

4.8. Vimentin

The intermediate filament protein vimentin is expressed in all primitive cell types, but not in their differentiated counterparts. In light of its role as a master regulator of EMT, it perhaps is not surprising to learn that TGF-β stimulation of EMT reactivates vimentin expression in dedifferentiating epithelial cells, an event that serves as a canonical marker for detecting fully transitioned epithelial cells and their acquisition of fibroblastoid-like phenotype [117].

4.9. α-Smooth Muscle Actin

A major component of contractile microfilaments is α-smooth muscle actin (α-SMA), which also serves as a canonical marker for detecting fibroblasts/mesenchymal cells, particularly myofibroblasts. Indeed, during its induction of EMT, TGF-β stimulates α-SMA expression in transitioning epithelial cells [118], an event associated with increased tumor invasion and decreased patient survival rates [119].

5. TRANSMEMBERANE & MEMBRANE PROXIMAL PROTEIN COMPLEXES THAT IMPACT TGF-β SIGNALING & EMT

Recent evidence suggests that cell surface signaling receptors, such as receptor tyrosine kinases (RTKs) and G-protein-coupled receptors, do not function in isolation and instead require accessory signaling inputs that arise from interacting receptors and signaling modules. As shown in Figure 3, the function and behavior of TGF-β receptors also are modulated *via* their association with an ever expanding array of receptor-interacting molecules and scaffolding proteins. Included in this growing list of TGF-β receptor regulators are members of the integrin superfamily of heterodimeric transmembrane adhesion receptors, which function as direct physical conduits that link the ECM to the cytoskeleton of the cell [120,121]. Integrin signaling commences upon their clustering and subsequent stimulation of the Ser/Thr protein kinase ILK (**i**ntegrin-**l**inked **k**inase), as well as members of the Src family of PTKs and FAK (**f**ocal **a**dhesion **k**inase), leading to the activation of a vast array of downstream effectors, including members of the MAP kinase family of protein kinases, members of the Ras/Rho family of small GTPases, and members of the PI3K and AKT signaling axes [120-124]. Integrins also regulate cell behavior through their ability to form complexes with RTKs [125,126]. For instance, β1 integrins form FAK-dependent complexes with the receptors for EGF, PDGF, and HGF [126,127], and in doing so, enables growth factor-mediated induction of cell migration and invasion [126]. Interestingly, the scaffolding function of FAK is independent of its PTK activity, but does require its N-terminal FERM (**F**AK **E**zrin **R**adixin **M**oesin) domain and C-terminal FAT (**F**ocal **A**dhesion **T**argeting) domain to bind RTKs and β1 integrins, respectively [126]. Lastly, the establishment of EMT phenotypes in cultured cells, as well as the development of late stage cancers and their acquisition of invasive and metastatic phenotypes both have been linked to dramatic changes in the expression and localization of integrins in epithelial cells [104,128].

In addition to its regulation of cell cycle progression, TGF-β also figures prominently in mediating ECM remodeling and repair *via* its ability to regulate integrin expression [129-131]. Moreover, αvβ6 and αvβ8 integrin ligation promotes the activation of TGF-β1 and TGF-β3 from inactive ECM depots [132-137], which regulates alveolar development,

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

wound closure, fibrosis, and EMT [130,132,138-140]. In addition, epidermal transgenic expression of α6β4 integrin also elicits elevated development of metastatic papillomas and carcinomas in a chemical carcinogenesis model of skin cancer. Importantly, the tumorigenicity associated with α6β4 integrin expression was linked to its ability to uncouple TGF-β from activating Smad2/3 and preventing cell cycle progression [141]. Similar reciprocity between integrins and TGF-β is observed in cancers of the prostate, whose metastasis to bone is stimulated by TGF-β and its induction of α2β1 integrin, which binds to bone-derived type I collagen [142]. Thus, the ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins. Accordingly, integrins have been found to associate with TGF-β receptors and play a critical function in coupling TGF-β to activation of its noncanonical effectors, and to its induction of EMT. For instance, neutralizing β1 integrin antibodies abrogated the ability of TGF-β to activate p38 MAPK and induce EMT in MECs [39]. Similarly, hepatocellular carcinoma cells elevate their expression of α3β1 integrin in response to TGF-β, an event that enhances their motility and invasiveness [143]. Moreover, administering laminin-5 together with TGF-β stimulated hepatocellular carcinoma cells to undergo EMT in an α3 integrin-dependent manner [144], further demonstrating the necessity of integrins to cooperate with TGF-β to induce EMT and invasion in transitioning cells.

We also described the functional cooperation between integrins and TGF-β in promoting EMT, as well as in stimulating the development and progression of mammary tumors. For instance, we found the expression and activity of αvβ3 integrin and its downstream effector Src to be essential for TGF-β stimulation of MEC proliferation, invasion, and EMT [43-45]. In addition, transgenic expression of αvβ3 integrin not only negated the cytostatic response of normal MECs to TGF-β, but also enhanced its stimulation of MEC invasion and p38 MAPK activation. Importantly, inactivation of either αvβ3 integrin or Src function abolished the ability of TGF-β to stimulate EMT and invasion in normal and malignant MECs [43,44]. Mechanistically, β3 integrin interacts physically with TβR-II, leading to its *(i)* phosphorylation on Y284 by Src; *(ii)* interaction with Grb2 and Shc at phosphorylated Y284; *(iii)* activation of p38 MAPK; and *(iv)* stimulation of EMT and invasive migration in normal and malignant MECs [44]. Along these lines, the growth and metastasis of breast cancer cells in mice absolutely required TβR-II to be phosphorylated on Y284, a phosphotransferase reaction that disrupts the delicate balance between canonical and noncanonical TGF-β signaling inputs activated during mammary tumorigenesis [45]. In addition to its ability to promote pulmonary metastasis stimulated by TGF-β [45], αvβ3 integrin expression also directs breast cancer cell metastasis to bone [145,146] and lung [146] in part through a TGF-β-dependent pathway. Collectively, these findings suggest that pharmacological targeting of noncanonical TGF-β effectors, particularly αvβ3 integrin, Src, and p38 MAPK, may prove efficacious in treating metastatic breast cancers.

Besides integrins, a growing number of intracellular proteins also have been shown to interact with and regulate the activity of TGF-β receptors. For instance, two members of the focal adhesion complex, namely FAK and its downstream effector p130Cas (p130**C**rk-**a**ssociated **s**ubstrate), both influence the cellular response to TGF-β through dramatically different mechanisms. Indeed, TGF-β stimulates FAK and its relative PYK2 during EMT [147], leading to the activation of JNK and the subsequent upregulation α-SMA in fibroblasts [148]. In addition, FAK activation in hepatocytes is necessary for the transcription of mesenchymal and invasive gene expression profiles, as well as for the delocalization of E-cadherin from the plasma membrane [149]. Finally, we recently established FAK as a molecular scaffold that facilitates the formation of oncogenic β3 integrin:TβR-II complexes and their activation of Src and interaction with Grb2 (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Moreover, the ability of FAK to

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

form these signaling complexes is essential for TGF-β stimulation of p38 MAPK in breast cancer cells, as well as for their induction of EMT and metastasis stimulated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*; [45]). Thus, the aberrant recruitment of FAK to TGF-β receptors readily influences the oncogenic conversion of TGF-β from a tumor suppressor to a tumor promoter, including its stimulation of pathophysiological EMT in carcinoma cells.

In stark contrast to FAK, the incorporation of p130Cas into active TGF-β receptor complexes alters the coupling of TGF-β to the canonical Smad2/3 pathway. Indeed, the activation and phosphorylation of p130Cas following cellular adhesion to ECM matrices led to its association and inactivation of Smad3, and to diminished cytostatic activity of TGF-β [150]. Similarly, we find that rendering malignant, metastatic MECs deficient in p130Cas enhances Smad2/3 activation by TGF-β, but fails to alter its coupling to p38 MAPK; however, this same cellular condition selectively inhibited breast cancer metastasis only in cells that possessed heightened TGF-β signaling (M.K. Wendt and W.P. Schiemann, *unpublished observation*), suggesting that p130Cas acts as a molecular integrator of canonical Smad2/3 signaling when confronted with elevated oncogenic behavior mediated by the receptors for TGF-β or EGF [151].

Recently, the regulation of TGF-β signaling has been shown to be modulated by two additional adapter proteins that localize to focal adhesions, namely Hic5 and Disabled-2 (Dab2). Indeed, Hic5 is a member of the paxillin superfamily and, like paxillin, functions as an adapter protein at focal adhesions [152], as well as resides in the nucleus where functions as a transcriptional coactivator in regulating gene expression induced by the androgen [153] and glucocorticoid [154,155]. Moreover, Hic5 expression is low in quiescent MECs, but is induced rapidly *via* a RhoA/ROCK-dependent pathway following administration of TGF-β [152]. In addition, uncoupling Hic5 from TGF-β regulation prevents its induction of EMT in normal MECs [156]. Thus, Hic5 plays an essential role in coupling TGF-β receptors to activation of RhoA/ROCK and, consequently, to the induction of EMT. Along these lines, Dab2 was identified originally as an ovarian tumor suppressor gene [157,158] that regulates the actin cytoskeletal architecture during cell migration and adhesion [159]. More recently, Prunier *et al* [160] established Dab2 as a novel gene target of TGF-β in MECs undergoing EMT in part *via* its ability to *(i)* associate with TGF-β receptor complexes [26]; *(ii)* promote Smad2/3 activation by TGF-β receptors [26]; and *(iii)* stimulate the activation of TAK1 and JNK, which induced fibronectin expression and enhanced cell motility [161]. Along these lines, TGF-β stimulates Dab2 expression in MECs undergoing EMT, which promotes the formation of Dab2:β1 integrin complexes and their activation of FAK [160]. Importantly, measures capable of disrupting Dab2 function prevents EMT stimulated by TGF-β, as well as promotes its ability to induce apoptosis in MECs. Although the molecular mechanisms underlying the ability of TGF-β to stimulate Dab2 expression remains to be defined, these studies do provide interesting insights into the connections that govern alterations in cell survival and morphology regulated by TGF-β.

Finally, two laboratories recently identified a novel collaboration between signaling molecules activated by TNF-α and those activated by TGF-β. Indeed, both studies demonstrated the ability of TGF-β to induce the physical association of its receptors with that of TRAF6 (**T**NF **r**eceptor-**a**ssociated **f**actor 6) [162,163], leading to K63-linked polyubiquitination and activation of TAK1 and its subsequent stimulation of p38 MAPK and JNK. Moreover, whereas TRAF6-deficiency had no effect on the coupling of TGF-β to Smad2/3, this same cellular condition uncoupled TGF-β from activation of MAP kinases and prevented this cytokine from inducing EMT in normal MECs [163]. Taken together, these studies reinforce the notion that imbalances in the TGF-β signaling system that favor its activation of noncanonical effectors over that of its canonical Smads are crucial to its

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

induction of EMT in normal and malignant epithelial cells. These findings also point to the need for additional studies to define precisely how these aberrant protein complexes and modules impact the epithelial cell response to TGF-β, and how science and medicine can better target these effector molecules that promote oncogenic signaling and EMT initiation by TGF-β.

6. SIGNALING SYSTEMS INVOLVED IN EMT STIMULATED BY TGF-β

Transmembrane signaling by TGF-β traditionally is associated with its activation of Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which clearly play an important role in mediating the ability of TGF-β to induce EMT, tumor formation, and cancer cell metastasis [164]. The necessity of Smads 2 and 3 for TGF-β stimulation of EMT has been reviewed extensively in the scientific literature, and readers desiring a more in depth description of Smad2/3 function in regulating EMT in normal and malignant cells are directed to several recent reviews [4,5,11]. As alluded to above, the enhanced coupling of TGF-β to its noncanonical effectors figures prominently in mediating its biological and pathological behaviors, particularly its ability to induce EMT and cancer cell metastasis. Table 2 lists a variety of noncanonical effectors targeted by TGF-β during its activation of EMT, while the role of these signaling molecules during epithelial cell EMT induced by TGF-β is discussed below.

6.1. Rho Family of Small GTPases

The Rho family of small GTPases is comprised of RhoA, Rac1, and Cdc42, which regulate the formation of stress fibers, lamellipodia, or filopodia, respectively [165,166]. Indeed, Rac1 is an established inducer of cell-cell adhesions in epithelial cells [167], which contrasts sharply with the ability of RhoA to dissolve these adhesive complexes to facilitate times of cell migration [62]. Given the importance of these small GTPases in overseeing cell adhesion, morphology, and migration, it is fitting to find that these effectors are intimately involved in EMT stimulated by TGF-β. For instance, the activation of RhoA by TGF-β enables MECs to undergo EMT, while measures capable of inhibiting RhoA function or that of its downstream effector, $p160^{ROCK}$, uncouple TGF-β from EMT in MECs [65]. Moreover, RhoA activation also is essential for TGF-β stimulation of α-SMA expression in renal epithelial cells undergoing EMT [118]; however, completion of this same cellular event in lens epithelial cells requires signaling inputs from both RhoA/ROCK and Smad2/3 [168]. Taken together, these studies point to the overall importance of noncanonical TGF-β signaling, particularly that induced by RhoA/ROCK, to induce EMT in epithelial cells.

6.2. PI3K/AKT

The tumor suppressing activity of TGF-β not only reflects its ability to induce cytostasis, but also its propensity to activate apoptosis in epithelial cells [10,11,13,169]. Interestingly, the ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems by TGF-β. Indeed, administration of PI3K inhibitors to MECs inhibits their activation of AKT and ability to undergo EMT in response to TGF-β [38]. The activation of AKT by TGF-β can transpire directly *via* TGF-β receptors or indirectly *via* the transactivation of EGF [170] and PDGF [171] receptors, which induces the expression of genes operant in mediating cancer cell EMT, metastasis, and survival. In addition to altering gene expression profiles, AKT also regulates mRNA translation when impacting the response of epithelial cells to TGF-β. For instance, TGF-β stimulation of EMT in MECs is accompanied by an increase in cell size and protein content, both of which correlate with the rapid activation of mTOR (**m**ammalian **t**arget **o**f **r**apamycin) in transitioning MECs [40]. Somewhat unexpectedly, administering the mTOR inhibitor, rapamycin, to MECs failed to affect their acquisition of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

an EMT morphology in response to TGF-β; however, this same cellular condition completely prevented the ability of TGF-β to increase MEC size and protein production, as well as inhibited their migration and invasion [40]. Taken together, these findings highlight an important bifurcation in the TGF-β signaling system that dissociates the ability of TGF-β to alter cell morphology from its ability to elevate cell motility. Future studies need to identify the transcriptional and translational objectives targeted by TGF-β, as well as determine their relative contribution to oncogenic signaling stimulated by TGF-β in normal and malignant cells.

6.3. Integrin-linked Kinase (ILK)

In addition to their stimulation of PTKs, the ECM engagement of β1 and β3 integrins also activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, PI3K/AKT, and small GTPases; and *(ii)* the inhibition of GSK3β [172-174]. Accordingly, targeting ILK expression to mouse mammary glands elicited a hyperplastic reaction that progressed to full-blown breast cancer in part *via* constitutive activation of ERK1/2 and AKT, which inactivated GSK3β [175]. Elevated ILK expression is associated with the acquisition of EMT phenotypes by MECs, including reductions in their expression of E-cadherin and adhesion, as well as increases in their formation of actin stress fibers and invasion [176]. ILK also participates in EMT stimulated by TGF-β by coupling this cytokine to its activation of AKT [177], and to its elevated expression of MMP-2 and uPA [91]. Collectively, these findings suggest that ILK may function analogously to FAK in mediating oncogenic signaling by TGF-β, and as such, suggest that ILK interfaces integrin signaling with that stimulated by TGF-β in epithelial cells undergoing EMT.

6.4. NF-κB

NF-κB is a principal player involved in regulating the production of proinflammatory cytokines [178], and in stimulating tumor growth, vascularization, survival, and invasion [178]. In addition, NF-κB activity was observed to be essential in mediating the ability of Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β [48,179]. Along these lines, NF-κB activity also associates with several hallmarks of EMT, including downregulated E-cadherin expression and upregulated expression of vimentin [180]. It is interesting to note that TGF-β typically represses NF-κB activity in normal epithelial cells [47,181,182], but readily induces the activation of this transcription factor in their malignant counterparts [47,182]. Recently, we demonstrated that the activation of NF-κB by TGF-β transpires *via* the aberrant formation of a TAB1:xIAP:TAK1:IKKβ signaling module that only materializes in malignant MECs, or in normal MECs following their induction of EMT by TGF-β [47]. Functionally, the formation of TAB1:xIAP:TAK1:IKKβ complexes is essential for TGF-β stimulation of *(i)* Cox-2 expression and its induction of EMT and invasion in normal and malignant MECs [47,182]; and *(ii)* mammary tumor growth in immunocompetent and immunocompromised mice [47], suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β. Collectively, these findings demonstrate the role of NF-κB in supporting the development of oncogenic signaling by TGF-β in normal and malignant cells, particularly its ability to drive the growth, metastasis, and EMT of tumors in response to TGF-β.

6.5. MAP Kinases

Members of the MAP kinase family of protein kinases, which includes ERK1/2, JNKs, and p38 MAPKs, all have been implicated in mediating EMT and metastasis stimulated by TGF-β [39,183,184]. For instance, pharmacological inhibition of ERK1/2 in MECs uncouples TGF-β from inducing EMT and its associated formation of stress fibers and delocalization of ZO-1 and E-cadherin [183]. Similarly, inactivation of JNK also prevents the ability of TGF-β to stimulate the morphological and transcriptional changes that drive EMT in epithelial

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cells [93,161]. Indeed, the activation of JNK by TGF-β induces fibronectin expression during EMT, and during fibroproliferative disorders that may progress to carcinoma [185]. Along these lines, collagen I and other ECM proteins can promote EMT *via* their activation of PI3K, Rac1, and JNK [186]; however, while it remains to be determined whether TGF-β is intimately involved in this ECM-dependent induction of EMT, it seems likely that the ability of TGF-β to stimulate the synthesis and secretion of ECM components is reciprocated by the ability of the ECM to establish paracrine and autocrine TGF-β signaling loops that perpetuate EMT in normal and malignant epithelial cells.

Besides its ability to activate ERK1/2 and JNK, TGF-β also stimulates p38 MAPK during its induction of EMT in normal and malignant cells [39]. Interestingly, the activation of p38 MAPK by TGF-β requires the expression and activity of either β1 or β3 integrins [39,43,44]. Indeed, we established the necessity of β3 integrin to form oncogenic signaling complexes with TβR-II, resulting in its phosphorylation on Y284 by Src [43,44]. Once phosphorylated, Y284 functions as a SH2-binding site that coordinates the recruitment of either ShcA or Grb2, as well as their subsequent activation of p38 MAPK [44]. Most importantly, pharmacologic or genetic inactivation of this oncogenic signaling axis prevented TGF-β from stimulating the growth and pulmonary metastasis of breast cancers produced in mice [45]. Finally, the activation of p38 MAPK not only induces EMT, but it also stimulates the expression of prometastatic genes, particularly TβR-II and MMPs 2 and 9 [187,188], which collectively points to the importance of inappropriate p38 MAPK activation in mediating the conversion of TGF-β from a tumor suppressor to a tumor promoter.

7. MECHANISMS OF GENE REGULATION BY TGF-β

The importance of aberrant genetic and epigenetic events in promoting tumorigenesis is highlighted by the consistent and repeated finding that cancer cells that have lost their ability to regulate various rate-limiting steps that normally suppress malignant development. These untoward events typically are associated with the *(i)* mutational activation of oncogenes, *(ii)* mutational inactivation of tumor suppressor genes, or *(iii)* amplified or silenced expression of genes coupled to the development of cancer hallmarks [189]. Although many of the signaling systems and genes targeted by TGF-β during its activation of EMT have been discussed above, the succeeding sections focus on the transcriptional mechanisms that orchestrate its transitioning of epithelial cells into their mesenchymal counterparts (Figure 3).

7.1. Nuclear Factors

The Snail family of transcription factors are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* two ZEB factors, ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2 [110,190]. Indeed, the binding of Snail to conserved E-box sequences present in E-cadherin promoter is classically associated with EMT and the repression of E-cadherin expression, as well as that of the aforementioned cell polarity genes, occludin and claudin [191]. The essential function of various Snail family members in mediating EMT and cancer metastasis have been extensively reviewed, and as such, readers desiring a more in-depth description of their functions and behaviors in governing EMT are directed several recent reviews [109,190]. Besides Snail family members, emerging evidence also implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. Indeed, the tumor suppressing activity of TGF-β is intimately linked to its ability to rapidly repress Myc expression in epithelial cells [11,13]. Accordingly, uncoupling TGF-β from regulation of Myc expression is a common occurrence in developing carcinomas, resulting in their insensitivity to cytostasis mediated by TGF-β [192,193]. Somewhat unexpectedly, Myc recently was observed to function cooperatively with Smad4 to induce Snail expression during TGF-β stimulation of EMT in MECs [194].

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Taken together, these findings suggest the Myc functions as a molecular detector that enables epithelial cells to sense TGF-β as a mediator of cytostasis or EMT.

7.2. STAT3

Signal **t**ransducer and **a**ctivator of **t**ranscription 3 (STAT3) is a critical component of cell survival and proliferative responses, and its inappropriate activation can endow this transcription factor with oncogene-like properties in developing and progressing neoplasms [195]. A recent study has suggested that TGF-β couples to STAT3 phosphorylation and activation *via* a PKA-dependent mechanism [196]. Moreover, STAT3 activation by TGF-β is necessary for its ability to induce apoptosis and EMT [196], and to stimulate the invasion and metastasis of Smad4-deficient pancreatic cancer cells [197]. In addition, carcinoma cells that overexpressed EGFR readily acquired EMT phenotypes when stimulated with EGF, a cellular reaction that required EGF/EGFR to activate STAT3 and its subsequent upregulation of TWIST [198]. Thus, while several studies have shown EGF to cooperate with TGF-β in mediating tumorigenesis, the extent to which this tumor-and EMT-promoting alliance requires STAT3 remains to be determined definitively.

7.3. Estrogen Receptor-α

Aberrant repression of the nuclear hormone receptor, estrogen receptor-α ER-α) has long been recognized as a major event that promotes the development and progression of mammary tumors, as well as significantly worsens the clinical prognosis of patients with metastatic breast cancer [199,200]. In addition to its prominent role in regulating mammary gland development and homeostasis, ER-α also prevents the ability of malignant MECs to acquire EMT and metastatic phenotypes, doing so *via* its stimulation of MTA3 (metastasis tumor antigen 3) expression, which in turn represses the expression of Snail [201]. Thus, inactivation or loss of ER-α in MECs promotes their EMT and invasion by allowing for their expression of Snail. Somewhat surprisingly, constitutive Snail expression in breast cancer cells was observed to inhibit ER-α expression [202], leading to enhanced invasion of these ER-α-deficient breast cancer cells. It is interesting to note that physiological actions of estrogen in mammary tissues typically oppose those of TGF-β. Accordingly, inactivation of ER-α signaling led to elevated expression of components of the TGF-β signaling system and, presumably, to enhanced EMT in breast cancer cells [202]. Thus, Snail appears to function as a novel molecular sensor that integrates the opposing cellular functions of ER-α and TGF-β, particularly their ability to inhibit and stimulate EMT, respectively.

7.4. TGF-β, microRNAs, & EMT

A number of recent studies have established microRNAs as important players that participate in cell and tissue development, as well as control cell proliferation and motility through their ability to repress mRNA translation, or to induce mRNA degradation [203-206]. These studies also have shown that a single microRNA can repress the translation of multiple transcripts, and as such, dysregulated expression of a single microRNA, either positively or negatively, could initiate a cascade of gene silencing events capable of eliciting disease development in humans, including cancer. Accordingly, aberrant regulation of several microRNAs (or miRs) is observed in human cancers (see [207]), especially in those of the breast, which can in fact be subtyped based on their differential expression of various microRNAs [205,208]. Along these lines, microRNAs also play a prominent role in regulating the expression of EMT-related genes. For instance, members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2 (SIP1), which as mentioned above function in repressing the expression of E-cadherin [209-211]. Indeed, miR-200 family member expression marks epithelial cells that express E-cadherin and not vimentin, as well as identifies cancer cells that are poorly motile [212]. With respect to EMT and its regulation by TGF-β, a recent study established that this

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cytokine downregulates the expression of microRNA-200 family members and miR-205, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [211]. In addition, these same microRNAs are frequently downregulated in invasive human breast cancer cells that exhibit a mesenchymal-like morphology [211]. Somewhat surprisingly, elevated ZEB1 expression also was found to repress that of miR-41 and miR-200c, both of which belong to the miR-200 family and whose absence establishes a negative feedback loop that stabilizes the acquisition of EMT phenotypes in epithelial cells [213].

In contrast to the miR-200 family of microRNAs, metastatic breast cancers were found to preferentially upregulate their expression of miR-10b, which promotes the invasion and metastasis of malignant MECs both *in vitro* and *in vivo* [214]. Mechanistically, Twist was observed to induce miR-10b expression that results in the *(i)* diminished translation of HoxD10 transcripts, and *(ii)* induction of the prometastatic gene, RhoC [214]. More recently, administration of TGF-β to normal MECs induced miR-155 expression *via* a Smad4-dependent mechanism, an event that elicited EMT in cytokine-stimulated MECs [215]. Once expressed, miR-155 abrogated MEC expression of RhoA and prevented their ability to undergo EMT in response to TGF-β [215]. Similar overexpression of miR-21 also is observed in human cancers and results in the repression of the tumor suppressor, tropomyosin-1 [216,217]. The net effect of these events is the enhanced ability of breast cancer cells to grow in an anchorage-independent fashion [218], and to resist apoptotic stimuli in part *via* upregulated expression of the survival factor, Bcl-2 [216-218]. As above, the ability of TGF-β to induce EMT has been linked to its induction of miR-21 [219], which enhances cancer cell motility and invasive migration by downregulating tropomyosin expression [220-222].

Taken together, these findings suggest that the ability of TGF-β to govern microRNA expression plays an important role in dictating whether this cytokine propagates tumor suppressing or promoting signals to responsive cells; they also suggest that the development of chemotherapeutic agents capable of targeting microRNAs may function in “normalizing” carcinoma cells and, consequently, rendering them insensitive to the oncogenic activities of TGF-β.

7.5. DNA Hypermethylation

DNA hypermethylation is well established in its ability to aberrantly silence the expression of tumor suppressor genes in developing and progressing carcinomas [107]. Importantly, epigenetic silencing of the E-cadherin promoter *via* hypermethylation leads to morphological and differential gene expression profiles indicative of EMT phenotypes [107,223]. Besides silencing of the E-cadherin promoter, EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to promote expanded DNA hypermethylation. Indeed, Roberts *et al* [224] observed the loss of p16INH4a expression to depress that of the polycomb genes, EZH2 and SUZ12, which collectively enhance DNA hypermethylation and the generation of MECs locked into a perpetual plastic state. Interestingly, the repression of E-cadherin expression during EMT appears to function as a prerequisite for directed gene hypermethylation during the development and progression of mammary tumorigenesis [225]. Moreover, hypermethylation of the E-cadherin promoter served to mark stable EMT in Ras-transformed MECs that was induced by serum *versus* a transient EMT induced in these same MECs by TGF-β [225]. Clearly, additional investigations are warranted to further our understanding of the linkages between TGF-β and DNA hypermethylation in mediating EMT in normal and malignant cells. Indeed, upregulated ZEB1 expression and its ability to induce EMT is tightly correlated with the loss of E-cadherin expression in cultured epithelial cells, and in metastatic carcinoma cells *in vivo* [226]. Based on these findings, it is tempting to speculate that initiation of EMT results in *(i)* the expression of Snail family members that collectively function in repressing

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

that of E-cadherin, and *(ii)* the subsequent recruitment of DNA methyltransferases that potentiate and stabilize the EMT phenotype.

8. FUTURE PERSPECTIVE

Embryogenesis and its associated EMT creates progenitor cells that ultimately give rise to every cell- and tissue-type within mature organisms. For instance, EMT underlying gastrulation results in the generation of the mesoderm, which subsequently develops along distinct differentiation pathways that elicit the production of muscle, bone, and connective tissues [7]. Similarly, a single mammary stem cell can give rise to both the outer myoepithelial and inner luminal layers that comprise the branched structure of these glands[227-229]. These and other studies suggest an important link between physiologic EMT and the generation and maintenance of stem cells, of which both phenomena require signaling inputs elicited by the TGF-β signaling system [230]. Given the parallels between physiologic and pathophysiologic EMT, it is fitting to find that the inappropriate reactivation of EMT in malignant tissues also promotes the selection and expansion of cancer stem cells. For instance, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [231]. In addition, TGF-β stimulation of EMT in human and mouse MECs established a population of transitioning cells that possessed stem cell-like properties [232,233], suggesting that EMT induced by TGF-β promotes “stemness.” Along these lines, inactivation of TGF-β signaling in cancer stem cells induced a mesenchymal-epithelial transition that reestablished a more epithelial-like morphology in aggressive cancer cells [234]. Thus, these intriguing findings suggest that the ability of TGF-β to stimulate the selection and expansion of stem cell-like progenitors in post-EMT epithelial cells may represent the molecular crux that endows TGF-β with oncogenic activity. Clinically, these findings also suggest that the development of chemoresistance may reflect the induction of EMT and its expansion of cancer stem cells by TGF-β. If correct, then the studies reviewed herein offer important insights into how science and medicine may one day target the TGF-β signaling system and its coupling to EMT in order to *(i)* regulate the behaviors and activities of normal and cancer stem cells, and *(ii)* alleviate the devastating effects of TGF-β in promoting the acquisition of invasive and metastatic phenotypes in human cancers.

Executive Summary

Defining EMT

- EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells.
- The major cell-cell junctions include tight junctions, adherens junctions, and desmosomes.
- Tight junctions are composed of claudins, occludins, and JAMs, which are linked to the actin cytoskeleton *via* ZO-1, −2, and −3.
- During EMT, Par6 recruits the E3 ubiquitin ligase, Smurf1, which ubiquitinates RhoA, leading to its degradation and subsequent dissolution of tight junctions.
- Adherens junctions consist E-cadherin that is linked to the actin cytoskeleton by α- and β-catenins.
- EMT represses E-cadherin transcription and disrupts its localization at the plasma membrane.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

EMT, TGF-β, and Cell Microenvironments

- Tumors house a mixture of malignant and normal cells, including fibroblasts, endothelial, and infiltrating immune cells, which collectively comprise the tumor microenvironment.
- Alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β.
- Transient disruption of the ECM and epithelial cell microenvironments are characteristic of physiological EMT. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis.
- MMPs comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, wound healing, and cell growth. MMPs also degrade nearly all ECM and basement membrane components, leading to the development and progression human malignancies.
- NCAM is a member of the immunoglobulin superfamily whose expression is increased during EMT.
- The extracellular portion of NCAM is cleaved by MMP-28.
- uPA is a serine protease whose expression is elevated during EMT and associates with advanced disease states and poor clinical outcomes.
- PAI-1 antagonizes tPA and uPA; its expression also is increased during EMT and associates with advanced disease states and poor clinical outcomes.
- EMT leads to the upregulation of collagen and fibronectin, whose expression drastically alters cell microenvironments.
- Vimentin is an intermediate filament protein, while α-smooth muscle actin is a component of contractile microfilaments. Upregulated expression of both proteins are considered to be markers of fully transitioned cells.

Transmembrane and Membrane Proximal Protein Complexes the Impact TGF-β Signaling and EMT

- α and β integrin heterodimers function in linking the ECM to intracellular signaling pathways, and to the cellular cytoskeletal system.
- Integrins interact with several intracellular kinases, as well as several transmembrane RTKs.
- Integrin β1 and β3 interact with TβR-II and profoundly affect downstream signaling events stimulated by TGF-β.
- β3 integrin is upregulated dramatically during EMT induced by TGF-β.
- Interaction between αvβ3 integrin and TβR-II leads to Src-mediated phosphorylation of TβR-II at Tyr284, which binds Grb2 and promotes the activation of downstream MAP kinases.
- The ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins.
- FAK is required for EMT stimulated by TGF-β.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

- p130Cas inhibits Smad3 activity and alters cytostasis induced by TGF-β.
- Hic5 is a member of the paxillin superfamily that is induced by and required for EMT stimulated by TGF-β.
- TRAF6 interacts physically with both TβR-I and TβR-II, leading to TAK1 activation and the stimulation of p38 MAPK and JNK.

Signaling Systems Involved in EMT Stimulated by TGF-β

- Transmembrane signaling by TGF-β activates Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which play important roles during TGF-β stimulation of cancer cell EMT and metastasis.
- Small GTPases RhoA, Rac1, and Cdc42 regulate the formation of stress fibers, lamellipodia, or filopodia, respectively, and are intimately involved in EMT stimulated by TGF-β.
- The ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems.
- ECM engagement of β1 and β3 integrins activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, and *(ii)* the inhibition of GSK3β, PI3K/AKT, and small GTPases.
- ILK participates in EMT stimulated by TGF-β by coupling this cytokine the activation of AKT, and to the elevated expression of MMP-2 and uPA.
- NF-κB activity enables Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β.
- Activation of NF-κB also associates with several hallmarks of EMT, including the downregulated expression of E-cadherin and upregulated expression of vimentin.
- MAP kinase family members, including ERK1/2, JNK, and p38 MAPK, have all been implicated in mediating EMT and metastasis stimulated by TGF-β.

Mechanisms of Gene Regulation by TGF-β

- Aberrant genetic and epigenetic events promote tumorigenesis by circumventing various rate-limiting cellular steps that normally suppress neoplastic development. These key steps are known as the “Hallmarks of Cancer” and include *(i)* oncogene activation; *(ii)* tumor suppressor inactivation; *(iii)* apoptosis resistance; *(iv)* angiogenesis activation; *(v)* invasion and metastasis initiation; and *(vi)* immortality acquisition.
- Snail transcription factor family members are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2.
- Evidence implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. The tumor suppressing activity of TGF-β is intimately linked to its repression of Myc expression in epithelial cells.
- STAT3 mediates cell survival and proliferative signals, and serves as an oncogene in several human cancers.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

- TGF-β activates STAT3 *via* a PKA-dependent mechanism, leading to the induction of EMT.
- ER-α promotes mammary gland develop and homeostasis, and suppresses EMT by inducing the expression of MTA3 (metastasis tumor antigen 3), which represses the expression of Snail.
- microRNAs are essential mediators of all facets of cell and tissue development, and of cell proliferation, motility, and survival.
- Members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2.
- Epigenetic silencing of the E-cadherin promoter *via* hypermethylation promotes the acquisition of EMT phenotypes and gene expression profiles.
- EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to expand aberrant DNA hypermethylation.

Redefining EMT Induced by TGF-β

- Inappropriate reactivation of EMT by TGF-β in malignant tissues promotes the selection and expansion of cancer stem and progenitor cells.
- Targeting the molecular links between TGF-β, EMT, and stemness reduces breast cancer tumorigenicity.
- The development of pharmacological agents that inhibit EMT stimulated by TGF-β may provide new avenues to manipulate the behaviors of normal and cancer stem cells, and to alleviate the acquisition of cancer metastasis.

Acknowledgments

We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA114039 and CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651); M.K.W. was supported by the American Cancer Society (PF-09-120-01-CS); and T.M.A was supported by the Department of Defense (BC083323).

REFERENCES

1. Baum B, Settleman J, Quinlan MP. Transitions between epithelial and mesenchymal states in development and disease. Semin. Cell Dev. Biol 2008;19:294–308. [PubMed: 18343170]
2. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell 2008;14:818–829. [PubMed: 18539112]
3. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp. Cell Res 2001;264:169–184. [PubMed: 11237532]
4. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007;98:1512–1520. [PubMed: 17645776]
5. Zavadil J, Bottinger EP. TGF-β and epithelial-to-mesenchymal transitions. Oncogene 2005;24:5764–5774. [PubMed: 16123809]
6. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr. Opin. Cell Biol 2003;15:740–746. [PubMed: 14644200]
7. Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech, Dev 2003;120:1351–1383. [PubMed: 14623443]
8. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cance 2002;2:442–454.
9. Willis BC, Borok Z. TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. Am. J. Physiol. Lung Cell. Mol. Physiol 2007;293:L525–534. [PubMed: 17631612]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

10. Blobe GC, Schiemann WP, Lodish HF. Role of TGF-β in human disease. N. Engl. J. Med 2000;342:1350–1358. [PubMed: 10793168]

11. Galliher AJ, Neil JR, Schiemann WP. Role of TGF-β in cancer progression. Future Oncol 2006;2:743–763. [PubMed: 17155901]

12. Massague J, Gomis RR. The logic of TGF-β signaling. FEBS Lett 2006;580:2811–2820. [PubMed: 16678165]

13. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat. Rev. Cancer 2003;3:807–821. [PubMed: 14557817]

14. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001;23:912–923. [PubMed: 11598958]

15. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J. Cell Biol 1994;127:2021–2036. [PubMed: 7806579]

*16. Kaartinen V, Voncken JW, Shuler C, et al. Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nature Genetics 1995;11:415–421. [PubMed: 7493022] Study established the critical role for TGF-β3 in regulating developmental EMT

17. Romano LA, Runyan RB. Slug is an essential target of TGF-β2 signaling in the developing chicken heart. Dev. Biol 2000;223:91–102. [PubMed: 10864463]

18. Saika S, Kono-Saika S, Ohnishi Y, et al. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am. J. Pathol 2004;164:651–663. [PubMed: 14742269]

19. Saika S, Kono-Saika S, Tanaka T, et al. Smad3 is required for dedifferentiation of retinal pigment epithelium following retinal detachment in mice. Lab. Invest 2004;84:1245–1258. [PubMed: 15273699]

20. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF-β 1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J. Clin. Invest 2003;112:1486–1494. [PubMed: 14617750]

21. Feng XH, Derynck R. Specificity and versatility in TGF-β signaling through Smads. Annu. Rev. Cell Dev. Biol 2005;21:659–693. [PubMed: 16212511]

22. Moustakas A, Heldin CH. Non-Smad TGF-β signals. J. Cell Sci 2005;118:3573–3584. [PubMed: 16105881]

23. Shi Y, Massague J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 2003;113:685–700. [PubMed: 12809600]

*24. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGF-β receptor. Cell 1998;95:779–791. [PubMed: 9865696] Identified SARA as a novel adapter molecule that facilitates Smad2/3 recruitment and activation by TGF-β receptors

25. Miura S, Takeshita T, Asao H, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol. Cell Biol 2000;20:9346–9355. [PubMed: 11094085]

26. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the TGF-β receptors to the Smad pathway. EMBO J 2001;20:2789–2801. [PubMed: 11387212]

*27. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGF-β receptor and functions as an antagonist of TGF-β signaling. Cell 1997;89:1165–1173. [PubMed: 9215638]

*28. Nakao A, Afrakht M, Moren A, et al. Identification of Smad7, a TGF-β-inducible antagonist of TGF-β signalling. Nature 1997;389:631–635. [PubMed: 9335507] Together with Ref [27], identified Smad7 as an inhibitory molecule of the TGF-β signaling system

29. Souchelnytskyi S, Nakayama T, Nakao A, et al. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and TGF-β receptors. J. Biol. Chem 1998;273:25364–25370. [PubMed: 9738003]

30. Ebisawa T, Fukuchi M, Murakami G, et al. Smurf1 interacts with TGF-β type I receptor through Smad7 and induces receptor degradation. J. Biol. Chem 2001;276:12477–12480. [PubMed: 11278251]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

31. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol. Cell 2000;6:1365–1375. [PubMed: 11163210]

32. Datta PK, Moses HL. STRAP and Smad7 synergize in the inhibition of TGF-β signaling. Mol. Cell. Biol 2000;20:3157–3167. [PubMed: 10757800]

33. Ibarrola N, Kratchmarova I, Nakajima D, et al. Cloning of a novel signaling molecule, AMSH-2, that potentiates TGF-β signaling. BMC Cell Biol 2004;5:2. [PubMed: 14728725]

34. Koinuma D, Shinozaki M, Komuro A, et al. Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7. EMBO J 2003;22:6458–6470. [PubMed: 14657019]

35. Liu FY, Li XZ, Peng YM, Liu H, Liu YH. Arkadia-Smad7-mediated positive regulation of TGF-β signaling in a rat model of tubulointerstitial fibrosis. Am. J. Nephrol 2007;27:176–183. [PubMed: 17347560]

36. Liu W, Rui H, Wang J, et al. Axin is a scaffold protein in TGF-β signaling that promotes degradation of Smad7 by Arkadia. EMBO J 2006;25:1646–1658. [PubMed: 16601693]

*37. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGF-β-mediated fibroblastic transdifferentiation and cell migration. J. Cell Sci 2002;115:3193–3206. [PubMed: 12118074]

*38. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for TGF-β-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem 2000;275:36803–36810. [PubMed: 10969078]

*39. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin β1 signaling is necessary for TGF-β activation of p38MAPK and epithelial plasticity. J. Biol. Chem 2001;276:46707–46713. [PubMed: 11590169]

*40. Lamouille S, Derynck R. Cell size and invasion in TGF-β induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J. Cell Biol 2007;178:437–451. [PubMed: 17646396]

*41. Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. TGF-β-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat. Cell Biol 2001;3:708–714. [PubMed: 11483955]

*42. Zavadil J, Bitzer M, Liang D, et al. Genetic programs of epithelial cell plasticity directed by TGF-β. Proc. Natl. Acad. Sci. USA 2001;98:6686–6691. [PubMed: 11390996]

*43. Galliher AJ, Schiemann WP. β3 integrin and Src facilitate TGF-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 2006;8:R42. [PubMed: 16859511]

44. Galliher AJ, Schiemann WP. Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res 2007;67:3752–3758. [PubMed: 17440088]

45. Galliher-Beckley AJ, Schiemann WP. Grb2 binding to Tyr284 in TGF-β is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis 2008;29:244–251. [PubMed: 18174260]

46. Azuma M, Motegi K, Aota K, Yamashita T, Yoshida H, Sato M. TGF-β1 inhibits NF-κB activity through induction of IκBα expression in human salivary gland cells: a possible mechanism of growth suppression by TGF-β1. Exp. Cell Res 1999;250:213–222. [PubMed: 10388535]

*47. Neil JR, Schiemann WP. Altered TAB1:IκB kinase interaction promotes TGF-β-mediated NF-κB activation during breast cancer progression. Cancer Res 2008;68:1462–1470. [PubMed: 18316610] Together with References [37]-[42], these studies established various noncanonical TGF-β effectors as critical mediators of EMT and oncogenic signaling stimulated by TGF-β.

48. Arsura M, Panta GR, Bilyeu JD, et al. Transient activation of NF-κB through a TAK1//IKK kinase pathway by TGF-β1 inhibits AP-1//SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene 2003;22:412–425. [PubMed: 12545162]

49. Kim DW, Sovak MA, Zanieski G, et al. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis 2000;21:871–879. [PubMed: 10783306]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

50. Park J-I, Lee M-G, Cho K, et al. TGF-β1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF-κB, JNK, and Ras signaling pathways. Oncogene 2003;22:4314–4332. [PubMed: 12853969]
51. Rayet B, Gelinas C. Aberrant Rel/NF-κB genes and activity in human cancer. Oncogene 1999;18:6938–6947. [PubMed: 10602468]
52. Horowitz JC, Rogers DS, Sharma V, et al. Combinatorial activation of FAK and AKT by TGF-β1 confers an anoikis-resistant phenotype to myofibroblasts. Cell Signal 2007;19:761–771. [PubMed: 17113264]
53. Thannickal VJ, Lee DY, White ES, et al. Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J. Biol. Chem 2003;278:12384–12389. [PubMed: 12531888]
54. Park SS, Eom YW, Kim EH, et al. Involvement of c-Src kinase in the regulation of TGF-β1-induced apoptosis. Oncogene 2004;23:6272–6281. [PubMed: 15208664]
55. Wang S, Wilkes MC, Leof EB, Hirschberg R. Imatinib mesylate blocks a non-Smad TGF-β pathway and reduces renal fibrogenesis *in vivo*. FASEB J 2005;19:1–11. [PubMed: 15629889]
56. Wilkes MC, Leof EB. TGF-β activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures. J. Biol. Chem 2006;281:27846–27854. [PubMed: 16867995]
57. Ebnet K, Suzuki A, Ohno S, Vestweber D. Junctional adhesion molecules (JAMs): more molecules with dual functions? J. Cell Sci 2004;117:19–29. [PubMed: 14657270]
58. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am. J. Physiol. Cell. Physiol 2004;286:C1213–1228. [PubMed: 15151915]
59. Itoh M, Bissell MJ. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. J. Mammary Gland Biol. Neoplasia 2003;8:449–462. [PubMed: 14985640]
60. Bose R, Wrana JL. Regulation of Par6 by extracellular signals. Curr. Opin. Cell Biol 2006;18:206–212. [PubMed: 16490351]
61. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGF-β receptors controls epithelial cell plasticity. Science 2005;307:1603–1609. [PubMed: 15761148] Identified the novel interaction between Par6 and TGF-β receptors, which promote EMT *via* the ubiquitination and degradation of RhoA
62. Takaishi K, Sasaki T, Kotani H, Nishioka H, Takai Y. Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK Cells. J. Cell Biol 1997;139:1047–1059. [PubMed: 9362522]
63. Niessen CM. Tight junctions/adherens junctions: basic structure and function. J. Invest. Dermatol 2007;127:2525–2532. [PubMed: 17934504]
64. Ridley AJ, Hall A. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992;70:389–399. [PubMed: 1643657]
65. Bhowmick NA, Ghiassi M, Bakin A, et al. TGF-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell 2001;12:27–36. [PubMed: 11160820]
66. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat. Rev. Cancer 2004;4:839–849. [PubMed: 15516957]
67. Kaplan RN, Rafii S, Lyden D. Preparing the “soil”: the premetastatic niche. Cancer Res 2006;66:11089–11093. [PubMed: 17145848]
68. Tlsty TD, Coussens LM. Tumor stroma and regulation of cancer development. Annu. Rev. Pathol 2006;1:119–150. [PubMed: 18039110]
69. Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. Mol. Med. Today 2000;6:324–329. [PubMed: 10904250]
70. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2002;2:161–174. [PubMed: 11990853]
71. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr. Opin. Cell Biol 2004;16:558–564. [PubMed: 15363807]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

72. Duivenvoorden WC, Hirte HW, Singh G. TGF-β1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. Clin. Exp. Metastasis 1999;17:27–34. [PubMed: 10390144]

73. Kim ES, Sohn YW, Moon A. TGF-β-induced transcriptional activation of MMP-2 is mediated by activating transcription factor (ATF)2 in human breast epithelial cells. Cancer Lett 2007;252:147–156. [PubMed: 17258390]

74. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 2000;103:481–490. [PubMed: 11081634]

75. Paszek MJ, Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. J. Mammary Gland Biol Neoplasia 2004;9:325–342. [PubMed: 15838603]

*76. Anderson AR, Weaver AM, Cummings PT, Quaranta V. Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment. Cell 2006;127:905–915. [PubMed: 17129778] Very interesting study that used a multiscale mathematical modeling approach to predict how altered microenvironmental factors and conditions impact tumor development and progression.

77. Sternlicht MD, Bissell MJ, Werb Z. The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter. Oncogene 2000;19:1102–1113. [PubMed: 10713697]

*78. Sternlicht MD, Lochter A, Sympson CJ, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 1999;98:137–146. [PubMed: 10428026]

*79. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 2005;436:123–127. [PubMed: 16001073] Together with Reference [78], established the essential role of MMPs in regulating the interactions between cells and their microenvironments, and in stimulating EMT.

80. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? J. Cell Biochem 2007;101:830–839. [PubMed: 17211838]

81. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr. Opin. Cell Biol 2005;17:548–558. [PubMed: 16098727]

82. Gotzmann J, Mikula M, Eger A, et al. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. Mutat. Res 2004;566:9–20. [PubMed: 14706509]

83. Jechlinger M, Grunert S, Beug H. Mechanisms in epithelial plasticity and metastasis: insights from 3D cultures and expression profiling. J. Mammary Gland Biol. Neoplasia 2002;7:415–432. [PubMed: 12882526]

84. Cavallaro U, Niedermeyer J, Fuxa M, Christofori G. N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. Nat. Cell Biol 2001;3:650–657. [PubMed: 11433297]

85. Lehembre F, Yilmaz M, Wicki A, et al. NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. EMBO J 2008;27:2603–2615. [PubMed: 18772882]

86. Illman SA, Lehti K, Keski-Oja J, Lohi J. Epilysin (MMP-28) induces TGF-β mediated epithelial to mesenchymal transition in lung carcinoma cells. J. Cell Sci 2006;119:3856–3865. [PubMed: 16940349]

87. Illman SA, Lohi J, Keski-Oja J. Epilysin (MMP-28)--structure, expression and potential functions. Exp. Dermatol 2008;17:897–907. [PubMed: 18803661]

88. Harbeck N, Kates RE, Schmitt M, et al. Urokinase-type plasminogen activator and its inhibitor type 1 predict disease outcome and therapy response in primary breast cancer. Clin. Breast Cancer 2004;5:348–352. [PubMed: 15585071]

89. Duffy MJ, Duggan C. The urokinase plasminogen activator system: a rich source of tumour markers for the individualised management of patients with cancer. Clin. Biochem 2004;37:541–548. [PubMed: 15234235]

90. Mitra SK, Lim ST, Chi A, Schlaepfer DD. Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. Oncogene 2006;25:4429–4440. [PubMed: 16547501]

91. Lin SW, Ke FC, Hsiao PW, Lee PP, Lee MT, Hwang JJ. Critical involvement of ILK in TGF-β1-stimulated invasion/migration of human ovarian cancer cells is associated with urokinase plasminogen activator system. Exp. Cell Res 2007;313:602–613. [PubMed: 17187779]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

92. Lester RD, Jo M, Montel V, Takimoto S, Gonias SL. uPAR induces epithelial mesenchymal transition in hypoxic breast cancer cells. J. Cell Biol 2007;178:425–436. [PubMed: 17664334]

93. Santibanez JF. JNK mediates TGF-β1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes. FEBS Lett 2006;580:5385–5391. [PubMed: 16989819]

94. Whitley BR, Church FC. Wound-induced migration of MDA-MB-435 and SKOV-3 cancer cells is regulated by plasminogen activator inhibitor-1. Int. J. Oncol 2005;27:749–757. [PubMed: 16077925]

95. Binder BR, Christ G, Gruber F, et al. Plasminogen activator inhibitor 1: physiological and pathophysiological roles. News Physiol. Sci 2002;17:56–61. [PubMed: 11909993]

96. Shetty S, Shetty P, Idell S, Velusamy T, Bhandary YP, Shetty RS. Regulation of plasminogen activator inhibitor-1 expression by tumor suppressor protein p53. J. Biol. Chem 2008;283:19570–19580. [PubMed: 18469003]

97. Kortlever RM, Bernards R. Senescence, wound healing and cancer: the PAI-1 connection. Cell Cycle 2006;5:2697–2703. [PubMed: 17172853]

98. Whitley BR, Palmieri D, Twerdi CD, Church FC. Expression of active plasminogen activator inhibitor-1 reduces cell migration and invasion in breast and gynecological cancer cells. Exp. Cell Res 2004;296:151–162. [PubMed: 15149846]

99. Descotes F, Riche B, Saez S, et al. Plasminogen activator inhibitor type 1 is the most significant of the usual tissue prognostic factors in node-negative breast ductal adenocarcinoma independent of urokinase-type plasminogen activator. Clin. Breast Cancer 2008;8:168–177. [PubMed: 18621614]

100. Wienke D, Davies GC, Johnson DA, et al. The collagen receptor Endo180 (CD280) Is expressed on basal-like breast tumor cells and promotes tumor growth *in vivo*. Cancer Res 2007;67:10230–10240. [PubMed: 17974964]

101. Kim ES, Kim MS, Moon A. TGF-β in conjunction with H-Ras activation promotes malignant progression of MCF10A breast epithelial cells. Cytokine 2005;29:84–91. [PubMed: 15598443]

102. Ignotz RA, Massague J. TGF-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem 1986;261:4337–4345. [PubMed: 3456347]

103. Xie L, Law B, Aakre M, et al. TGF-β-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res 2003;5:R187–R198. [PubMed: 14580254]

104. Maschler S, Wirl G, Spring H, et al. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene 2005;24:2032–2041. [PubMed: 15688013]

105. Kang Y, Massague J. Epithelial-mesenchymal transitions: Twist in development and metastasis. Cell 2004;118:277–279. [PubMed: 15294153]

106. Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer 2002;36:115–124. [PubMed: 11955645]

107. Graff JR, Greenberg VE, Herman JG, et al. Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. Cancer Res 1998;58:2063–2066. [PubMed: 9605742]

108. Comijn J, Berx G, Vermassen P, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell 2001;7:1267–1278. [PubMed: 11430829]

109. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 2007;7:415–428.

110. Cano A, Perez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol 2000;2:76–83. [PubMed: 10655586]

111. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A. TGF-β employs HMGA2 to elicit epithelial-mesenchymal transition. J. Cell Biol 2006;174:175–183. [PubMed: 16831886]

112. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous Expression of N-Cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J. Cell Biol 2000;148:779–790. [PubMed: 10684258]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

113. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin. Cancer Res 2007;13:7003–7011. [PubMed: 18056176]

114. Pyo SW, Hashimoto M, Kim YS, et al. Expression of E-cadherin, P-cadherin and N-cadherin in oral squamous cell carcinoma: correlation with the clinicopathologic features and patient outcome. J. Craniomaxillofac. Surg 2007;35:1–9. [PubMed: 17296306]

115. Yang L, Huang J, Ren X, et al. Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell 2008;13:23–35. [PubMed: 18167337]

116. Nam JS, Terabe M, Mamura M, et al. An anti-TGF-β antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Res 2008;68:3835–3843. [PubMed: 18483268]

117. Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat. Rev. Mol. Cell Biol 2003;4:657. [PubMed: 12923528]

118. Masszi A, Di Ciano C, Sirokmany G, et al. Central role for Rho in TGF-β1-induced α-smooth muscle actin expression during epithelial-mesenchymal transition. Am. J. Physiol. Renal Physiol 2003;284:F911–924. [PubMed: 12505862]

119. Yazhou C, Wenlv S, Weidong Z, Licun W. Clinicopathological significance of stromal myofibroblasts in invasive ductal carcinoma of the breast. Tumour Biol 2004;25:290–295. [PubMed: 15627894]

120. Cary LA, Guan JL. Focal adhesion kinase in integrin-mediated signaling. Front. Biosci 1999;4:D102–113. [PubMed: 9889179]

121. Cary LA, Han DC, Guan JL. Integrin-mediated signal transduction pathways. Histol. Histopathol 1999;14:1001–1009. [PubMed: 10425567]

122. Schwartz MA, Ginsberg MH. Networks and crosstalk: integrin signalling spreads. Nat. Cell Biol 2002;4:E65–68. [PubMed: 11944032]

123. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat. Rev Cancer 2002;2:91–100. [PubMed: 12635172]

124. Ginsberg MH, Partridge A, Shattil SJ. Integrin regulation. Curr. Opin. Cell Biol 2005;17:509–516. [PubMed: 16099636]

125. Guo W, Giancotti FG. Integrin signalling during tumour progression. Nat. Rev. Mol Cell Biol 2004;5:816–826. [PubMed: 15459662]

126. Sieg DJ, Hauck CR, Ilic D, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat. Cell Biol 2000;2:249–256. [PubMed: 10806474]

127. Chen SY, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol. Cell. Biol 2006;26:5155–5167. [PubMed: 16782899]

128. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc. Soc. Exp. Biol. Med 1999;222:124–138. [PubMed: 10564536]

129. Sheppard D, Cohen DS, Wang A, Busk M. TGF-β differentially regulates expression of integrin subunits in guinea pig airway epithelial cells. J. Biol. Chem 1992;267:17409–17414. [PubMed: 1512272]

130. Kumar NM, Sigurdson SL, Sheppard D, Lwebuga-Mukasa JS. Differential modulation of integrin receptors and extracellular matrix laminin by TGF-β1 in rat alveolar epithelial cells. Exp. Cell Res 1995;221:385–394. [PubMed: 7493638]

131. Wang A, Yokosaki Y, Ferrando R, Balmes J, Sheppard D. Differential regulation of airway epithelial integrins by growth factors. Am. J. Respir. Cell Mol. Biol 1996;15:664–672. [PubMed: 8918373]

*132. Munger JS, Huang X, Kawakatsu H, et al. The integrin αvβ6 binds and activates latent TGF-β1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999;96:319–328. [PubMed: 10025398] First established the importance of integrins in promoting the activation of latent TGF-β complexes.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

133. Jenkins RG, Su X, Su G, et al. Ligation of protease-activated receptor 1 enhances αvβ6 integrin-dependent TGF-β activation and promotes acute lung injury. J. Clin. Invest 2006;116:1606–1614. [PubMed: 16710477]
134. Neurohr C, Nishimura SL, Sheppard D. Activation of TGF-β by the integrin αvβ8 delays epithelial wound closure. Am. J. Respir. Cell Mol. Biol 2006;35:252–259. [PubMed: 16574941]
135. Morris DG, Huang X, Kaminski N, et al. Loss of integrin αvβ6-mediated TGF-β activation causes MMP12-dependent emphysema. Nature 2003;422:169–173. [PubMed: 12634787]
136. Mu D, Cambier S, Fjellbirkeland L, et al. The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. J Cell Biol 2002;157:493–507. [PubMed: 11970960]
137. Knight PA, Wright SH, Brown JK, Huang X, Sheppard D, Miller HR. Enteric expression of the integrin αvβ6 is essential for nematode-induced mucosal mast cell hyperplasia and expression of the granule chymase, mouse mast cell protease-1. Am. J. Pathol 2002;161:771–779. [PubMed: 12213704]
138. Kim KK, Kugler MC, Wolters PJ, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc. Natl. Acad. Sci. USA 2006;103:13180–13185. [PubMed: 16924102]
139. Bates RC, Bellovin DI, Brown C, et al. Transcriptional activation of integrin β6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. J. Clin. Invest 2005;115:339–347. [PubMed: 15668738]
140. Ma LJ, Yang H, Gaspert A, et al. TGF-β-dependent and -independent pathways of induction of tubulointerstitial fibrosis in β6(-/-) mice. Am. J. Pathol 2003;163:1261–1273. [PubMed: 14507636]
141. Owens DM, Broad S, Yan X, Benitah SA, Watt FM. Suprabasal α5β1 integrin expression stimulates formation of epidermal squamous cell carcinomas without disrupting TGF-β signaling or inducing spindle cell tumors. Mol. Carcinog 2005;44:60–66. [PubMed: 15924349]
142. Kostenuik PJ, Singh G, Orr FW. TGF-β upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. Clin. Exp. Metastasis 1997;15:41–52. [PubMed: 9009105]
143. Giannelli G, Fransvea E, Marinosci F, et al. TGF-β1 triggers hepatocellular carcinoma invasiveness via α3β1 integrin. Am. J. Pathol 2002;161:183–193. [PubMed: 12107103]
144. Giannelli G, Bergamini C, Fransvea E, Sgarra C, Antonaci S. Laminin-5 with TGF-β1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology 2005;129:1375–1383. [PubMed: 16285938]
145. Sloan EK, Pouliot N, Stanley KL, et al. Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. Breast Cancer Res 2006;8:R20. [PubMed: 16608535]
146. Bandyopadhyay A, Agyin JK, Wang L, et al. Inhibition of pulmonary and skeletal metastasis by a TGF-β type I receptor kinase inhibitor. Cancer Res 2006;66:6714–6721. [PubMed: 16818646]
147. Nakamura K, Yano H, Schaefer E, Sabe H. Different modes and qualities of tyrosine phosphorylation of Fak and Pyk2 during epithelial-mesenchymal transdifferentiation and cell migration: analysis of specific phosphorylation events using site-directed antibodies. Oncogene 2001;20:2626–2635. [PubMed: 11420674]
148. Liu S, Shi-wen X, Kennedy L, et al. FAK is required for TGF-β-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. Mol. Biol. Cell 2007;18:2169–2178. [PubMed: 17409352]
149. Cicchini C, Laudadio I, Citarella F, et al. TGF-β-induced EMT requires focal adhesion kinase (FAK) signaling. Exp. Cell Res 2008;314:143. [PubMed: 17949712]
150. Kim W, Kang Y Seok, Kim J Soo, Shin N-Y, Hanks SK, Song WK. The integrin-coupled signaling adaptor p130Cas suppresses Smad3 function in TGF-β signaling. Mol. Biol. Cell 2008;19:2135–2146. [PubMed: 18321991]
151. Cabodi S, Tinnirello A, Di Stefano P, et al. p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-Neu oncogene-dependent breast tumorigenesis. Cancer Res 2006;66:4672–4680. [PubMed: 16651418]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

152. Tumbarello DA, Brown MC, Hetey SE, Turner CE. Regulation of paxillin family members during epithelial-mesenchymal transformation: a putative role for paxillin δ. J. Cell Sci 2005;118:4849–4863. [PubMed: 16219691]

153. Fujimoto N, Yeh S, Kang HY, et al. Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J. Biol. Chem 1999;274:8316–8321. [PubMed: 10075738]

154. Guerrero-Santoro J, Yang L, Stallcup MR, DeFranco DB. Distinct LIM domains of Hic-5/ARA55 are required for nuclear matrix targeting and glucocorticoid receptor binding and coactivation. J. Cell Biochem 2004;92:810–819. [PubMed: 15211577]

155. Yang L, Guerrero J, Hong H, DeFranco DB, Stallcup MR. Interaction of the Tau2 transcriptional activation domain of glucocorticoid receptor with a novel steroid receptor coactivator, Hic-5, which localizes to both focal adhesions and the nuclear matrix. Mol. Biol. Cell 2000;11:2007–2018. [PubMed: 10848625]

156. Tumbarello DA, Turner CE. Hic-5 contributes to epithelial-mesenchymal transformation through a RhoA/ROCK-dependent pathway. J. Cell Physiol 2007;211:736–747. [PubMed: 17299801]

157. Mok SC, Wong KK, Chan RK, et al. Molecular cloning of differentially expressed genes in human epithelial ovarian cancer. Gynecol. Oncol 1994;52:247–252. [PubMed: 8314147]

158. Mok SC, Chan WY, Wong KK, et al. DOC-2, a candidate tumor suppressor gene in human epithelial ovarian cancer. Oncogene 1998;16:2381–2387. [PubMed: 9620555]

159. Prunier C, Hocevar BA, Howe PH. Wnt signaling: physiology and pathology. Growth Factors 2004;22:141–150. [PubMed: 15518237]

160. Prunier C, Howe PH. Disabled-2 (Dab2) is required for TGF-β-induced epithelial to mesenchymal transition (EMT). J. Biol. Chem 2005;280:17540–17548. [PubMed: 15734730]

161. Hocevar BA, Prunier C, Howe PH. Disabled-2 (Dab2) mediates TGF-β-stimulated fibronectin synthesis through TGF-β-activated kinase 1 and activation of the JNK pathway. J. Biol. Chem 2005;280:25920–25927. [PubMed: 15894542]

*162. Sorrentino A, Thakur N, Grimsby S, et al. The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat. Cell Biol 2008;10:1199–1207. [PubMed: 18758450]

*163. Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol. Cell 2008;31:918–924. [PubMed: 18922473] Together with Reference [162], these studies demonstrated the importance of TRAF6 to interact physically with TGF-β receptors, leading to their activation of MAP kinases via ubiquitination of TAK1.

164. Gal A, Sjoblom T, Fedorova L, Imreh S, Beug H, Moustakas A. Sustained TGF-β exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. Oncogene 2008;27:1218–1230. [PubMed: 17724470]

165. Hall A. Rho GTPases and the control of cell behaviour. Biochem. Soc. Trans 2005;33:891–895. [PubMed: 16246005]

166. Hall A, Nobes CD. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos. Trans. R. Soc. Lond. B. Biol. Sci 2000;355:965–970. [PubMed: 11128990]

167. Sander EE, ten Klooster JP, van Delft S, van der Kammen RA, Collard JG. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. J. Cell Biol 1999;147:1009–1022. [PubMed: 10579721]

168. Cho HJ, Yoo J. Rho activation is required for TGF-β-induced epithelial-mesenchymal transition in lens epithelial cells. Cell Biol. Int 2007;31:1225–1230. [PubMed: 17537651]

169. Massague J. How cells read TGF-β signals. Nat. Rev. Mol. Cell. Biol 2000;1:169–178. [PubMed: 11252892]

170. Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. Oncogene 2005;24:4580–4587. [PubMed: 15856020]

171. Jechlinger M, Sommer A, Moriggl R, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. J. Clin. Invest 2006;116:1561–1570. [PubMed: 16741576]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

172. Dedhar S, Williams B, Hannigan G. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends. Cell Biol 1999;9:319–323. [PubMed: 10407411]

173. Hannigan G, Troussard A, ADedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. Nat. Rev. Cancer 2005;5:51–63. [PubMed: 15630415]

174. Hehlgans S, Haase M, Cordes N. Signalling via integrins: implications for cell survival and anticancer strategies. Biochim. Biophys. Acta 2007;1775:163–180. [PubMed: 17084981]

175. White DE, Cardiff RD, Dedhar S, Muller WJ. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene 2001;20:7064–7072. [PubMed: 11704830]

176. Somasiri A, Howarth A, Goswami D, Dedhar S, Roskelley CD. Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells. J. Cell Sci 2001;114:1125–1136. [PubMed: 11228156]

177. Lee YI, Kwon YJ, Joo CK. Integrin-linked kinase function is required for TGF-β-mediated epithelial to mesenchymal transition. Biochem. Biophys. Res. Commun 2004;316:997–1001. [PubMed: 15044083]

178. Karin M. NF-κB in cancer development and progression. Nature 2006;441:431. [PubMed: 16724054]

179. Huber MA, Azoitei N, Baumann B, et al. NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J. Clin. Invest 2004;114:569–581. [PubMed: 15314694]

180. Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-κB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene 2006;26:711. [PubMed: 16862183]

181. Sovak MA, Arsura M, Zanieski G, Kavanagh KT, Sonenshein GE. The inhibitory effects of TGF-β1 on breast cancer cell proliferation are mediated through regulation of aberrant NF-κB/Rel expression. Cell Growth Differ 1999;10:537–544. [PubMed: 10470853]

182. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis 2008;29:2227–2235. [PubMed: 18725385]

183. Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the ERK pathway is required for TGF-β1-induced EMT *in vitro*. Neoplasia 2004;6:603–610. [PubMed: 15548370]

184. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in TGF-β-mediated signaling. J. Biol. Chem 1997;272:1429–1432. [PubMed: 8999807]

185. Hocevar BA, Brown TL, Howe PH. TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J 1999;18:1345–1356. [PubMed: 10064600]

186. Shintani Y, Wheelock MJ, Johnson KR. Phosphoinositide-3 kinase-Rac1-c-Jun NH2-terminal kinase signaling mediates collagen I-induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells. Mol. Biol. Cell 2006;17:2963–2975. [PubMed: 16624865]

187. Ke Z, Lin H, Fan Z, et al. MMP-2 mediates ethanol-induced invasion of mammary epithelial cells over-expressing ErbB2. Int. J. Cancer 2006;119:8–16. [PubMed: 16450376]

188. Buck MB, Knabbe C. TGF-β signaling in breast cancer. Ann. NY Acad. Sci 2006;1089:119–126. [PubMed: 17261761]

189. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70. [PubMed: 10647931]

190. Moreno-Bueno G, Portillo F, Cano A. Transcriptional regulation of cell polarity in EMT and cancer. Oncogene 2008;27:6958–6969. [PubMed: 19029937]

191. Ikenouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J. Cell Sci 2003;116:1959–1967. [PubMed: 12668723]

*192. Alexandrow MG, Kawabata M, Aakre M, Moses HL. Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of TGF-β. Proc.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Natl. Acad. Sci. USA 1995;92:3239–3243. [PubMed: 7724545] Established c-Myc as an a molecule that possesses anti-TGF-β activity during tumorigenesis.

193. Chen CR, Kang Y, Massague J. Defective repression of c-Myc in breast cancer cells: A loss at the core of the TGF-β growth arrest program. Proc. Natl. Acad. Sci. USA 2001;98:992–999. [PubMed: 11158583]
194. Smith AP, Verrecchia A, Faga G, et al. A positive role for Myc in TGF-β-induced Snail transcription and epithelial-to-mesenchymal transition. Oncogene 2008;28:422–430. [PubMed: 18978814]
195. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303. [PubMed: 10458605]
196. Yang Y, Pan X, Lei W, et al. Regulation of TGF-β1-induced apoptosis and epithelial-to-mesenchymal transition by protein kinase A and signal transducers and activators of transcription 3. Cancer Res 2006;66:8617–8624. [PubMed: 16951175]
197. Zhao S, Venkatasubbarao K, Lazor JW, et al. Inhibition of STAT3Tyr705 phosphorylation by Smad4 suppresses TGF-β-mediated invasion and metastasis in pancreatic cancer cells. Cancer Res 2008;68:4221–4228. [PubMed: 18519681]
198. Lo HW, Hsu SC, Xia W, et al. Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. Cancer Res 2007;67:9066–9076. [PubMed: 17909010]
199. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. Nat. Rev. Cancer 2002;2:101–112. [PubMed: 12635173]
200. Coombes RC, Gibson L, Hall E, Emson M, Bliss J. Aromatase inhibitors as adjuvant therapies in patients with breast cancer. J. Steroid Biochem. Mol. Biol 2003;86:309–311. [PubMed: 14623526]
201. Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell 2003;113:207–219. [PubMed: 12705869]
202. Dhasarathy A, Kajita M, Wade PA. The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-α. Mol. Endocrinol 2007;21:2907–2918. [PubMed: 17761946]
203. Silveri L, Tilly G, Vilotte JL, Le Provost F. MicroRNA involvement in mammary gland development and breast cancer. Reprod. Nutr. Dev 2006;46:549–556. [PubMed: 17107644]
204. Croce CM, Calin GA. miRNAs, cancer, and stem cell division. Cell 2005;122:6–7. [PubMed: 16009126]
205. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–7070. [PubMed: 16103053]
206. Blenkiron C, Miska EA. miRNAs in cancer: approaches, aetiology, diagnostics and therapy. Hum. Mol. Genet 2007;16:R106–113. [PubMed: 17613543]
207. Dalmay T, Edwards DR. MicroRNAs and the hallmarks of cancer. Oncogene 2006;25:6170–6175. [PubMed: 17028596]
208. Blenkiron C, Goldstein LD, Thorne NP, et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 2007;8:R214. [PubMed: 17922911]
209. Hurteau GJ, Carlson JA, Spivack SD, Brock GJ. Overexpression of the microRNA Hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res 2007;67:7972–7976. [PubMed: 17804704]
210. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J. Biol. Chem 2008;283:14910–14914. [PubMed: 18411277]
*211. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol 2008;10:593–601. [PubMed: 18376396]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

*212. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 2008;22:894–907. [PubMed: 18381893]

*213. Burk U, Schubert J, Wellner U, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 2008;9:582–589. [PubMed: 18483486] Together with References [211] and [212], these studies provide the first evidence linking altered microRNA expression to EMT stimulated by TGF-β.

214. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007;449:682–688. [PubMed: 17898713]

215. Kong W, Yang H, He L, et al. MicroRNA-155 is regulated by the TGF-β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol. Cell. Biol 2008;28:6773–6784. [PubMed: 18794355]

216. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. Oncogene 2007;26:2799–2803. [PubMed: 17072344]

217. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J. Biol. Chem 2007;282:14328–14336. [PubMed: 17363372]

218. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008;18:350–359. [PubMed: 18270520]

219. Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ. TGF-β and microRNA:mRNA regulatory networks in epithelial plasticity. Cells Tissues Organs 2007;185:157–161. [PubMed: 17587821]

220. Bakin AV, Safina A, Rinehart C, Daroqui C, Darbary H, Helfman DM. A critical role of tropomyosins in TGF-β regulation of the actin cytoskeleton and cell motility in epithelial cells. Mol. Biol. Cell 2004;15:4682–4694. [PubMed: 15317845]

221. Varga AE, Stourman NV, Zheng Q, et al. Silencing of the tropomyosin-1 gene by DNA methylation alters tumor suppressor function of TGF-β. Oncogene 2005;24:5043–5052. [PubMed: 15897890]

222. Zheng Q, Safina A, Bakin AV. Role of high-molecular weight tropomyosins in TGF-β-mediated control of cell motility. Int. J. Cancer 2008;122:78–90. [PubMed: 17721995]

223. Lombaerts M, van Wezel T, Philippo K, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br. J. Cancer 2006;94:661. [PubMed: 16495925]

224. Reynolds PA, Sigaroudinia M, Zardo G, et al. Tumor suppressor p16INK4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells. J. Biol. Chem 2006;281:24790–24802. [PubMed: 16766534]

225. Dumont N, Wilson MB, Crawford YG, Reynolds PA, Sigaroudinia M, Tlsty TD. Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. Proc. Natl. Acad Sci. USA 2008;105:14867–14872. [PubMed: 18806226]

226. Singh M, Spoelstra NS, Jean A, et al. ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease. Mod. Pathol 2008;21:912. [PubMed: 18487993]

227. Shackleton M, Vaillant F, Simpson KJ, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84–88. [PubMed: 16397499]

228. Stingl J, Raouf A, Eirew P, Eaves CJ. Deciphering the mammary epithelial cell hierarchy. Cell Cycle 2006;5:1519–1522. [PubMed: 16861925]

229. Villadsen R, Fridriksdottir AJ, Ronnov-Jessen L, et al. Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 2007;177:87–101. [PubMed: 17420292]

230. Mishra L, Derynck R, Mishra B. TGF-β signaling in stem cells and cancer. Science 2005;310:68–71. [PubMed: 16210527]

231. Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat. Genet 2008;40:499–507. [PubMed: 18443585]

*232. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–715. [PubMed: 18485877]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

*233. Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS ONE 2008;3:e2888. [PubMed: 18682804]

*234. Shipitsin M, Campbell LL, Argani P, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259–273. [PubMed: 17349583] Together with References [232] and [233], these studies provide the first evidence linking EMT to the acquisition of "stemness." Importantly, TGF-β signaling plays a major role in overseeing EMT and the appearance of cancer stem cells.

235. Farina AR, Coppa A, Tiberio A, et al. TGF-β1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity. Int. J. Cancer 1998;75:721–730. [PubMed: 9495240]

236. Piek E, Ju WJ, Heyer J, et al. Functional characterization of TGF-β signaling in Smad2- and Smad3-deficient fibroblasts. J. Biol. Chem 2001;276:19945–19953. [PubMed: 11262418]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

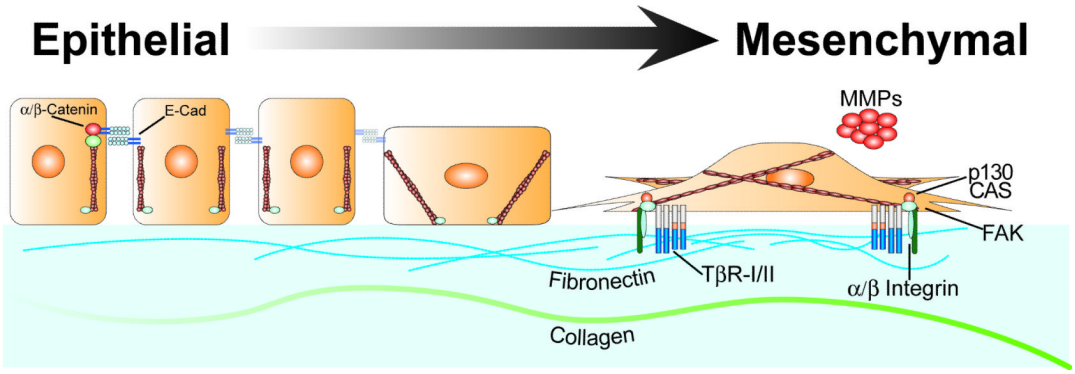


Figure 1. Epithelial Cells Transition to Mesenchymal-like Cells in Response to TGF-β

This schematic depicts polarized epithelial cells and their cuboidal structure that is maintained *via* cell-cell junctions comprised of homotypic E-cadherin molecules that are linked to the cortical actin cytoskeleton by α- and β-catenins. TGF-β stimulation of EMT during wound healing or tumor invasive migration results in the delocalization, degradation, and/or downregulation of cell-cell junctions and, consequently, a loss of epithelial integrity. In addition, the morphologic transition of epithelial cells also is supported by the simultaneous formation of actin stress fibers, the upregulation of integrins, and the activation of focal adhesion complexes. Moreover, the increased production and section of ECM proteins, such as fibronectin and collagen, coupled with the elevated expression and activation of MMPs enables transitioned fibroblastoid-like cells to exhibit invasive and motile phenotypes.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

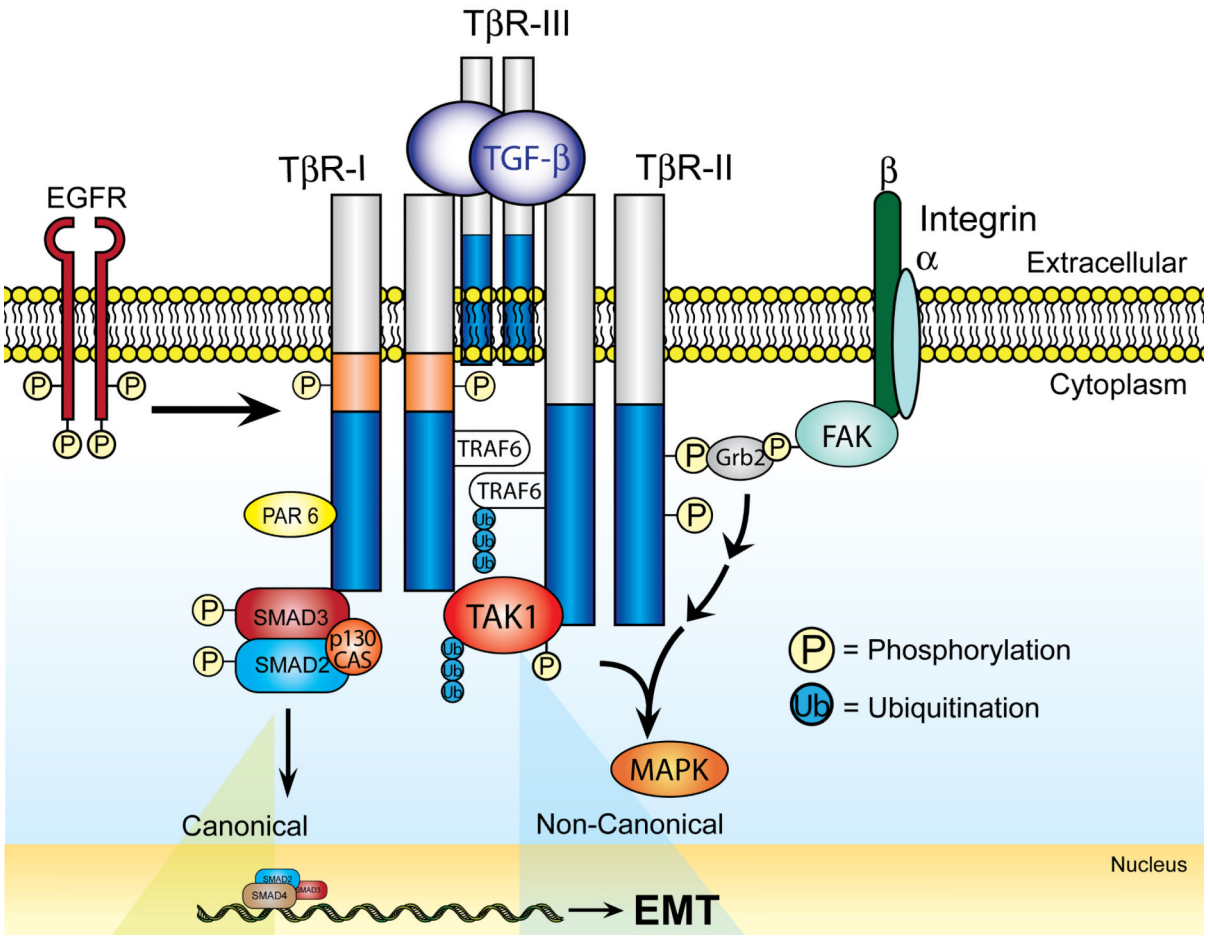


Figure 2. Differential Interactions of TGF-β Receptors with Transmembrane and Membrane Proximal Proteins Complexes Facilitate the Diversity TGF-β Signaling

β1 and β3 integrins interact physically with TβR-II [43-45]. The association of TβR-II with β3 integrin is mediated by FAK, which facilitates the binding of TβR-II to the SH2-binding protein, Grb2. In addition, TβR-II also interacts physically with EGFR (M.K. Wendt and W.P. Schiemann, *unpublished observation*), which also is activated indirectly by TGF-β through its increased synthesis and secretion of EGFR ligands. The cytoplasmic tails of both TβR-I and TβR-II interact with TRAF6, which ubiquitinates itself and the MAPKKK, TAK1. Additional interactions include the binding of p130Cas to Smad3, as well as that of PAR6 with TβR-I. Importantly, the differential composition of TGF-β receptor and scaffolding complexes directs the coupling of TGF-β to canonical and noncanonical effector activation, as well as underlies the pathophysiological conversion of TGF-β signaling and EMT in malignant epithelial cells. The biological outcomes of these various protein-protein interactions are discussed in the text.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

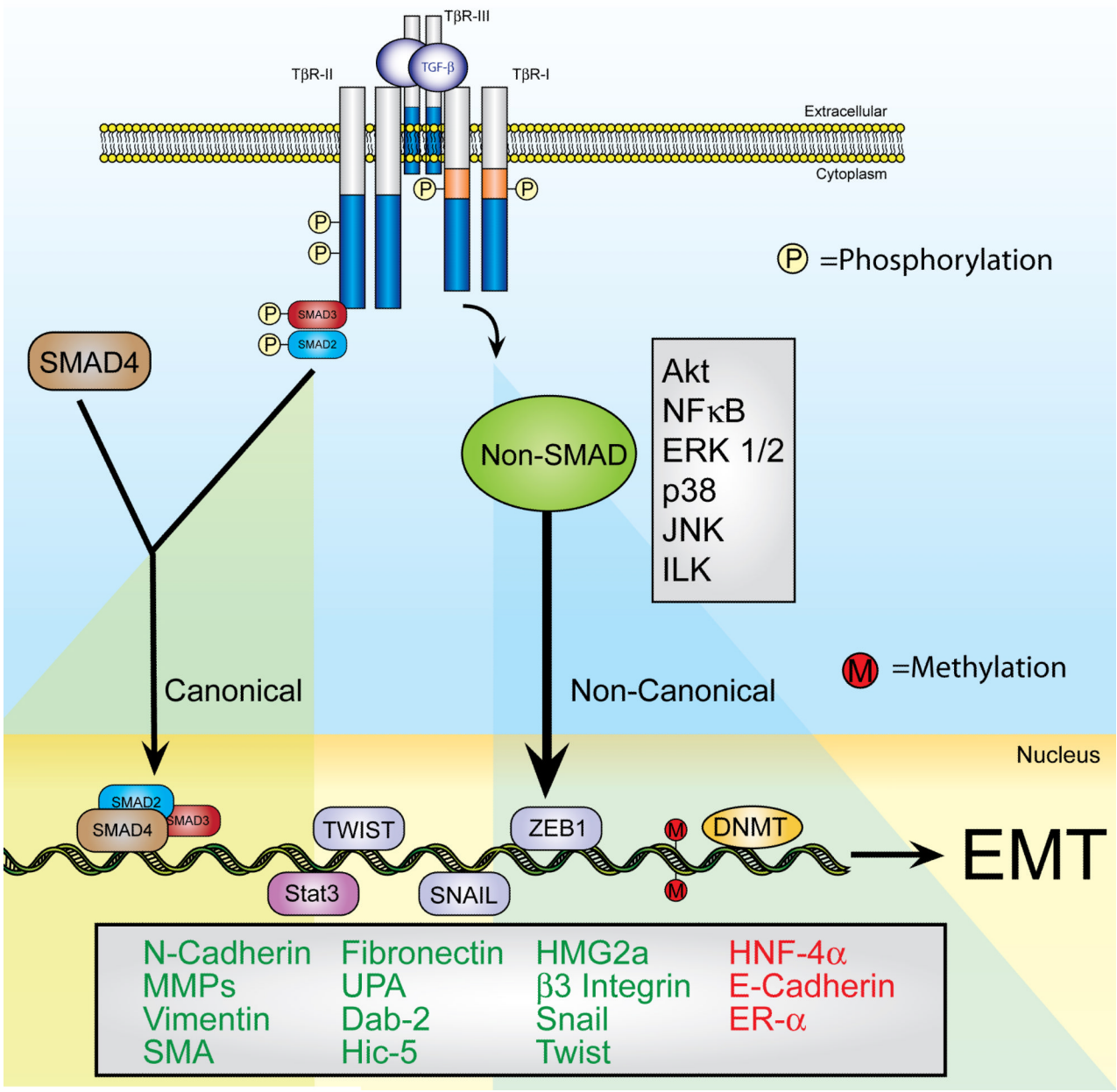


Figure 3. Diverse TGF-β Signaling Pathways Support a Complex Transcriptional Response During EMT

TGF-β stimulates epithelial cells by binding and activating two transmembrane Ser/Thr protein kinase receptors, namely TGF-β type I (TβR-I) and type II (TβR-II). Activation of these ligand:receptor ternary complexes requires TβR-II to transphosphorylate TβR-I, which phosphorylates and activates Smad2/3. Once activated, Smad2/3 form heterocomplexes with Smad4, which collectively translocate to the nucleus to mediate canonical signaling events by TGF-β (left panel). Noncanonical (right panel) TGF-β signaling takes place through its ability to stimulate various alternate signaling pathways discussed in detail herein. Activation of canonical Smad2/3 signaling results in their nuclear translocation with Smad4 and subsequent regulation of gene expression through their numerous interactions with additional transcriptional activators and repressors. Alternatively, activation of noncanonical TGF-β signaling, such as MAP kinases, small GTPases, PI3K/AKT, and NF-κB, also

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

couples TGF-β to its regulation of gene expression profiles operant in mediating EMT. Finally, activation of the transcription factors belonging to the Snail family (*e.g.*, Snail, Twist, or ZEB1), or of Stat3 elicit EMT-gene expression, which ultimately promotes the prolonged induction of EMT and fibroblastoid-like phenotypes of carcinoma cells *via* DNA methylation-mediated silencing of E-cadherin expression. Altered coupling of TGF-β to its canonical and noncanonical effector pathways leads to differential gene expression patterns that ultimately contribute to the development of oncogenic signaling by TGF-β. Indeed, the initiation of oncogenic signaling by TGF-β converts its regulation of physiological EMT in normal epithelial cells to one of pathophysiologic EMT in their malignant counterparts.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Expression of EMT-associated Genes Targeted by TGF-β

Gene Name	Expression Change	Reference
E-cadherin	Decrease	Miettinen *et al* [15]
β3 integrin	Increase	Galliher *et al* [43]
N-cadherin	Increase	Hazan *et al* [112]
NCAM	Increase	Lehembre *et al* [85]
MMP-2	Increase	Duivenvoorden *et al* [72]
MMP-3	Increase	Farina *et al* [235]; Radisky et al [79]
MMP-9	Increase	Farina *et al* [235]; Kim *et al* [73]
Vimentin	Increase	Grunert *et al* [117]
α-Smooth Muscle Actin	Increase	Masszi *et al* [118]
Fibronectin	Increase	Ignotz *et al* [102]
Estrogen Receptor-α	Decrease	Dhasarathy *et al* [202]
Urokinase Plasminogen Activator	Increase	Farina *et al* [235]
Dab2	Increase	Hocevar *et al* [26]
Hic5	Increase	Tumbarello *et al* [156]
HMG2A	Increase	Thuault *et al* [111]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 2

Signaling Pathways Activated During EMT Stimulated by TGF-β

Pathway	Reference
Smad2/3	Piek *et al* [236]
Rho family of small GTPases	Bhowmick *et al* [65]
PI3K and AKT	Bakin *et al* [38]
NF-κB	Huber *et al* [179]
ERK1/2	Xie *et al* [183]
p38 MAPK	Bhowmick *et al* [39]; Galliher and Schiemann [43-45]
JNK	Hocevar *et al* [185]
Integrin-linked kinase	Lee *et al* [177]; Lin *et al* [91]

NIH Public Access
Author Manuscript
Future Oncol. Author manuscript; available in PMC 2009 July 15.

Published in final edited form as:
Future Oncol. 2009 March ; 5(2): 259–271. doi:10.2217/14796694.5.2.259.

The TGF-β Paradox in Human Cancer: An Update

Maozhen Tian and **William P. Schiemann**
Department of Pharmacology, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045

Summary

Transforming growth factor-β (TGF-β) plays an essential role in maintaining tissue homeostasis through its ability to induce cell cycle arrest, differentiation, apoptosis, and to preserve genomic stability. Thus, TGF-β is a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. Interestingly, tumorigenesis typically elicits aberrations in the TGF-β signaling pathway that engenders resistance to the cytostatic activities of TGF-β, thereby enhancing the development and progression of human malignances. Moreover, these genetic and epigenetic events conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, invasion, and metastasis. The dichotomous nature of TGF-β during tumorigenesis is known as the "TGF-β Paradox," which remains the most critical and mysterious question concerning the physiopathological role of this multifunctional cytokine. Here we review recent findings that directly impact our understanding of the "TGF-β Paradox" and discuss their importance to targeting the oncogenic activities of TGF-β in developing and progressing neoplasms.

Keywords

Angiogenesis; Cancer; Cell Invasion; Epithelial-mesenchymal Transition; Metastasis; Signal Transduction; Transforming growth factor-β

1. TGF-β and the Tumor Microenvironment

1.1. TGF-β and Fibroblasts

Tumor development in many respects mirrors that of an organ, albeit in a highly dysfunctional and disorganized manner. For instance, whereas normal tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts and endothelial cells (ECs), as well as a variety of infiltrating immune and progenitor cells [1,2]. Moreover, tumor reactive stroma not only plays an important role during cancer initiation and progression, but also in determining whether TGF-β suppresses or promotes tumor formation (Figure 1; [3–5]). Along these lines, TGF-β exerts its anti-tumor activities by regulating epithelial cell behavior, and by regulating that of adjacent fibroblasts, which synthesize and secrete a variety of cytokines, growth factors, and extracellular matrix (ECM) proteins that mediate tissue homeostasis and suppress cancer development. Thus, inactivating paracrine TGF-β signaling between adjacent epithelial and stromal compartments promotes cellular transformation, as well as induces the growth, survival, and motility of developing neoplasms [6,7]. For instance, rendering fibroblasts deficient in the expression of the TGF-β type II receptor (TβR-II), which manifests as

Correspondence: William P. Schiemann, Department of Pharmacology, MS-8303, University of Colorado Denver, Anschutz Medical Campus, RC1 South Tower, Room L18-6110, 12801 East 17th Avenue, PO Box 6511, Aurora, CO 80045. Phone: (303)724-1541. Fax: (303)724-3663. E-mail: E-mail: Bill.Schiemann@uchsc.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

insensitivity to TGF-β, results in the formation of prostate intraepithelial neoplasia and invasive carcinoma of the forestomach [3]. Conditional deletion of TβR-II in mammary gland fibroblasts enhanced their proliferation and abundance within abnormally developed ductal units [8]. Interestingly, grafting a mixture of TβR-II-deficient mammary fibroblasts with mammary carcinoma cells under the subrenal capsule significantly enhanced the growth and invasion of breast cancer cells. The enhanced tumorigenicity of implanted mammary carcinoma cells was not recapitulated in grafts containing TGF-β-responsive fibroblasts, which failed to synthesize and secrete the high levels of TGF-α, MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor) produced by their TβR-II-deficient counterparts [3–5,8]. Thus, TGF-β signaling in fibroblasts suppresses their activation of cancer-promoting paracrine signaling axes that target adjacent epithelial cells. Somewhat surprisingly, TβR-II-deletion in mammary carcinoma cells resulted in the activation of two tumorigenic paracrine signaling axes comprised of SDF-1:CXCR4 and CXCL5:CXCR2, which collectively function in recruiting immature $GR1^+CD11b^+$ myeloid cells to developing mammary tumors [9]. Upon their arrival within mammary tumor microenvironments, $GR1^+CD11b^+$ cells promote breast cancer cell invasion and metastasis by attenuating host tumor immunosurveillance, and by stimulating MMP expression [9]. Recently, the ability of TGF-β to induce cell cycle progression in glioma cells required initiation of autocrine PDGF-B signaling. Importantly, the proliferation promoting properties of TGF-β and Smad2/3 only occurred in glioma lacking methylation of the PDGF-B gene, suggesting that the methylation status of PDGF-B determines the oncogenic activities of TGF-β in part *via* autocrine PDGF-B signaling within tumor microenvironments [10].

Tumorigenesis often is accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that enhance the selection and expansion of metastatic cells [11,12]. Lysyl oxidases (LOX) belong to a 5 gene family of copper-dependent amine oxidases (*i.e.*, LOX, LOXL, LOXL2, LOXL3, and LOXL4) that function in cross-linking collagens to elastin in the ECM [13,14]. Mechanistically, the activation of these cross-linking reactions by LOXs secreted by fibroblasts and epithelial cells serves to increase the tensile strength and structural integrity of tissues during embryonic development and organogenesis, as well as during the maintenance of normal tissue homeostasis [13,14]. Similar to TGF-β, members of the LOX family have been associated with tumor suppression and tumor promotion. Indeed, the transformation of fibroblasts by oncogenic Ras is suppressed by LOX and its ability to bind, oxidize, and inactivate growth factors housed in cell microenvironments, which presumably contributes the loss of cyclin D1 expression observed in LOX-expressing fibroblasts [15,16]. More recently, LOX was observed to interact physically with TGF-β1 and alter its ability to stimulate Smad3 in cultured osteoblasts [17], while LOXL4 expression inhibited TGF-β stimulation of liver cancer cell invasion through synthetic basement membranes [18]. Thus, these findings implicate LOXs as potential suppressive agents within tumor microenvironments. In stark contrast, aberrant LOX activity also is associated with cancer progression, particularly the selection, expansion, and dissemination of metastatic cells [13–15,19–22]. Indeed, upregulated LOX expression is *(i)* essential for hypoxia-induced metastasis of human MDA-MB-231 breast cancer cells in mice [19]; and *(ii)* is observed most frequently in poorly differentiated, high grade mammary tumors and, consequently, predicts for increased disease recurrence and decreased patient survival [15,19]. Recently, we observed LOX expression to be induced strongly by TGF-β in normal and malignant MECs, and in mammary tumors produced in mice. Moreover, inhibiting LOX activity or degrading its metabolic byproduct, hydrogen peroxide, both antagonize the ability of TGF-β to induce the proliferation, EMT, and invasion in normal and malignant MECs. Furthermore, we find that LOX antagonism uncouples TGF-β from stimulating Src and p38 MAPK (M. Taylor and W.P. Schiemann, *unpublished findings*), whose activities are essential for mediating oncogenic signaling by TGF-β in breast cancer cells [23–25]. Along these lines, future studies need to enhance our understanding of *(i)* the role of tumor reactive fibroblasts and their production of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

TGF-β in protecting carcinoma cells from tumoricidal radiotherapies, and *(ii)* the molecular and cellular mechanisms whereby anti-TGF-β therapies selectively sensitize carcinoma cells, not their adjacent normal counterparts, to ionizing radiation treatments [26–28].

Collectively, these findings highlight the important role TGF-β plays in governing autocrine and paracrine signaling networks, and more importantly, demonstrate how disrupting the delicate balance between these systems contributes to carcinoma development and progression.

1.2. TGF-β and Immunosurveillance

In addition to its regulation of stromal fibroblasts, TGF-β present in cell microenvironments also plays an essential role in governing the delicate balance between host immunosurveillance and inflammation, which collectively can determine whether tumor development and progression is induced or inhibited [29,30]. The importance of TGF-β in regulating immune system function and homeostasis is underscored by the finding that *(i)* TGF-β1-deficient mice exhibit lethal multifocal inflammatory disease [31,32]; *(ii)* Smad3-deficient mice exhibit defects in the responsiveness and chemotaxis of their neutrophils, and their T and B cells [33]; and *(iii)* transgenic expression of truncated TβR-II specifically in T cells results in severe autoimmune reactions characterized by multifocal inflammation and autoantibody production [31]. Furthermore, T cell-specific deletion of Smad4 in mice drives T cell differentiation towards a Th2 phenotype and their elevated secretion of interleukins (ILs) 4, 5, 6, and 13 [34]. Similar to fibroblasts, the net effect of disrupting paracrine T cell signaling networks is the development of gastrointestinal carcinomas in these genetically engineered animals [34]. In addition, cancer cells typically increase their production and secretion of TGF-β into tumor microenvironments, as well as into the general circulation of cancer patients [35–37]. Abnormally elevated TGF-β concentrations also are detected within the tumor milieu in response to ECM degradation mediated by resident and recruited leukocytes – *i.e.*, monocytes/macrophages, dendritic cells, granulocytes, mast cells, T cells, and natural killer (NK) cells – that either promote or suppress tumor development in a context-specific manner [38].

1.2.1. TGF-β and Adaptive Immunity—TGF-β suppresses host immunosurveillance by inhibiting the proliferation and differentiation of NK and T cells, and by diminishing their synthesis and secretion of cytotoxic effector molecules, including interferon-γ, lymphotoxin-α, perforin/granzyme, and Fas ligand [30,39,40]. TGF-β also inhibits the tumor-targeting activities of T and NK cells through its stimulation of Tregs housed within tumor microenvironments [41]. Whereas TGF-β potently inhibits the proliferation of naïve $CD8^+$ T cells, this cytokine elicits little-to-no activity in fully differentiated $CD8^+$ T cells due to their downregulation of TβR-II. Administration of ILs 2 or 10 to differentiated $CD8^+$ T cells restores their responsiveness to TGF-β, as does expression of the co-stimulatory molecule CD28, which promotes the survival of memory/effector phenotypes in thymic and peripheral T cell populations [30,39,42]. Mechanistically, the immunosuppressive effects of TGF-β transpire in part *via* Smad3, whose phosphorylation and activation prevents the mitogenesis of $CD8^+$ T cells by *(i)* inhibiting their production of IL-2; *(ii)* repressing their expression of c-Myc, cyclin D2, and cyclin E; and *(iii)* stimulating the expression of the CDKIs p15, p21, and p27 [30, 39,40]. In contrast to its stimulation of cytostasis in $CD8^+$ T cells, TGF-β has no effect on the proliferation of $CD4^+$ T cells, but does inhibit the differentiation of $CD4^+$ T cells into Th1 and Th2 lineages by *(i)* downregulating T cell receptor expression; *(ii)* reducing intracellular Ca^{++} signaling; and *(iii)* repressing the expression and activation of transcription factors [30, 39,40], all of which weaken host immunosurveillance. Collectively, these findings predict that inactivating TGF-β signaling in $CD8^+$ or $CD4^+$ T cells will inhibit tumor formation by elevating host immunosurveillance, a supposition shown to occur during T cell-mediated eradication of skin [43] and prostate [44] cancers in mice. More recently, TGF-β was observed to promote the development and progression of breast and colon cancers by inducing CD8+ T cells to

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

secrete IL-17, which exerts pro-survival signaling in carcinoma cells [45]. Thus, in addition to improving host immunosurveillance, neutralizing TGF-β function in T cells also will improve tumor resolution by suppressing the activation of carcinoma survival pathways.

1.2.2. TGF-β and Innate Immunity—In addition to its role in regulating adaptive immunity, TGF-β also plays an essential role in directing activities and behaviors of components of the innate immune system, including NK cells, dendritic cells, mast cells, monocytes, and macrophages. Indeed, we defined a novel TAB1:xIAP:TAK1:IKKβ:NF-κB signaling axis that forms aberrantly in breast cancer cells, and in normal MECs following their induction of EMT by TGF-β. Once formed, this signaling axis enables oncogenic signaling by TGF-β in part *via* activation of NF-κB and its consequential production of proinflammatory cytokines, which promote breast cancer growth in mice in a manner consistent with regulation of innate immunity by TGF-β [46]. Along these lines, TGF-β receptors were observed to associate with those for IL-1β, thereby enabling *(i)* TGF-β to activate NF-κB; *(ii)* IL-1β to activate Smad2; and *(iii)* both pathways to potentiate inflammatory cytokine production [47] their ability to promote inflammation and the enhanced the survival of tumor-associated monocytes [48,49]. In addition, transgenic expression of IL-1β in the stomachs of mice promoted the activation of myeloid-derived suppressor cells (MDSCs) *via* an IL-1R/NF-κB signaling axis, whose inappropriate and constitutive activation results in the formation of stomach neoplasias [50]. TGF-β is a potent inhibitor of the cytolytic activity of NK cells, presumably by attenuating the activation of their NKp30 and NKD2D receptors, and by inhibiting their production of interferon-γ. In addition, TGF-β also represses the activities of dendritic cells by inhibiting their expression of MHC class II, CD40, CD80, and CD86, and TNF-α, IL-12, and CCL5/Rantes [30,39,40,51]. Mast cells are actively recruited to tumor microenvironments by TGF-β where they synthesize and secrete numerous tumor promoting factors, including histamine, proteases, and cytokines (*e.g.*, VEGF and TGF-β) [40,52]. Lastly, TGF-β stimulates monocytes and macrophage chemotaxis to tumor microenvironments, leading to enhanced tumor invasion, angiogenesis, and metastasis, and to diminished antigen presentation and immunosurveillance towards developing neoplasms [53,54].

1.3. TGF-β and Endothelial Cells

Angiogenesis is the process whereby new blood vessels sprout and form from preexisting vessels; it also is an essential physiological process that transpires during embryonic development, wound healing, and the female reproductive cycle [55,56]. The initiation of pathological angiogenesis has been linked to numerous human diseases, including rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration [56,57]. Interestingly, all solid tumors larger than 1 cm^3 suffer from hypoxia [58], and as such, initiate angiogenesis as a means of acquiring an efficient supply of nutrients and waste removal, as well as a route for their metastasis to distant locales. Two distinct phases are involved in angiogenesis, namely angiogenic activation and resolution. During the activation phase of angiogenesis, ECs initially exhibit increased vessel permeability and elevated rates of cell proliferation, migration, and invasion. In addition, new vessel sprouting is further enhanced by a reduction in EC adhesion, coupled to an alteration in basement membrane integrity. In contrast, angiogenic resolution essentially restores activated ECs to their resting, quiescent phenotypes, as well as promotes the recruitment of perivascular cells that maintain vessel stability and hemodynamics [55–57].

TGF-β plays critical roles in regulating both the activation and resolution phases of angiogenesis [59–62]. Indeed, homozygous deletion of various components of the TGF-β signaling system in mice routinely results in the appearance of vascular and EC defects, particularly in animals lacking TGF-β1 [63], TβR-I [64], TβR-II [65,66], TβR-III [67,68], Smad1 [69], or Smad5 [70]. In humans, loss or inactivation of endoglin leads to hereditary

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

hemorrhagic telangiectasia type 1 (HHT1) [71,72], while that of ALK1 results in HHT2 [73, 74]. Moreover, the defects associated with HHT1 and HHT2 in humans are phenocopied in knockout mice lacking expression of either endoglin [75,76] or ALK1 [77–79], respectively. Thus, altered expression and/or activity of TGF-β in tumor microenvironments clearly will impact the ability of hypoxic tumors overcome this impediment to their growth and survival.

ECs have been reported to express two distinct TβR-Is, namely TβR-I/Alk5 and ALK1. The importance of these two receptors in mediating vessel development by TGF-β is evidenced by the embryonic lethality observed at day E11.5 and E10.5 in mice lacking ALK1 [79] or ALK5 [64], respectively. Recent evidence also suggests that these two type I receptors differentially regulate the coupling of TGF-β to angiogenic activation and resolution. For instance, TβRI/ALK5 activation stimulates Smad2/3 and the subsequent expression of genes operant in mediating vessel maturation, including plasminogen activator inhibitor 1 (PAI-1) and fibronectin [78,80,81]. Moreover, microarray gene expression analyses of EC cells before and after their stimulation with TGF-β confirmed that the activation of a TGF-β:TβR-I/ALK5:Smad2/3 signaling axis does indeed promote angiogenic resolution [61,82]. In contrast, ALK1 activation stimulates Smad1/5 and the subsequent expression of genes operant in mediating angiogenesis activation, including Id1 and interleukin 1 receptor-like 1 [78,80–82]. Moreover, ALK-1 signaling stimulated by TGF-β requires this cytokine to initially activate TβR-II and ALK-5, which then recruit and activate ALK-1 following its association with TβR-II:ALK-5:TGF-β ternary complexes [78]. Thus, activation of ALK-1 and the induction of angiogenesis by TGF-β must first proceed through its assembly of angiostatic TGF-β receptor complexes (*i.e.*, TβR-II:ALK-5). At present, the molecular mechanisms that initially exclude and then recruit ALK-1 to angiostatic TGF-β receptor complexes remain unknown, but may reflect a delicate balance between TGF-β and other angiogenic factors located within tumor microenvironments. Indeed, low TGF-β concentrations enhance the ability of bFGF and VEGF to stimulate EC proliferation and angiogenic sprouting, while high TGF-β concentrations inhibit these angiogenic activities [62,83]. Along these lines, the pro-angiogenic functions of TGF-β also have been linked to its ability to regulate the expression and/or activities of other angiogenic factors, such as bFGF and VEGF [84]. It is interesting to note that inclusion of TGF-β to Matrigel plugs implanted into mice only promotes angiogenesis and vessel development in the presence of bFGF and its ability to create a pro-angiogenic microenvironment (M. Tian and W.P. Schiemann, *unpublished observation*). Thus, it is plausible that the recruitment of ALK-1 to angiostatic TGF-β receptor complexes may first require the stimulation of accessory angiogenic signals or proteins within activated EC microenvironments. Along these lines, the coupling of TGF-β to angiogenesis is controlled by the presence of endoglin, whose expression is induced by ALK1 and serves to promote EC proliferation, migration, and tubulogenesis by antagonizing the activities of TβR-I/ALK5 [60,82].

Collectively, these studies highlight the complexities associated with the ability of TGF-β to regulate EC activities coupled to angiogenesis. Future studies clearly need to *(i)* better define the precise mechanisms that enable TGF-β and its downstream effectors to govern the induction of angiogenic or angiostatic gene expression profiles; *(ii)* establish the impact of EC and perivascular cell differentiation states to influence the angiogenic response to TGF-β; and *(iii)* identify the microenvironmental cues and signals the cooperate with TGF-β in mediating angiogenesis activation and resolution.

2. TGF-β, EMT, and Metastasis

The acquisition of invasive and metastatic phenotypes by carcinomas ushers in their transition from indolent to aggressive disease states, during which time immotile, polarized epithelial cells undergo EMT and transdifferentiate into highly motile, apolar fibroblastoid-like cells

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

[85–87]. In doing so, post-EMT carcinoma cells remodel their ECM and microenvironments in a manner that facilitates their intravasation into the vascular or lymphatic systems, as well as their extravasation at distant locales to form micrometastases that ultimately develop into secondary carcinomas [88]. Interestingly, a recent study identified a set of potential metastatic gene signature whose expression is highly associated with the acquisition of pulmonary metastasis by human breast cancers [89]. Included in this metastatic gene signatures are IDs (**I**nhibitor of **D**ifferentiation) 1 and 3, which mediate constitutive proliferative signals in newly established pulmonary micrometastases [89]. In addition, the ability of TGF-β to induce ANGPTL4 (angiopoietin-like 4) expression in breast cancer cells enables their retention, extravasation, and colonization specifically in the lungs, not the bone [90]. Pathological reactivation of EMT programs in differentiated cells and tissues not only promotes their invasion and metastasis, but also underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders, all of which are characterized by dysregulated microenvironmental signaling [85–88,91,92]. In the following sections, we summarize recent developments linking TGF-β to the induction of EMT and metastasis, to the selection and expansion of cancer stem cells, and to the regulation of microRNA expression in developing and progressing neoplasms.

2.1. TGF-β Signaling and EMT

2.1.1. Canonical TGF-β Effectors and EMT—The ability of TGF-β to induce EMT and metastasis transpires through the activation of canonical (*i.e.*, Smad2/3-dependent) and noncanonical (*i.e.*, Smad2/3-independent) TGF-β signaling inputs. For instance, inactivating canonical TGF-β signaling in human MCF10ACA1a breast cancer cells by engineering their expression of a dominant-negative Smad3 construct [93] or a TβR-I mutant incapable of activating Smad2/3 (*i.e.*, L45 mutant) [94] significantly reduced their ability to colonize the lung. Along these lines, Smad4-deficiency not only diminished the expression of PTHrP, IL-11, and CTGF in human MDA-MB-231 breast cancer cells, but also abrogated their metastasis to bone in response to TGF-β [95–98]. Interestingly, whereas Smad4-deficiency cooperates with oncogenic K-Ras to induce the initiation and development of pancreatic cancer, the expression and activity of Smad4 is essential for TGF-β stimulation of pancreatic cancer EMT and growth [99]. Similar inactivation of canonical TGF-β signaling by overexpression of Smad7 [100, 101] prevents the invasion of breast [102] and head and neck cancers [103,104], as well as the pulmonary metastasis of melanomas [105]. Collectively, these findings highlight the importance of Smad2/3/4 signaling in mediating EMT and metastasis stimulated by TGF-β, and suggest the potential benefit of Smad2/3 antagonists to improve the clinical course of patients with metastatic disease.

2.1.2. Noncanonical TGF-β Effectors and EMT—Noncanonical TGF-β signaling also plays an essential role in mediating TGF-β stimulation of EMT, invasion, and metastasis [106]. Included in this growing list of noncanonical effectors targeted by TGF-β are Ras/MAP kinase [107–115], PI3K/AKT [116], Rho/ROCK [117], Jagged/Notch [118], mTOR [119], and Wnt/β-catenin [120]. Collaborative signaling events occurring between NF-κB and oncogenic Ras also mediate EMT and pulmonary extravasation of breast cancer cells in response to TGF-β [121]. Similarly, we identified TGF-β stimulation of NF-κB as an essential pathway operant in coupling TGF-β to the expression of Cox-2, whose activity and production of PGE2 are critical for EMT induced by TGF-β in normal and malignant MECs [122]. We [23–25] and others [108] also established integrins as key players in mediating EMT, invasion, and p38 MAPK activation by TGF-β, as well as its ability to stimulate the growth and pulmonary metastasis of breast cancers in mice [25]. Essential effectors targeted by the formation of integrin:TGF-β receptor signaling complexes are *(i)* the protein proto-oncogene Src and its phosphorylation of TβR-II at Y284, which creates a docking site for Grb2 and ShcA [23–25]; *(ii)* the adapter molecule Dab2, which facilitates TGF-β stimulation of Smad2/3 and FAK

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

[123,124]; and *(iii)* the protein tyrosine kinase FAK, which coordinates the formation of αvβ3 integrin:TβR-II complexes and, together with its effector p130Cas, is essential for TGF-β stimulation of breast cancer pulmonary metastasis in mice (M.K. Wendt and W.P. Schiemann, *unpublished observation*). In addition, αvβ3 integrin also mediates TGF-β-dependent metastasis of breast cancer cells to bone [125,126]. Collectively, these findings implicate TβR-II as an essential mediator of oncogenic signaling by TGF-β, particularly its ability to promote the acquisition of invasive and metastatic phenotypes at the expense of significantly impacting primary tumor growth [127]. Along these lines, a missense mutation in TβR-II identified in human head and neck carcinomas was observed to promote their EMT and invasion in part *via (i)* hyperactive protein kinase activity in mutant TβR-II proteins, and *(ii)* inappropriate coupling of TGF-β receptors to Smad1/5 activation, as opposed to Smad2/3 [128]. Interestingly, following its phosphorylation by TβR-II, the tight-junction assembly protein, PAR-6, associates with TβR-I and coordinates the ubiquitination and degradation of RhoA by Smurf1 [129]. The net effect of these TGF-β-dependent events results in the dissolution of epithelial cell tight junctions and the disassembly of their actin cytoskeleton, leading to the induction of EMT.

2.2. TGF-β and Cancer Stem Cells

It is important to note that EMT is a normal and essential physiological process that directs tissue development and morphogenesis in the embryo, as well as promotes the healing, remodeling, and repair of injured tissues in adults [85–87]. Thus, tumorigenic EMT in many respects reflects the inappropriate reactivation of embryonic and morphologic gene expression programs, and as such, points towards a potential link between EMT and the maintenance of stem cell properties. Accordingly, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [130], while human and mouse MECs induced to undergo EMT acquire stem cell-like properties in part *via* activation of the TGF-β signaling system [131]. Because TGF-β is a master regulator of physiological and pathological EMT [91], these findings suggest that the conversion of TGF-β from a tumor suppressor to a tumor promoter mirrors its ability to induce the selection and expansion of stem cell-like progenitors in post-EMT cells. In fact, TGF-β treatment of malignant, but nonmetastatic human breast cancer cells suppressed their tumorigenicity by diminishing the size of the cancer stem cell pool, and by reducing ID1 expression that results in the differentiation of the progenitor pool [132]. Thus, uncoupling TGF-β from regulation of ID1 expression may dictate whether TGF-β either promotes or suppresses the maintenance and/or expansion of cancer stem cells. Indeed, pharmacological inhibition of TGF-β signaling in cancer stem cells induced a mesenchymal-epithelial transition that resulted in their acquisition of a more epithelial-like morphology [133]. Along these lines, Future studies clearly need to *(i)* identify the molecular mechanisms that link TGF-β and EMT to the generation of cancer stem cells, and *(ii)* establish the therapeutic impact of TGF-β in promoting chemoresistance *via* its stimulation of EMT and the expansion of cancer stem cells.

2.3. TGF-β and microRNAs

Finally, accumulating evidence now positions microRNAs as potentially important regulators of the “TGF-β Paradox.” Indeed, expression of miR-21 in breast cancers predicts for elevated TGF-β1 expression and a poor clinical prognosis [134], while that in gliomas results in the suppression of multiple components of the TGF-β signaling system, including its ligands (*e.g.*, TGF-βs 1 and 3), its receptors (*e.g.*, TβR-II and TβR-III), and its effector molecules (*e.g.*, Smad3, Daxx, and PDCD4) [135,136]. Recently, TGF-β was shown to promote contractile phenotypes in vascular smooth muscle cells by stimulating the processing of primary miR-21 transcripts into their pre-miR-21 counterparts *via* the formation of Smad2/3:DROSHA complexes. In doing so, cellular levels of miR-21 accumulate rapidly, resulting in diminished expression of PDCD4 (**p**rogrammed **c**ell **d**eath **4**) and its inability to

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

suppress contractile machinery expression in vascular smooth muscle cells [136]. Similar induction of miR-21 expression took place in Smad4-deficient carcinoma cells, suggesting that TGF-β-regulated miR processing also takes place in epithelial cells in a manner independent of Smad4 [136]. Moreover, miR-21 expression also functions to promote EMT stimulated by TGF-β [137], although the molecular mechanisms underlying this event remain to be determined definitively. In contrast to miR-21 and its role in promoting EMT by TGF-β, microRNA-200 family members and miR-205 function in maintaining epithelial cell polarity and, consequently, in suppressing EMT. Importantly, the ability of TGF-β to induce EMT first requires this cytokine to downregulate microRNA-200 family member and miR-205 expression, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [138]. Thus, aberrant microRNA expression may play a significant role in determining whether epithelial cells sense and respond to the tumor suppressing functions of TGF-β, or rather to its oncogenic activities.

Conclusions and Future Perspectives

Despite considerable progress over the last decade in defining the molecular mechanisms that underlie the initiation and maintenance of the "TGF-β Paradox," science and medicine still lack the necessary knowledge and wherewithal to explain and, more importantly, to manipulate the physiopathological actions of TGF-β to improve the clinical course of human malignancies. While it is abundantly clear that TGF-β plays a major role, both directly and indirectly, in regulating the ability of cancer cells to acquire each of the 6 hallmarks necessary for their malignant progression [139], it remains unclear as to how these events conspire in regulating the response of developing and progressing neoplasms to TGF-β. For instance, defects in TGF-β function rarely effect primary tumor growth, but more commonly play a significant role in enabling cancer cells to acquire EMT and invasive/metastatic phenotypes. Thus, while it is easy to rationalize why tumors require TGF-β to provide them with a selective EMT and metastatic advantage, teleologically it remains troublesome to assume that these phenotypic changes induced by TGF-β are permanently ingrained in aggressive carcinoma cells. Indeed, cancer cells perpetually locked into a "vagabond" mentally is counterintuitive to the processes underlying metastasis development and the formation of secondary carcinomas at distant locales. Instead, it appears that the exquisite balance between the functions and behaviors of TGF-β in distinct tissue types become unbalanced and incapable of suppressing disease development, particularly that of neoplastic transformation. Along these lines, the processes underlying the maintenance of normal tissue and cellular homeostasis have been liken to those necessary in facilitating the existence of a well-balanced and harmonious society [140]. The studies highlighted herein are consistent with a role for TGF-β in serving either as a benevolent or corrupt village manager, one whose ultimate agenda is dictated by the prevailing mood of the village's stromal and microenvironmental constituents. Thus, these findings also underscore and reinforce the need to develop novel pharmacological agents designed to antagonize the oncogenic activities of TGF-β in cancer cells, as well as in their supporting stromal compartments.

Executive Summary

TGF-β and the Tumor Microenvironment Fibroblasts

- Tumor reactive stroma plays a critical role in determining whether TGF-β suppresses or promotes tumor formation.
- Loss and/or disruption of paracrine signaling systems between fibroblast and adjacent epithelial cells results in cellular transformation, and in the progression of developing neoplasms.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

- Similar inactivation of TGF-β function in epithelial cells also elicits aberrant epithelial:fibroblast paracrine signaling networks that drives malignancy.
- TGF-β stimulation of desmoplastic and fibrotic reactions promotes the formation of stiff, noncompliant microenvironments that select for the expansion of metastatic cells.
- LOX family members are essential for desmoplasia induced by TGF-β, and for stimulating breast cancer metastasis in hypoxic tumor environments.
- Fibrotic reactions enhance TGF-β signaling and may facilitate tumor protection to radiotherapies.

TGF-β and Immunosurveillance

- TGF-β is a potent suppressor of inflammation and immune suppression.
- Similar to fibroblasts, altered paracrine signaling by immune cell contributes to tumor formation, particularly that in the gastrointestinal track.
- TGF-β is a potent inhibitor of adaptive immunity, which contributes to weaken host immunosurveillance.
- TGF-β also is a potent activator of innate immunity, which contributes to carcinoma progression and metastasis.

TGF-β and Endothelial Cells

- Angiogenesis is the process whereby new blood vessels develop from pre-existing vessels.
- Angiogenesis also provides cancer cells a route for their metastatic spread.
- Aberrant TGF-β signaling elicits developmental vascular defects that typically result in embryonic lethality.
- Human hereditary hemorrhagic telangiectasia (HTT) is phenocopied in mice lacking expression of either ALK1 or endoglin.
- TGF-β regulates both the activation and resolution of angiogenesis by differential activation of ALK1 (*i.e*., pro-angiogenic *via* Smad1/5/8 activation) or TβR-I/ALK5 (*i.e*., anti-angiogenic *via* Smad2/3 activation).
- Activation of ALK1 by TGF-β requires the presence of ALK5 and TβR-II.

TGF-β, EMT, and Metastasis

- EMT is a process whereby polarized, immotile epithelial cells transdifferentiate into apolar, highly motile fibroblastoid-like cells.
- TGF-β is a master regulator of normal and tumorigenic EMT.
- EMT is essential for the acquisition of invasive and metastatic phenotypes in carcinoma cells.
- TGF-β induces EMT *via* stimulation of canonical (*i.e*., Smad2/3) and noncanonical (*i.e*., Ras/MAP kinases, PI3K, AKT, and Rho/ROCK) pathways.
- β3 integrin, Src, and p38 MAPK are essential in facilitating EMT stimulated by TGF-β.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

- Aberrant expression of microRNAs in response to TGF-β may drive EMT and metastasis.
- EMT induced by TGF-β may play important roles in generating chemoresistant cancer stem cells.

Acknowledgements

Members of the Schiemann Laboratory are thanked for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA114039 and CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651).

References

1. Bierie B, Moses HL. Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer 2006;6:506–520. [PubMed: 16794634]
2. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat. Rev. Cancer 2004;4:839–849. [PubMed: 15516957]
3. Bhowmick NA, Chytil A, Plieth D, et al. TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 2004;303:848–851. [PubMed: 14764882]
4. Bhowmick NA, Moses HL. Tumor-stroma interactions. Curr. Opin. Genet. Dev 2005;15:97–101. [PubMed: 15661539]
5. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–337. [PubMed: 15549095]
6. Fidler IJ. Critical determinants of metastasis. Semin. Cancer Biol 2002;12:89–96. [PubMed: 12027580]
7. Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002;70:498–505. [PubMed: 12492492]
8. Cheng N, Bhowmick NA, Chytil A, et al. Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP-and HGF-mediated signaling networks. Oncogene 2005;24:5053–5068. [PubMed: 15856015]
9. Yang L, Huang J, Ren X, et al. Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1 +CD11b+ myeloid cells that promote metastasis. Cancer Cell 2008;13:23–35. [PubMed: 18167337]
10. Bruna A, Darken RS, Rojo F, et al. High TGF-β-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. Cancer Cell 2007;11:147–160. [PubMed: 17292826]
11. Paszek MJ, Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. J. Mammary Gland Biol. Neoplasia 2004;9:325–342. [PubMed: 15838603]
12. Anderson AR, Weaver AM, Cummings PT, Quaranta V. Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment. Cell 2006;127:905–915. [PubMed: 17129778]
13. Lucero HA, Kagan HM. Lysyl oxidase: an oxidative enzyme and effector of cell function. Cell. Mol. Life Sci 2006;63:2304–2316. [PubMed: 16909208]
14. Payne SL, Hendrix MJ, Kirschmann DA. Paradoxical roles for lysyl oxidases in cancer--a prospect. J. Cell. Biochem 2007;101:1338–1354. [PubMed: 17471532]
15. Erler JT, Giaccia AJ. Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res 2006;66:10238–10241. [PubMed: 17079439]
16. Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. J. Cell. Biochem 2003;88:660–672. [PubMed: 12577300]
17. Atsawasuwan P, Mochida Y, Katafuchi M, et al. Lysyl oxidase binds TGF-β and regulates its signaling *via* amine oxidase activity. J. Biol. Chem 2008;283:34229–34240. [PubMed: 18835815]
18. Kim DJ, Lee DC, Yang SJ, et al. Lysyl oxidase like 4, a novel target gene of TGF-β1 signaling, can negatively regulate TGF-beta1-induced cell motility in PLC/PRF/5 hepatoma cells. Biochem. Biophys. Res. Commun 2008;373:521–527. [PubMed: 18586005]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

19. Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006;440:1222–1226. [PubMed: 16642001]
20. Kirschmann DA, Seftor EA, Fong SF, et al. A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 2002;62:4478–4483. [PubMed: 12154058]
21. Payne SL, Fogelgren B, Hess AR, et al. Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism. Cancer Res 2005;65:11429–11436. [PubMed: 16357151]
22. Payne SL, Hendrix MJ, Kirschmann DA. Lysyl oxidase regulates actin filament formation through the p130(Cas)/Crk/DOCK180 signaling complex. J. Cell. Biochem 2006;98:827–837. [PubMed: 16440329]
23. Galliher AJ, Schiemann WP. β3 integrin and Src facilitate TGF-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 2006;8:R42. [PubMed: 16859511]
24. Galliher AJ, Schiemann WP. Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res 2007;67:3752–3758. [PubMed: 17440088]
25. Galliher-Beckley AJ, Schiemann WP. Grb2 Binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis 2008;29:244–251. [PubMed: 18174260]
26. Andarawewa KL, Paupert J, Pal A, Barcellos-Hoff MH. New rationales for using TGF-β inhibitors in radiotherapy. Int. J. Radiat. Biol 2007;83:803–811. [PubMed: 18058368]
27. Barcellos-Hoff MH, Medina D. New highlights on stroma-epithelial interactions in breast cancer. Breast Cancer Res 2005;7:33–36. [PubMed: 15642180]
28. Biswas S, Guix M, Rinehart C, et al. Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. J. Clin. Invest 2007;117:1305–1313. [PubMed: 17415413]
29. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J. Clin. Invest 2007;117:1175–1183. [PubMed: 17476347]
30. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. TGF-β regulation of immune responses. Annu. Rev. Immunol 2006;24:99–146. [PubMed: 16551245]
31. Gorelik L, Flavell RA. Abrogation of TGF-β signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity 2000;12:171–181. [PubMed: 10714683]
32. Kulkarni AB, Huh CG, Becker D, et al. TGF-β1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. U.S.A 1993;90:770–774. [PubMed: 8421714]
33. Yang X, Letterio JJ, Lechleider RJ, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J 1999;18:1280–1291. [PubMed: 10064594]
34. Kim BG, Li C, Qiao W, et al. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature 2006;441:1015–1019. [PubMed: 16791201]
35. Dalal BI, Keown PA, Greenberg AH. Immunohistochemical localization of secreted TGF-β1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. Am. J. Pathol 1993;143:381–389. [PubMed: 8393616]
36. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for TGF-β1 associates with disease progression in human breast cancer. Cancer Res 1992;52:6949–6952. [PubMed: 1458485]
37. Ivanovic V, Todorovic-Rakovic N, Demajo M, et al. Elevated plasma levels of TGF-β1 in patients with advanced breast cancer: Association with disease progression. Eur. J. Cancer 2003;39:454–461. [PubMed: 12751375]
38. Lin SJ, Chang C, Ng AK, Wang SH, Li JJ, Hu CP. Prevention of TGF-β-induced apoptosis by interlukin-4 through Akt activation and p70S6K survival signaling pathways. Apoptosis 2007;12:1659–1670. [PubMed: 17624592]
39. Teicher BA. TGF-β and the immune response to malignant disease. Clin. Cancer Res 2007;13:6247–6251. [PubMed: 17975134]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

40. Wrzesinski SH, Wan YY, Flavell RA. TGF-β and the immune response: implications for anticancer therapy. Clin. Cancer Res 2007;13:5262–5270. [PubMed: 17875754]
41. Chen, Ml; Pittet, MJ.; Gorelik, L., et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals *in vivo*. Cancer Res 2006;66:8617–8624. [PubMed: 16951175]
42. Gorelik L, Flavell RA. TGF-β in T-cell biology. Nat. Rev. Immunol 2002;2:46–53. [PubMed: 11905837]
43. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of TGF-β signaling in T cells. Nat. Med 2001;7:1118–1122. [PubMed: 11590434]
44. Zhang Q, Yang X, Pins M, et al. Adoptive transfer of tumor-reactive TGF-β-insensitive CD8+ T cells: Eradication of autologous mouse prostate cancer. Cancer Res 2005;65:1761–1769. [PubMed: 15753372]
45. Nam JS, Terabe M, Kang MJ, et al. TGF-β subverts the immune system into directly promoting tumor growth through interleukin-17. Cancer Res 2008;68:3915–3923. [PubMed: 18483277]
46. Neil JR, Schiemann WP. Altered TAB1:IκB kinase interaction promotes TGF-β-mediated NF-κB activation during breast cancer progression. Cancer Res 2008;68:1462–1470. [PubMed: 18316610]
47. Lu T, Tian L, Han Y, Vogelbaum M, Stark GR. Dose-dependent cross-talk between the TGF-β and interleukin-1 signaling pathways. Proc. Natl. Acad. Sci. U.S.A 2007;104:4365–4370. [PubMed: 17360530]
48. Fries G, Perneczky A, Kempski O. Enhanced interleukin-1β release and longevity of glioma-associated peripheral blood monocytes in vitro. Neurosurgery 1994;35:264–270. [PubMed: 7969834]
49. Griffin BD, Moynagh PN. Persistent interleukin-1β signaling causes long term activation of NF-κB in a promoter-specific manner in human glial cells. J. Biol. Chem 2006;281:10316–10326. [PubMed: 16455661]
50. Tu S, Bhagat G, Cui G, et al. Overexpression of interleukin-1β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell 2008;14:408–419. [PubMed: 18977329]
51. Larmonier N, Cathelin D, Larmonier C, et al. The inhibition of TNF-α anti-tumoral properties by blocking antibodies promotes tumor growth in a rat model. Exp. Cell Res 2007;313:2345–2355. [PubMed: 17466973]
52. Conti P, Castellani MI, Kempuraj D, et al. Role of mast cells in tumor growth. Ann. Clin. Lab. Sci 2007;37:315–321. [PubMed: 18000287]
53. Lin EY, Li JF, Gnatovskiy L, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006;66:11238–11246. [PubMed: 17114237]
54. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer 2004;4:71–78. [PubMed: 14708027]
55. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat. Med 2000;6:389–395. [PubMed: 10742145]
56. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249–257. [PubMed: 11001068]
57. Carmeliet P. Angiogenesis in health and disease. Nat. Med 2003;9:653–660. [PubMed: 12778163]
58. Erler JT, Weaver VM. Three-dimensional context regulation of metastasis. Clin. Exp. Metastasis. 2008PMID: 18814043
59. Bertolino P, Deckers M, Lebrin F, ten Dijke P. TGF-β signal transduction in angiogenesis and vascular disorders. Chest 2005;128:585S–590S. [PubMed: 16373850]
60. Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-β receptor function in the endothelium. Cardiovasc. Res 2005;65:599–608. [PubMed: 15664386]
61. Pepper MS. TGF-β: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev 1997;8:21–43. [PubMed: 9174661]
62. Pepper MS, Vassalli JD, Orci L, Montesano R. Biphasic effect of TGF-β1 on *in vitro* angiogenesis. Exp. Cell Res 1993;204:356–363. [PubMed: 7679998]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

63. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in TGF-β1 knock out mice. Development 1995;121:1845–1854. [PubMed: 7600998]
64. Larsson J, Goumans MJ, Sjostrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-β type I receptor-deficient mice. EMBO J 2001;20:1663–1673. [PubMed: 11285230]
65. Goumans MJ, Zwijsen A, van Rooijen MA, Huylebroeck D, Roelen BA, Mummery CL. TGF-β signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice. Development 1999;126:3473–3483. [PubMed: 10409495]
66. Oshima M, Oshima H, Taketo MM. TGF-β receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev. Biol 1996;179:297–302. [PubMed: 8873772]
67. Compton LA, Potash DA, Brown CB, Barnett JV. Coronary vessel development is dependent on the type III TGF-β receptor. Circ. Res 2007;101:784–791. [PubMed: 17704211]
68. Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-β receptor for endocardial cell transformation in the heart. Science 1999;283:2080–2082. [PubMed: 10092230]
69. Lechleider RJ, Ryan JL, Garrett L, et al. Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. Dev. Biol 2001;240:157–167. [PubMed: 11784053]
70. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. Development 1999;126:1631–1642. [PubMed: 10079226]
71. McAllister KA, Grogg KM, Johnson DW, et al. Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat. Genet 1994;8:345–351. [PubMed: 7894484]
72. Shovlin CL, Hughes JM, Scott J, Seidman CE, Seidman JG. Characterization of endoglin and identification of novel mutations in hereditary hemorrhagic telangiectasia. Am. J. Hum. Genet 1997;61:68–79. [PubMed: 9245986]
73. Berg JN, Gallione CJ, Stenzel TT, et al. The activin receptor-like kinase 1 gene: Genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. Am. J. Hum. Genet 1997;61:60–67. [PubMed: 9245985]
74. Johnson DW, Berg JN, Baldwin MA, et al. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat. Genet 1996;13:189–195. [PubMed: 8640225]
75. Arthur HM, Ure J, Smith AJ, et al. Endoglin, an ancillary TGF-β receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev. Biol 2000;217:42–53. [PubMed: 10625534]
76. Bourdeau A, Dumont DJ, Letarte M. A murine model of hereditary hemorrhagic telangiectasia. J. Clin. Invest 1999;104:1343–1351. [PubMed: 10562296]
77. Srinivasan S, Hanes MA, Dickens T, et al. A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. Hum. Mol. Genet 2003;12:473–482. [PubMed: 12588795]
78. Goumans MJ, Valdimarsdottir G, Itoh S, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF-β/ALK5 signaling. Mol. Cell 2003;12:817–828. [PubMed: 14580334]
79. Oh SP, Seki T, Goss KA, et al. Activin receptor-like kinase 1 modulates TGF-β1 signaling in the regulation of angiogenesis. Proc. Natl. Acad. Sci. U.S.A 2000;97:2626–2631. [PubMed: 10716993]
80. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium *via* two distinct TGF-β type I receptors. EMBO J 2002;21:1743–1753. [PubMed: 11927558]
81. Wu X, Ma J, Han JD, Wang N, Chen YG. Distinct regulation of gene expression in human endothelial cells by TGF-β and its receptors. Microvasc. Res 2006;71:12–19. [PubMed: 16405919]
82. Ota T, Fujii M, Sugizaki T, et al. Targets of transcriptional regulation by two distinct type I receptors for TGF-β in human umbilical vein endothelial cells. J. Cell Physiol 2002;193:299–318. [PubMed: 12384983]
83. Fajardo LF, Prionas SD, Kwan HH, Kowalski J, Allison AC. TGF-β1 induces angiogenesis *in vivo* with a threshold pattern. Lab. Invest 1996;74:600–608. [PubMed: 8600310]
84. Goumans MJ, Lebrin F, Valdimarsdottir G. Controlling the angiogenic switch: a balance between two distinct TGF-β receptor signaling pathways. Trends Cardiovasc. Med 2003;13:301–307. [PubMed: 14522471]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

85. Savagner P. Leaving the neighborhood: Molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001;23:912–923. [PubMed: 11598958]

86. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer 2002;2:442–454. [PubMed: 12189386]

87. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr. Opin. Cell Biol 2003;15:740–746. [PubMed: 14644200]

88. Zavadil J, Bottinger EP. TGF-β and epithelial-to-mesenchymal transitions. Oncogene 2005;24:5764–5774. [PubMed: 16123809]

89. Gupta GP, Perk J, Acharyya S, et al. ID genes mediate tumor reinitiation during breast cancer lung metastasis. Proc. Natl. Acad. Sci. U.S.A 2007;104:19506–19511. [PubMed: 18048329]

90. Padua D, Zhang XH, Wang Q, et al. TGF-β primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 2008;133:66–77. [PubMed: 18394990]

91. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007;98:1512–1520. [PubMed: 17645776]

92. Willis BC, Borok Z. TGF-β-induced EMT: Mechanisms and implications for fibrotic lung disease. Am. J. Physiol. Lung Cell. Mol. Physiol 2007;293:L525–L534. [PubMed: 17631612]

93. Tian F, DaCosta Byfield S, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 2003;63:8284–8292. [PubMed: 14678987]

94. Tian F, Byfield SD, Parks WT, et al. Smad-binding defective mutant of TGF-β type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 2004;64:4523–4530. [PubMed: 15231662]

95. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc. Natl. Acad. Sci. U.S.A 2005;102:13909–13914. [PubMed: 16172383]

96. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537–549. [PubMed: 12842083]

97. Yin JJ, Selander K, Chirgwin JM, et al. TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J. Clin. Invest 1999;103:197–206. [PubMed: 9916131]

98. Deckers M, van Dinther M, Buijs J, et al. The tumor suppressor Smad4 is required for TGF-β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res 2006;66:2202–2209. [PubMed: 16489022]

99. Bardeesy N, Cheng KH, Berger JH, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev 2006;20:3130–3146. [PubMed: 17114584]

100. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGF-β receptor and functions as an antagonist of TGF-β signaling. Cell 1997;89:1165–1173. [PubMed: 9215638]

101. Souchelnytskyi S, Nakayama T, Nakao A, et al. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and TGF-β receptors. J. Biol. Chem 1998;273:25364–25370. [PubMed: 9738003]

102. Azuma H, Ehata S, Miyazaki H, et al. Effect of Smad7 expression on metastasis of mouse mammary carcinoma JygMC(A) cells. J. Natl. Cancer Inst 2005;97:1734–1746. [PubMed: 16333029]

103. Leivonen SK, Ala-Aho R, Koli K, Grenman R, Peltonen J, Kahari VM. Activation of Smad signaling enhances collagenase-3 (MMP-13) expression and invasion of head and neck squamous carcinoma cells. Oncogene 2006;25:2588–2600. [PubMed: 16407850]

104. Leivonen SK, Kahari VM. TGF-β signaling in cancer invasion and metastasis. Int. J. Cancer 2007;121:2119–2124. [PubMed: 17849476]

105. Javelaud D, Mohammad KS, McKenna CR, et al. Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis. Cancer Res 2007;67:2317–2324. [PubMed: 17332363]

106. Moustakas A, Heldin CH. Non-Smad TGF-β signals. J. Cell Sci 2005;118:3573–3584. [PubMed: 16105881]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

107. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGF-β-mediated fibroblastic transdifferentiation and cell migration. J. Cell Sci 2002;115:3193–3206. [PubMed: 12118074]
108. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin β1 signaling is necessary for TGF-β activation of p38MAPK and epithelial plasticity. J. Biol. Chem 2001;276:46707–46713. [PubMed: 11590169]
109. Cui W, Fowlis DJ, Bryson S, et al. TGF-β1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell 1996;86:531–542. [PubMed: 8752208]
110. Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-β1 involves MAPK, Smad and AP-1 signalling pathways. J. Cell. Biochem 2005;95:918–931. [PubMed: 15861394]
111. Ellenrieder V, Hendler SF, Boeck W, et al. TGF-β1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res 2001;61:4222–4228. [PubMed: 11358848]
112. Fowlis DJ, Cui W, Johnson SA, Balmain A, Akhurst RJ. Altered epidermal cell growth control *in vivo* by inducible expression of TGF-β1 in the skin of transgenic mice. Cell Growth Differ 1996;7:679–687. [PubMed: 8732677]
113. Janda E, Lehmann K, Killisch I, et al. Ras and TGF-β cooperatively regulate epithelial cell plasticity and metastasis: Dissection of Ras signaling pathways. J. Cell Biol 2002;156:299–313. [PubMed: 11790801]
114. Lehmann K, Janda E, Pierreux CE, et al. Raf induces TGF-β production while blocking its apoptotic but not invasive responses: A mechanism leading to increased malignancy in epithelial cells. Genes Dev 2000;14:2610–2622. [PubMed: 11040215]
115. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat. Cell Biol 2002;4:487–494. [PubMed: 12105419]
116. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for TGF-β-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem 2000;275:36803–36810. [PubMed: 10969078]
117. Bhowmick NA, Ghiassi M, Bakin A, et al. TGF-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell 2001;12:27–36. [PubMed: 11160820]
118. Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP. Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J 2004;23:1155–1165. [PubMed: 14976548]
119. Lamouille S, Derynck R. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J. Cell Biol 2007;178:437–451. [PubMed: 17646396]
120. Kim K, Lu Z, Hay ED. Direct evidence for a role of β-catenin/LEF-1 signaling pathway in induction of EMT. Cell Biol. Int 2002;26:463–476. [PubMed: 12095232]
121. Huber MA, Azoitei N, Baumann B, et al. NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J. Clin. Invest 2004;114:569–581. [PubMed: 15314694]
122. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanism. Carcinogenesis 2008;29:2227–2235. [PubMed: 18725385]
123. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the TGF-β receptors to the Smad pathway. EMBO J 2001;20:2789–2801. [PubMed: 11387212]
124. Prunier C, Howe PH. Disabled-2 (Dab2) is required for TGF-β-induced epithelial to mesenchymal transition (EMT). J. Biol. Chem 2005;280:17540–17548. [PubMed: 15734730]
125. Bandyopadhyay A, Agyin JK, Wang L, et al. Inhibition of pulmonary and skeletal metastasis by a TGF-β type I receptor kinase inhibitor. Cancer Res 2006;66:6714–6721. [PubMed: 16818646]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

126. Sloan EK, Pouliot N, Stanley KL, et al. Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. Breast Cancer Res 2006;8:R20. [PubMed: 16608535]

127. Muraoka-Cook RS, Kurokawa H, Koh Y, et al. Conditional overexpression of active TGF-β1 *in vivo* accelerates metastases of transgenic mammary tumors. Cancer Res 2004;64:9002–9011. [PubMed: 15604265]

128. Bharathy S, Xie W, Yingling JM, Reiss M. Cancer-associated TGF-β type II receptor gene mutant causes activation of bone morphogenic protein-Smads and invasive phenotype. Cancer Res 2008;68:1656–1666. [PubMed: 18339844]

129. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGF-β receptors controls epithelial cell plasticity. Science 2005;307:1603–1609. [PubMed: 15761148]

130. Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat. Genet 2008;40:499–507. [PubMed: 18443585]

131. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–715. [PubMed: 18485877]

132. Tang B, Yoo N, Vu M, et al. TGF-β can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res 2007;67:8643–8652. [PubMed: 17875704]

133. Shipitsin M, Campbell LL, Argani P, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259–273. [PubMed: 17349583]

134. Qian B, Katsaros D, Lu L, et al. High miR-21 expression in breast cancer associated with poor disease-free survival in early stage disease and high TGF-β1. Breast Cancer Res. Treat. 2008PMID: 18932017

135. Papagiannakopoulos T, Shapiro AKosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res 2008;68:8164–8172. [PubMed: 18829576]

136. Davis BN, Hilyard AC, Lagna GHata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 2008;454:56–61. [PubMed: 18548003]

137. Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ. TGF-β and microRNA:mRNA regulatory networks in epithelial plasticity. Cells Tissues Organs 2007;185:157–161. [PubMed: 17587821]

138. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol 2008;10:593–601. [PubMed: 18376396]

139. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57–70. [PubMed: 10647931]

140. Rizki A, Bissell MJ. Homeostasis in the breast: It takes a village. Cancer Cell 2004;6:1–2. [PubMed: 15261134]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

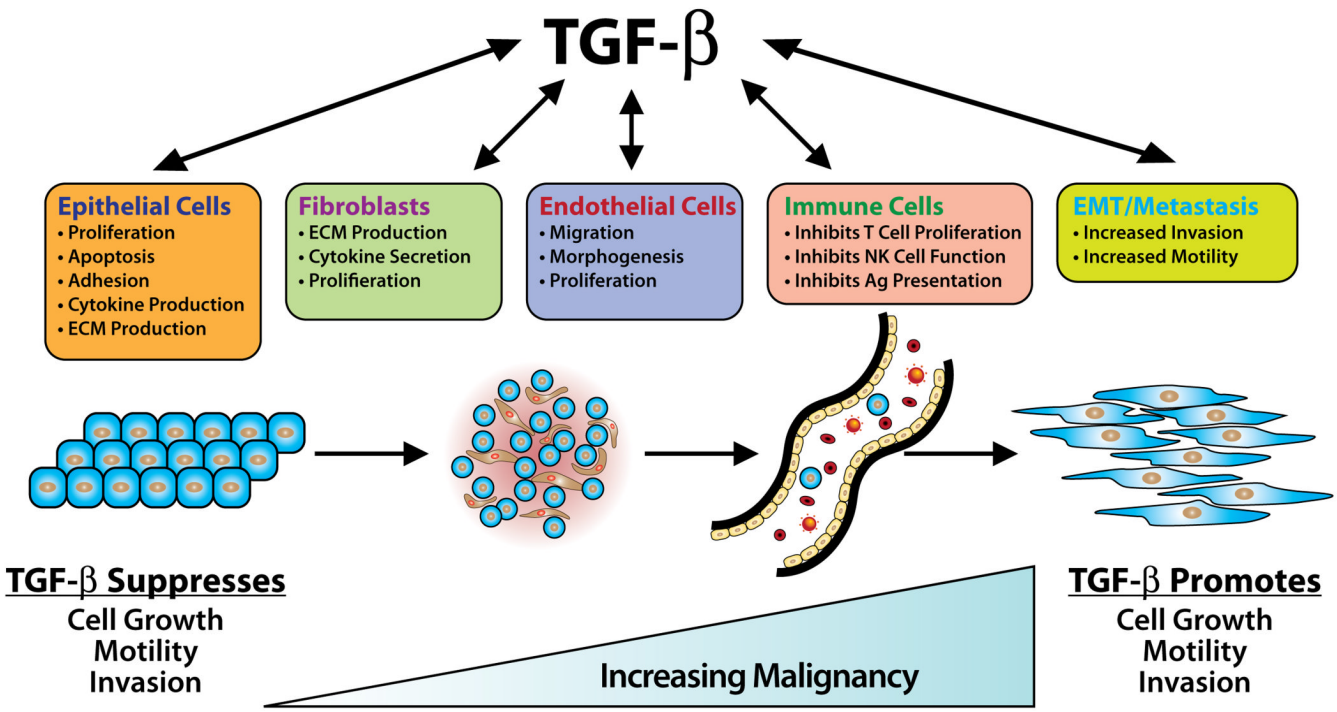


Figure 1. Cellular Targets of TGF-β During the Development and Progression of Human Cancers

TGF-β is a multifunctional cytokine that normally suppresses cell proliferation, differentiation, and apoptosis, as well as regulates cell and tissue homeostasis. Under normal physiological conditions, TGF-β functions as a tumor suppressor by preventing the ability of cells to progress through the cell cycle, or by stimulating the ability of cells to undergo apoptosis or differentiation. However, genetic and epigenetic events that transpire during tumorigenesis can convert TGF-β from a tumor suppressor to a tumor promoter, particularly the ability of cancer cells to acquire invasive and metastatic phenotypes. The oncogenic activities of TGF-β also are coordinated by dysregulated autocrine and paracrine signaling networks that take place between epithelial, fibroblasts, endothelial, and immune cells, that collectively promote tumor angiogenesis, invasion, and metastasis, and inhibit host immunosurveillance within tumor microenvironments. See text for specific examples of how TGF-β signaling becomes dysregulated during tumorigenesis

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 49, pp. 34145–34156, December 4, 2009
© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

p130Cas Is Required for Mammary Tumor Growth and Transforming Growth Factor-β-mediated Metastasis through Regulation of Smad2/3 Activity*[S]

Received for publication, May 20, 2009, and in revised form, October 10, 2009 Published, JBC Papers in Press, October 12, 2009, DOI 10.1074/jbc.M109.023614

Michael K. Wendt, Jason A. Smith, and William P. Schiemann[1]

From the Department of Pharmacology, University of Colorado, Denver, Anschutz Medical Campus, Aurora, Colorado 80045

During breast cancer progression, transforming growth factor-β (TGF-β) switches from a tumor suppressor to a pro-metastatic molecule. Several recent studies suggest that this conversion in TGF-β function depends upon fundamental changes in the TGF-β signaling system. We show here that these changes in TGF-β signaling are concomitant with aberrant expression of the focal adhesion protein, p130Cas. Indeed, elevating expression of either the full-length (FL) or just the carboxyl terminus (CT) of p130Cas in mammary epithelial cells (MECs) diminished the ability of TGF-β1 to activate Smad2/3, but increased its coupling to p38 MAPK. This shift in TGF-β signaling evoked (i) resistance to TGF-β-induced growth arrest, and (ii) acinar filling upon three-dimensional organotypic cultures of p130Cas-FL or -CT expressing MECs. Furthermore, rendering metastatic MECs deficient in p130Cas enhanced TGF-β-stimulated Smad2/3 activity, which restored TGF-β-induced growth inhibition both *in vitro* and in mammary tumors produced in mice. Additionally, whereas elevating TβR-II expression in metastatic MECs had no affect on their phosphorylation of Smad2/3, this event markedly enhanced their activation of p38 MAPK, leading to increased MEC invasion and metastasis. Importantly, depleting p130Cas expression in TβR-II-expressing metastatic MECs significantly increased their activation of Smad2/3, which (i) reestablished the physiologic balance between canonical and noncanonical TGF-β signaling, and (ii) reversed cellular invasion and early mammary tumor cell dissemination stimulated by TGF-β. Collectively, our findings identify p130Cas as a molecular rheostat that regulates the delicate balance between canonical and noncanonical TGF-β signaling, a balance that is critical to maintaining the tumor suppressor function of TGF-β during breast cancer progression.

Invasion and metastasis are the most lethal characteristics of breast cancer (1, 2). Transforming growth factor-β (TGF-β)[2] is a powerful suppressor of mammary tumorigenesis, doing so through its ability to repress mammary epithelial cell (MEC) proliferation, as well as through its creation of cell microenvironments that inhibit MEC motility, invasion, and metastasis (2). During breast cancer progression, the tumor suppressing function of TGF-β is frequently subverted, thus transforming TGF-β from a suppressor of breast cancer formation to a promoter of its growth and metastasis (2–4). Unfortunately, how mammary tumorigenesis overcomes the cytostatic function of TGF-β remains incompletely understood, as does the manner in which developing breast cancers ultimately sense TGF-β as a pro-metastatic factor.

Transmembrane signaling by TGF-β commences upon binding to its type II receptor (TβR-II), which recruits and activates its type I receptor (TβR-I), which then phosphorylates and activates Smads 2 and 3. Following their activation, Smads 2 and 3 form heteromeric complexes with Smad4, which collectively translocate to the nucleus to regulate a multitude of transcriptional events and cellular responses (*i.e.* apoptosis, cytostasis, and homeostasis, (5, 6)). In addition to stimulating Smad2/3, TGF-β also activates several noncanonical signaling systems, including members of the MAP kinase family (*e.g.* ERK1/2, JNK, and p38 MAPK (7)). Interestingly, several studies suggest that genetic and epigenetic events cooperate with aberrant Smad2/3 activities and functions to facilitate the conversion of TGF-β from tumor suppressor to a tumor promoter (8, 9). However, these and other studies also present strong evidence implicating dysregulated activation of several noncanonical TGF-β effectors during this same switch in TGF-β function (10). Thus, deciphering the relative contribution of signaling imbalances that arise between Smad2/3-dependent and -independent TGF-β signaling systems is essential to enhancing our understanding of how TGF-β ultimately promotes the development and progression of mammary tumorigenesis.

Recently, we identified a critical αvβ3 integrin:pY284-TβR-II:Grb2 signaling axis that mediates TGF-β stimulation of MAP kinases in normal and malignant MECs, leading to their acquisition of epithelial-mesenchymal transition, invasive, and metastatic phenotypes both *in vitro* and *in vivo* (11–13). Moreover, activation of this oncogenic signaling axis by TGF-β requires

* This work was supported, in whole or in part, by National Institutes of Health Grant CA129359, Komen Foundation Grant BCTR0706967, Department of Defense Grant BC084651 (to W. P. S.), and American Cancer Society Grant PF-09-120-01-CSM (to M. K. W.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1] To whom correspondence should be addressed: MS-8303, University of Colorado Denver, Anschutz Medical Campus, RC1 South Tower, Rm. L18-6110, 12801 E. 17th Ave., P. O. Box 6511, Aurora, CO 80045. Tel.: 303-724-1541; Fax: 303-724-3663; E-mail: Bill.Schiemann@ucdenver.edu.

[2] The abbreviations used are: TGF-β, transforming growth factor-β; FAK, focal adhesion kinase; MEC, mammary epithelial cell; TβR-I, TGF-β type I receptor; TβR-II, TGF-β type II receptor; p130Cas, Crk-associated substrate; PAI-1, plasminogen activator inhibitor-1; NmuMG, normal murine mammary epithelial; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CMV, cytomegalovirus; shRNA, short hairpin RNA; β-gal, β-galactosidase; FL, full-length; CT, carboxyl-terminal.

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

β3 integrin to form complexes with TβR-II (11–13). Unfortunately, it remains uncertain as to whether this interaction is direct or facilitated through another scaffolding protein. As such, we sought to identify members of focal adhesion complexes as potential integrin effectors capable of contributing to altered TGF-β signaling.

p130Cas (Crk-associated substrate) functions as a molecular scaffold within focal adhesion complexes, and is readily phosphorylated by focal adhesion kinase (FAK) and Src (14). Additionally, p130Cas binds stably to a variety of signaling molecules, including the (i) protein-tyrosine kinases FAK, PYK2, Src, Fyn, and Abl; (ii) adaptor molecules Crk, CrkL, Trip6, and AJUBA; (iii) guanine nucleotide exchange factors AND34 and CG3; and (iv) the MAPK family member, JNK (15, 16). The extensive interactome of p130Cas ideally positions and enables this molecule to interpret and integrate a variety of signaling inputs arising from numerous receptor systems. Indeed, the biological importance of p130Cas is emphasized by studies showing that its genetic ablation in mice elicits embryonic lethality, whereas fibroblasts derived from p130Cas-deficient embryos exhibit drastically altered cytoskeletal architectures (17). Moreover, fibroblasts transformed by Src become significantly more invasive when engineered to simultaneously overexpress p130Cas (15). Patients with primary breast tumors expressing high levels of p130Cas (also known as breast cancer resistance-1) experience a more rapid disease recurrence and have a greater risk of resistance to tamoxifen therapy (18). Recent studies also indicate that specific overexpression of p130Cas/breast cancer resistance-1 expression can confer breast cancer resistance to adriamycin (19). Moreover, directed overexpression of p130Case in murine MECs significantly increased their proliferative and survival indices *in vivo*, as well as greatly reduced the latency of mammary tumors arising from murine mammary tumor virus-driven Her2/Neu expression in mice (20). This study also observed the expression of p130Cas to be up-regulated significantly in a subset of human breast cancer samples (20). Collectively, these findings highlight the critical roles played by p130Cas in regulating normal tissue morphogenesis, and in promoting breast cancer progression. With respect to TGF-β, a recent study identified p130Cas as a potential inhibitor of Smad3 function (21). However, the pathophysiological importance of this event, if any, in mediating the oncogenic activities of TGF-β and/or p130Cas during breast cancer progression remains to be established.

The objective of the present study was to determine the role of p130Cas in facilitating the acquisition of oncogenic signaling by TGF-β during breast progression. We show here that p130Cas expression is up-regulated significantly in metastatic breast cancer cells (murine 4T1/human MCF10A-Ca1a) as compared with their nonmetastatic counterparts (murine 67NR/human MCF10A). Moreover, increased p130Cas expression was consistent with a decrease in TGF-β1-induced Smad2/3 signaling. Indeed, overexpression of p130Cas in nonmetastatic MECs led to a decrease in Smad2/3 activity, whereas depletion of p130Cas in metastatic MECs increased Smad2/3 activity. Most importantly, we show for the first time that p130Cas is essential for TGF-β stimulation of breast cancer growth, invasion, and pulmonary dissemination in mice. Taken together, our findings establish p130Cas as a novel molecular rheostat that regulates the balance between canonical and noncanonical TGF-β signaling in developing mammary tumors, whose acquisition of metastatic phenotypes is potentiated by elevated p130Cas expression and its consequential disruption of homeostatic TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Normal murine NMuMG and metastatic 4T1 cells were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 4T1 cells were engineered to stably express firefly luciferase by transfection with pNifty-CMV-luciferase (22), followed by their selection and isolation with Zeocin (500 μg/ml; Invitrogen). The creation of 4T1 cells lacking p130Cas was accomplished by their transduction with lentiviral particles encoding either a scrambled (*i.e.* non-silencing shRNA) or murine-directed p130Cas shRNA (pLKO.1; Thermo Scientific, Huntsville, AL). The production of pLKO.1 lentiviral particles and their transduction into target cells was accomplished as described previously (23). In addition, NMuMG and 4T1 cells also were transduced with murine ecotropic retroviral particles that encoded for full-length p130Cas (pMSCV-Cas-FL), the carboxyl terminus of p130Cas (amino acids 544–874) (pMSCV-Cas-CT), or TβR-II (pMSCV-TβR-II), and the resulting polyclonal populations were selected by yellow fluorescence protein, or hygromycin resistance (200 μg/ml).

Cell Proliferation Assays—NMuMG and 4T1 cells were seeded in 96-well plates (10,000 cells/well) and allowed to adhere for 4 h, whereupon varying concentrations of TGF-β1 (0–5 ng/ml) were administered. Agonist stimulations were allowed to proceed for 48 h at 37 °C and cellular DNA was radiolabeled by inclusion of [^{3}H]thymidine (1 μCi/well) during the final 6 h of TGF-β1 treatment. Afterward, the amount of [^{3}H]thymidine incorporated into cellular DNA was quantified by scintillation counting.

Immunoblot Assays—NMuMG and 4T1 cells were lysed on ice in three-dimensional RIPA buffer (50 mM Tris, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 0.1% SDS (v/v), pH 7.4) supplemented with (i) protease inhibitor mixture (Sigma), and (ii) the phosphatase inhibitors sodium orthovanadate (10 mM), β-glycerophosphate (40 mM), and sodium fluoride (20 mM). Afterward, the resulting whole cell extracts were clarified by microcentrifugation prior to being immunoblotted with the following primary antibodies (dilution): (*a*) anti-phospho-p38 MAPK (1:500; Cell Signaling, Danvers, MA); (*b*) anti-p38 MAPK (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (*c*) anti-phospho-Smad2 (1:1000; Cell Signaling); (*d*) anti-phospho-Smad3 (1:500; Cell Signaling); (*e*) anti-Smad 2/3 (1:1000; BD Biosciences); (*f*) anti-FAK (1:1000; Santa Cruz Biotechnology); (*g*) anti-p130Cas (1:1000; BD Biosciences); (*h*) phospho-p130Cas (1:1000; Cell Signaling); (*i*) anti-actin (1:1000; Santa Cruz Biotechnology); (*j*) lamin A/C (1:1000; Santa Cruz Biotechnology); and (*k*) E-Cadherin (1:2000; BD Biosciences).

Real-time PCR Analyses—Quiescent 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor SB208530 (10 μM) and total RNA was isolated using the RNeasy Plus Kit (Qiagen, Valen-

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

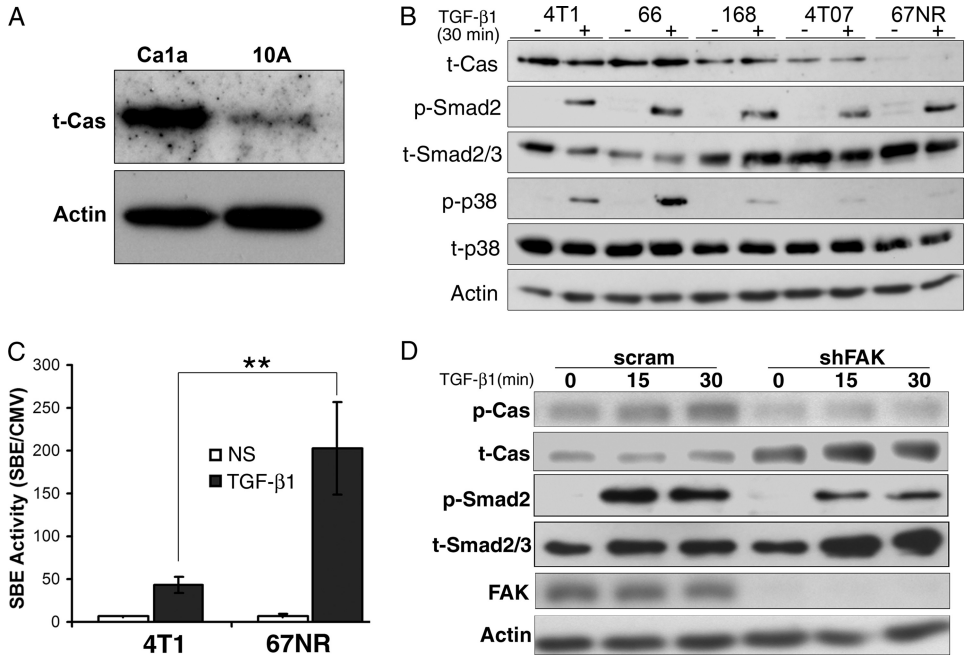


FIGURE 1. **Elevated p130Cas inhibits TGF-β-mediated Smad2/3 activation.** *A*, normal human MECs (*MCF-10A*) and their metastatic derivatives (*Ca1a*) were immunoblotted for p130Cas expression. β-Actin (*Actin*) is shown as loading control. Data are representative images from a representative experiment that was performed two times with identical results. *B*, murine breast cancer cells derived from the same primary Balb/c tumor, including the highly metastatic 4T1 and 66c14 (*66*) cells, the partially metastatic 168Farn (*168*) and 4T07 cells, and the nonmetastatic 67NR cells were immunoblotted for p130Cas expression, and the phosphorylation of Smad2 (*p-Smad2*) and p38 MAPK (*p-p38*) in response to TGF-β1 stimulation (5 ng/ml). Total Smad2/3 (*t-Smad2/3*), p38 MAPK (*t-p38*), and β-actin (*Actin*) were analyzed as loading controls. Data are from a representative experiment that was performed two times with identical results. *C*, 4T1 and 67NR cells were transiently transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated with TGF-β1 (5 ng/ml) for 18 h prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of luciferase/β-gal activity ratios. **, $p = 0.01$. *D*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or FAK-specific (*shFAK*) shRNA were stimulated with TGF-β1 (5 ng/ml) for varying times, and subsequently immunoblotted with phospho-specific antibodies against p130Cas (*p-Cas*) and Smad2 (*p-Smad2*) as indicated. Membranes were stripped and reprobed with antibodies against p130Cas (*t-Cas*), Smad2/3 (*t-Smad2/3*), β-actin (*Actin*), and FAK as indicated. Data are from a representative experiment that was performed at least three times with similar results.

cia, CA). Afterward, total RNA was reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad) and semi-quantitative real-time PCR was conducted for PAI-1 using iQ SYBR Green (Bio-Rad) according to the manufacturer's recommendations and as described previously (23). Differences in RNA concentrations were controlled by normalizing individual gene signals to their corresponding glyceraldehyde-3-phosphate dehydrogenase signal.

Cell Fractionation Studies—Unstimulated and TGF-β1 (5 ng/ml)-stimulated NMuMG cells were lysed on ice in Buffer C (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.004% Nonidet P-40, pH 7.9) supplemented with protease inhibitor mixture (Sigma). Afterward, the resulting whole cell extract was subjected to a single freeze-thaw cycle, followed by microcentrifugation to yield a clarified cytoplasmic fraction. The remaining pellet was resuspended in Buffer N (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, and 10% glycerol, pH 7.9) supplemented with protease inhibitor mixture, and shaken vigorously for 2 h at 4 °C. Afterward, this mixture was subjected to microcentrifugation to yield a clarified nuclear fraction.

Three-dimensional Culture Assays—NMuMG (1×10^4) and 4T1 (5×10^3) cells were diluted in complete medium supplemented with 5% Cultrex (R&D Systems, Minneapolis, MN), and subsequently seeded in 48-well plates on top of a Cultrex cushion. Where indicated, 4T1 cells were grown in the presence of TGF-β1 (5 ng/ml). The medium/Cultrex mixture was replaced at 7 days, and organoids were allowed to grow for a total length of 10 days, at which point they were monitored for hollowing by phase-contrast microscopy and quantified by three individuals who were blinded to the culture conditions. 4T1 acinar size was quantified using Image J software.

Cell Invasion Assays—The ability of TGF-β1 (5 ng/ml) to alter the invasion of 4T1 cells (50,000 cells/well) was analyzed using a modified Boyden Chamber assay as described previously (13).

Luciferase Reporter Gene Assays—NMuMG cells were transiently transfected overnight with LT1 liposomes (Mirus, Madison, WI) that contained 300 ng/well of pSBE-firefly luciferase (4X-CAGA) cDNA and 50 ng/well of pCMV-β-gal cDNA. Afterward, the cells were stimulated for 24 h with TGF-β1 (5 ng/ml), and subsequently harvested and assayed for firefly luciferase (Promega, Madison WI) and β-gal (Clontech, Mountain View, CA) activities. In addition, 4T1 cells that stably expressed firefly luciferase under control of the CMV promoter were similarly (i) transfected with 300 ng/well of pSBE-*Renilla* luciferase (4X-CAGA); (ii) stimulated with TGF-β1; and (iii) assayed for *Renilla* and firefly luciferase using the Dual-Glo Assay System as above (Promega).

Immunofluorescent Analyses—4T1 cells (25,000 cells/well) were allowed to adhere overnight to glass coverslips. Afterward, the cells were washed extensively in phosphate-buffered saline and immediately stimulated with TGF-β1 (5 ng/ml). Upon completion of agonist stimulation, the cells were (i) fixed in 4% paraformaldehyde; (ii) permeablized in 0.1% Triton X-100; (iii) stained with anti-Smad 2/3 antibodies (1:100; BD Biosciences); and (iv) visualized by addition of biotinylated anti-mouse antibodies (1:1000) in conjunction with the addition of rhodamine-conjugated streptavidin (1:2000).

Tumor Growth, in Vivo Bioluminescent Imaging, and Immunohistochemical Analyses—Control or various 4T1 derivatives engineered to stably express firefly luciferase were resuspended in sterile phosphate-buffered saline (50 μl) and injected orthotopically into the mammary fatpad (10,000 cells/injection) of 6-week-old female Balb/c mice (Jackson Laboratory, Bar Harbor, ME). Primary 4T1 tumor growth and metastasis development were assessed by (i) weekly bioluminescent imaging of

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

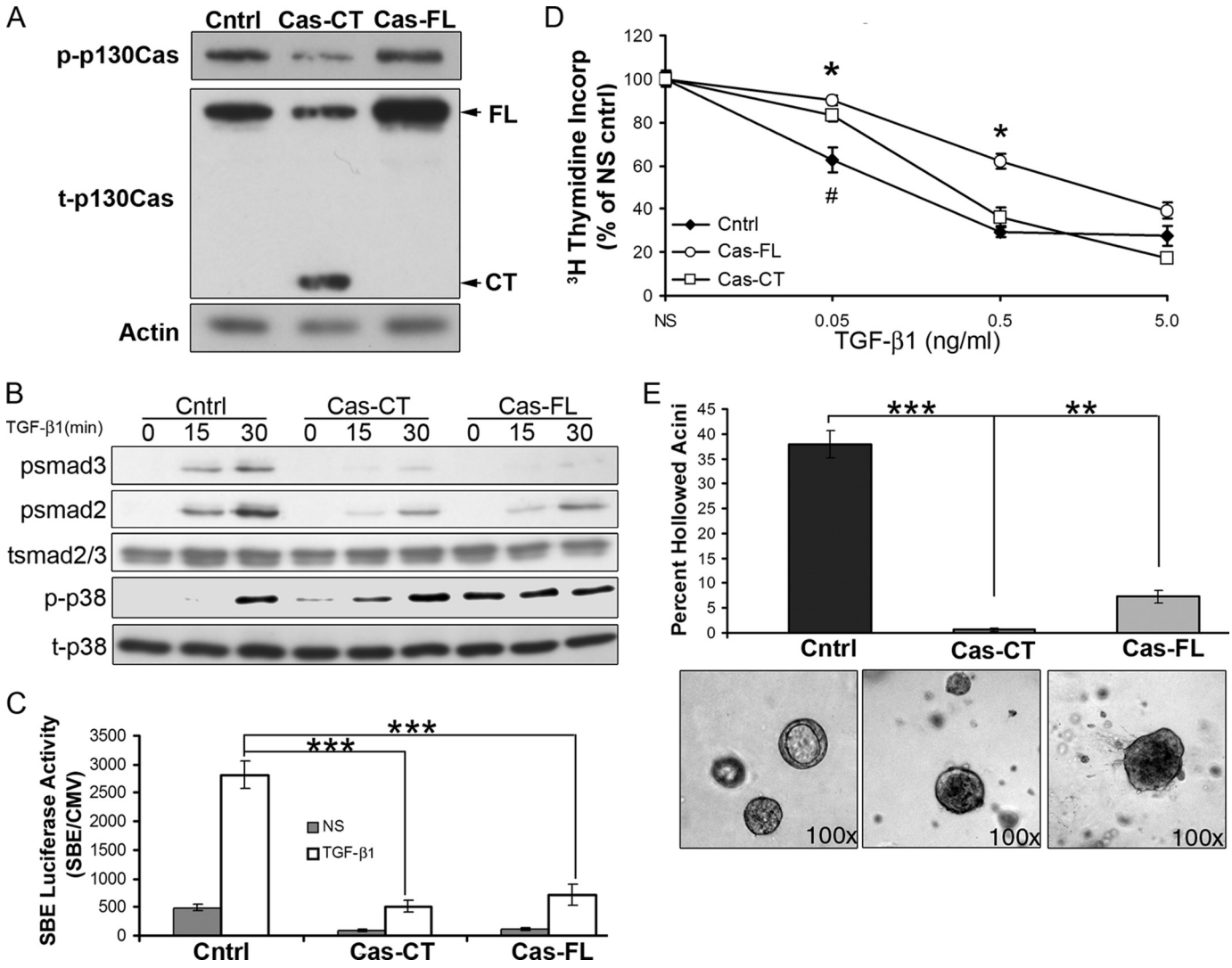


FIGURE 2. **Overexpression of p130Cas inhibits Smad2/3 activity and alters normal mammary epithelial acinar formation.** *A,* NMuMG cells were transduced with retroviral particles containing vector control (*cntrl*), full-length p130Cas (*Cas-FL*), or the carboxyl terminus of p130Cas (*Cas-CT*). Stable transgene expression was assessed by immunoblotting for phosphorylated (*p-p130Cas*) and total p130Cas (*t-p130Cas*). β-Actin (*Actin*) is shown as a loading control. *B*, the p130Cas-manipulated cell lines as described in *A* were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently analyzed for the phosphorylation of Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*). Membranes were stripped and reprobed for total Smad2/3 (*tsmad2/3*) and p38 MAPK (*t-p38*) as loading controls. Data are from representative experiments that were performed at least three times with identical results. *C,* control (cntrl) and p130Cas-expressing (Cas-CT and Cas-FL) NMuMG cells were transiently co-transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated overnight with TGF-β1 (5 ng/ml) prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. of SBE/CMV activity ratios observed in three independent experiments completed in triplicate. ***, $p < 0.001$). *D*, control (*cntrl*) and p130Cas-expressing (*Cas-CT* and *Cas-FL*) NMuMG cells were stimulated with increasing concentrations of TGF-β1 (0–5 ng/ml) for 48 h, and subsequently assayed for [^{3}H]thymidine incorporation into cellular DNA. Data are the mean ± S.E. quantities of incorporated [^{3}H]thymidine normalized to unstimulated controls observed in three independent experiments completed in triplicate (*, $p < 0.05$ between Cntrl and Cas-FL; #, $p < 0.05$ between Cntrl and Cas-CT). *E*, the p130Cas-manipulated NMuMG cells described in *A* were grown in three-dimensional organotypic cultures for 10 days, at which point the percentage of hollowed acini were quantified by phase-contrast microscopy (**, $p < 0.001$; ***, $p < 0.0001$). Representative acini are shown.

tumor bearing animals on a Xenogen IVIS-200 (Xenogen Corporation, Hopkinton, MA); (ii) calculating primary tumor volumes using digital calipers and the equation digital V = $(x^2)(y)(0.5)$, where x is the tumor width and y is tumor length; and (iii) measuring primary tumor weights following their surgical excision on days 21 or 26 post-inoculation. Finally, serial histological sections of control and p130Cas-deficient 4T1 tumors were stained with Ki67 antibodies, and counterstained with hematoxylin as described previously (11). Data were quantified using Image J software. All animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of University of Colorado.

Statistical Analysis—Statistical values were defined using an unpaired Student's *t* test, where a p value < 0.05 was considered significant.

RESULTS

Elevated p130Cas Expression Inhibits TGF-β-mediated Smad2/3 Activation—Elevated expression of p130Cas is associated with mammary tumor progression (20), and with the uncoupling of TGF-β to Smad3 activation in epithelial cells

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

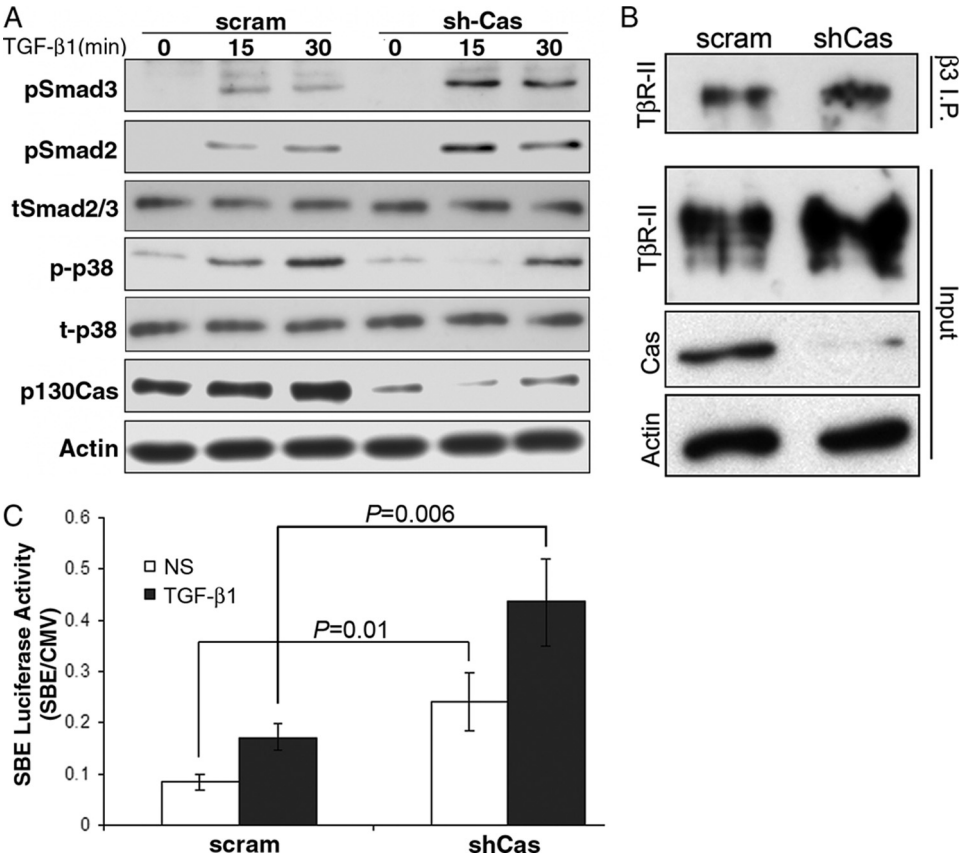


FIGURE 3. **p130Cas deficiency increases Smad2/3 activity in metastatic MECs.** *A*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or p130Cas-specific (*shCas*) shRNA were stimulated with TGF-β1 (5 ng/ml) as indicated, and subsequently immunoblotted with phosphospecific antibodies against Smad3 (*pSmad3*), Smad2 (*pSmad2*), or p38 MAPK (*p-p38*) as shown. Membranes were stripped and reprobed with antibodies against Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), β-actin (*Actin*), and p130Cas as loading controls. *B*, whole cell extracts prepared from control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were incubated with β3 integrin antibodies (*β3 I.P.*), and the resulting immunocomplexes were isolated and immunoblotted for TβR-II. Immunoblotting aliquots of the prepared cell extracts (*Input*) served to monitor the levels of TβR-II, p130Cas, and β-actin (*Actin*). Data are representative of three independent experiments and show that p130Cas deficiency does not affect carcinoma-specific formation of β3 integrin·TβR-II complexes. *C*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were transiently transfected with SBE-luciferase (*Renilla*), and subsequently stimulated overnight with TGF-β1 (5 ng/ml). *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of *Renilla*/firefly activity ratios.

(21). Unfortunately, the pathophysiological importance of these events, if any, in mediating oncogenic TGF-β signaling in normal and malignant MECs remains unknown. As an initial measure of potential changes in p130Cas expression during mammary tumor progression, we monitored p130Cas protein levels by immunoblotting whole cell extracts prepared from nontransformed human MECs (MCF10A) and their corresponding metastatic derivatives (CA1a) (24–26). As shown in Fig. 1*A*, p130Cas expression was readily increased in human metastatic CAla cells as compared with nontransformed isogenic counterparts. Along these lines, we also observed p130Cas expression to be up-regulated dramatically in the murine 4T1 model of mammary tumor progression (Fig. 1*B*) (26, 27). Indeed, the highly metastatic 4T1 and 66c14 cells expressed significantly more p130Cas than did their moderately metastatic counterparts, 168-Farn and 4T07 (Fig. 1*B*). Consistent with this trend, we found nonmetastatic 67NR cells to express very little p130Cas (Fig. 1*B*). Importantly, the increased expression of p130Cas was consistent with a shift in the balance of TGF-β signaling from primarily that of canonical Smad2 phosphorylation in nonmetastatic 67NR cells to one that included a marked activation of p38 MAPK in metastatic 4T1 and 66c14 cells (Fig. 1*B*). Furthermore, using a measure of the downstream transcriptional activity of Smad2/3, we also observed a drastic diminution in the ability of TGF-β1 to activate Smad2/3 in 4T1 *versus* 67NR cells (Fig. 1*C*). We also monitored changes in the phosphorylation status of p130Cas upon stimulation with TGF-β. As shown in Fig. 1*D*, stimulating 4T1 cells with TGF-β not only readily induced the phosphorylation of Smad2, but also that of p130Cas (Fig. 1*D*). Moreover, the basal levels as well as the ability of TGF-β to induce phosphorylation of p130Cas were abrogated by rendering 4T1 cells deficient in its upstream kinase, FAK (Fig. 1*D*). Interestingly, depletion of FAK elicited a compensatory up-regulation of total p130Cas expression that was consistent with diminished coupling of TGF-β to Smad2 phosphorylation (Fig. 1*D*). Together, these findings are consistent with the notion that p130Cas, irrespective of its phosphorylation status, functions to shift the balance of TGF-β signaling during breast cancer progression by suppressing Smad2/3 activity and supporting p38 MAPK activation in response to TGF-β.

Overexpression of p130Cas Inhibits Smad2/3 Activity and Alters Normal Mammary Epithelial Acinar Formation—To address the role p130Cas in regulating TGF-β signaling, we overexpressed either the full-length protein (Cas-FL) or just the carboxyl terminus of p130Cas (Cas-CT) in normal murine mammary epithelial (NMuMG) cells (Fig. 2*A*). Indeed, overexpression of either Cas-CT or Cas-FL readily decreased the phosphorylation of Smad2 and Smad3 in response to TGF-β1 (Fig. 2*B*). In contrast, TGF-β-induced p38 MAPK phosphorylation was readily increased upon Cas-CT expression, whereas expression of Cas-FL was sufficient to induce the phosphorylation of p38 MAPK even in the absence of added TGF-β (Fig. 2*B*). Furthermore, overexpression of either Cas-CT or Cas-FL also dramatically decreased the extent of basal and TGF-β-induced Smad2/3-dependent transcription (Fig. 2*C*). Functionally, we observed the p130Cas-dependent reduction in Smad2/3 activity to significantly inhibit the cytostatic response of NMuMG cells to TGF-β (Fig. 2*D*). Finally, because TGF-β is critically involved regulating normal mammary gland develop-

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

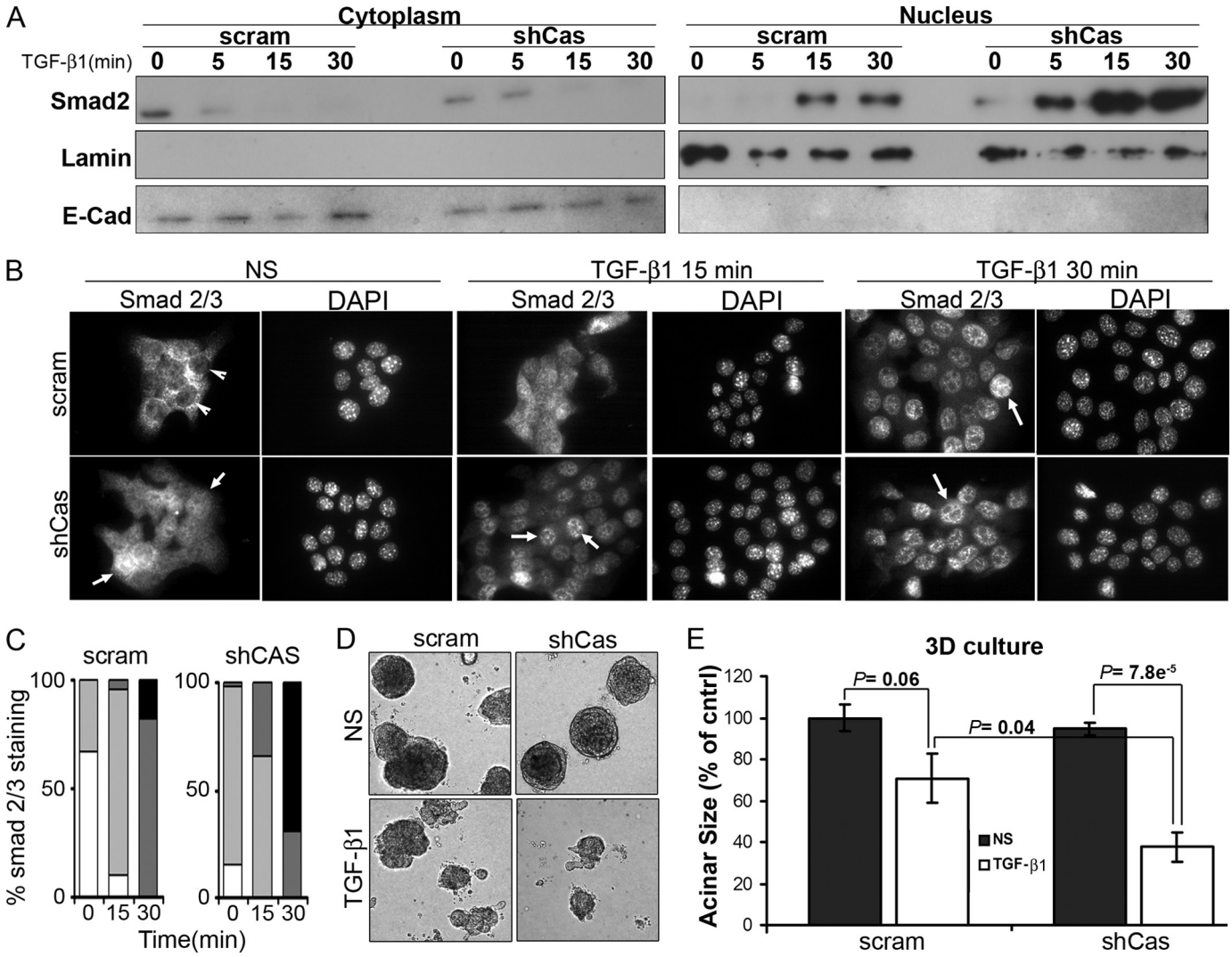


FIGURE 4. **p130Cas deficiency increases Smad2/3 nuclear localization and decreases the proliferation of metastatic MECs.** *A*, quiescent control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently lysed, fractionated, and immunoblotted for Smad2/3. Membranes were stripped and reprobed with antibodies against E-cadherin (*E-Cad*) and lamin A/C (*Lamin*) to monitor the integrity of the cytoplasmic and nuclear preparations, respectively. Data are from a representative experiment that was performed three times with similar results. *B*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were stimulated as described in *A* and subsequently fixed and processed for indirect Smad2/3 immunofluorescence, as well as counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*) to visualize cell nuclei. *Arrows* indicate the distinct absence or presence of Smad2/3 in 4T1 cell nuclei. Data are representative images from four independent experiments. *C*, Smad2/3 immunofluorescence data in *B* was quantified as follows: *white bars*, nuclear exclusion; *light gray bars*, cellular diffuse; *dark gray bars*, weak nuclear; *black bars*, strong nuclear staining for Smad2/3. Data are from 10 randomly selected fields for each time point obtained in two independent experiments. *D*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were grown in a three-dimensional organotypic culture for 10 days in the absence or presence of TGF-β1 (5 ng/ml). Representative acini from three independent experiments are shown. *E*, p130Cas depletion restored the cytostatic response of TGF-β in 4T1 cells, which were grown as in *D* prior to quantifying acinar size. Data are the mean ± S.E. of 9 randomly selected fields obtained from three independent experiments.

ment (28–30), we next sought to assess the affect of p130Cas overexpression on the formation of acini by NMuMG cells propagated in a three-dimensional organotypic culture system. Importantly, expression of either Cas-FL or Cas-CT readily invoked acinar filling, a phenotype that recapitulates *in vivo* mammary tumor progression (31). Taken together, these findings clearly indicate that inappropriate up-regulation (see Fig. 1) of p130Cas expression was sufficient to inhibit the physiologic activity of Smad2/3, thereby diminishing the tumor suppressive activities of TGF-β.

p130Cas Deficiency Increases Smad2/3 Activity in Metastatic MECs—Given that elevating p130Cas expression was sufficient to inhibit Smad2/3 signaling stimulated by TGF-β, we next examined how p130Cas deficiency would affect the coupling of Smad2/3 to TGF-β in MECs. To this end, we expressed and screened five independent p130Cas-specific shRNA sequences in NMuMG cells. The general importance of p130Cas in maintaining normal MEC physiology and homeostasis was readily apparent as NMuMG cells that expressed shCas#5 shRNA, which elicited the greatest degree of p130Cas depletion (see supplemental Fig. S1*A*), failed to thrive and survive under extended culture conditions (data not shown). Overall, p130Cas deficiency led to decreased Smad2 expression in NMuMG cells (see supplemental Fig. S1*A*). These findings are consistent with the notion that p130Cas deficiency augments the activity of Smad2/3, which elicits proteosome-directed degradation of Smad2/3 (see supplemental Fig. S1*B*) (23, 32, 33). However, this decrease in Smad2 expression precluded a direct

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

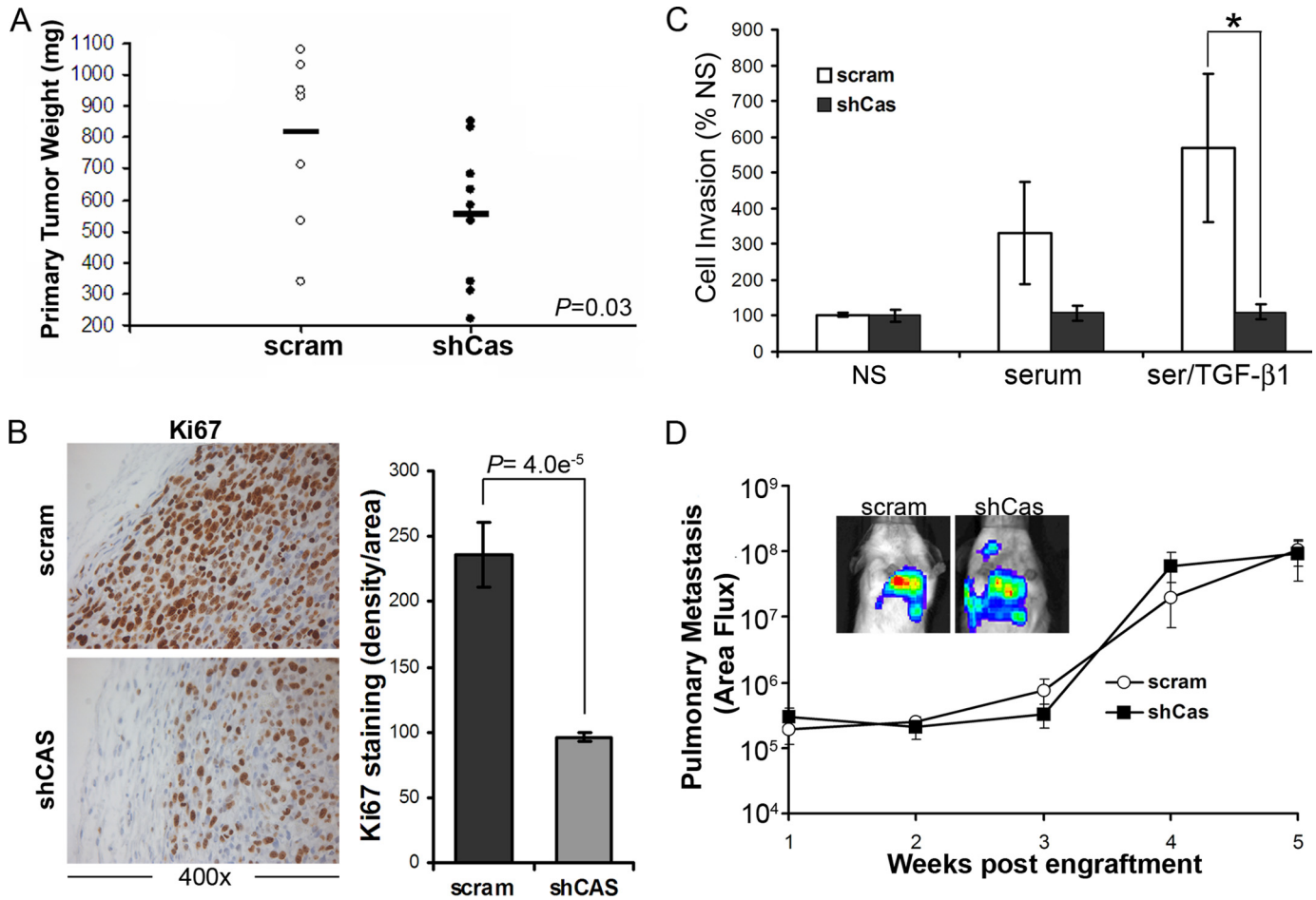


FIGURE 5. **p130Cas deficiency inhibits primary tumor growth and cell invasion.** *A*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were engrafted onto the mammary fat pad of Balb/c mice. Primary tumors were removed surgically 21 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (10 mice/group). $p = 0.03$. *B*, histological sections of primary tumors were stained with Ki67 to monitor the proliferative index of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 tumors. Staining intensity was quantified over nine fields of view from three separate tumors/group and showed a decrease in the proliferative index at the invasive front of primary tumors upon p130Cas depletion. *C*, the invasion of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells through Matrigel was stimulated by 2% fetal bovine serum (*serum*), or by 2% fetal bovine serum supplemented with TGF-β1 (5 ng/ml; *Ser/TGF-β1*). Data are the mean ± S.E. invasion to unstimulated MECs (*NS*) observed in three independent experiments completed in triplicate (*, $p < 0.05$). *D*, 4T1 cell derivatives were engrafted onto the mammary fat pads of female Balb/c mice as described in *A* and pulmonary photon flux readings were determined at the indicated time points post-engraftment. *Inset* shows representative bioluminescent signals of pulmonary metastases 4 weeks post-engraftment.

analysis of the affect p130Cas deficiency elicited on the activation of Smad2/3 by TGF-β in normal MECs. Despite this limitation, aberrant p130Cas expression has been associated with increased breast cancer progression and poorer clinical prognosis (18–20). Therefore, we sought to address the functional impact of p130Cas deficiency on the ability of TGF-β to initiate oncogenic signaling in the 4T1 metastatic model of breast cancer. Indeed, depletion of p130Cas greatly augmented the coupling of TGF-β to Smad2 and Smad3 in metastatic 4T1 cells without affecting total Smad2/3 levels (Fig. 3*A*). We previously demonstrated that the aberrant interaction of β3 integrin with TβR-II in post-epithelial-mesenchymal transition and malignant MECs is critical for the activation of p38 MAPK by TGF-β (11–13). Fig. 3*B* shows that p130Cas is not required for the formation of β3 integrin·TβR-II complexes in 4T1 cells, which serves to explain the slightly diminished coupling of TGF-β to p38 MAPK in p130Cas-depleted 4T1 cells (Fig. 3*A*). Furthermore, p130Cas deficiency not only elicited a significant increase in autocrine-driven SBE-luciferase activity in quiescent 4T1 cells (Fig. 3*C*), but also significantly augmented their induction of this Smad2/3-responsive reporter gene when stimulated by TGF-β (Fig. 3*C*). Accordingly, Smad2/3 localized primarily to the cytoplasm in quiescent parental 4T1 cells, as determined by (i) cellular fractionation coupled to Smad2/3 immunoblotting (Fig. 4*A*), and (ii) indirect Smad2/3 immunofluorescence (Fig. 4, *B* and *C*). In stark contrast, Smad2/3 was present in the cytoplasm and nuclear compartments in quiescent 4T1 cells that lacked p130Cas expression (Fig. 4, *A*–*C*), a finding consistent with their elevated basal levels of Smad2/3 activity. Moreover, p130Cas deficiency greatly accelerated the rate and extent of Smad2/3 that accumulated in the nucleus following TGF-β stimulation (Fig. 4, *A*–*C*). Collectively, these findings are consistent with the notion that aberrant p130Cas expression down-regulates the activity of Smad2/3 in metastatic breast cancer cells.

A characteristic phenotype of mammary carcinoma cells, including 4T1 cells, is their resistance to TGF-β-mediated growth arrest when grown in two-dimensional culture systems (12, 34, 35). Interestingly, the resultant increase in Smad2/3 activity elicited by p130Cas deficiency was unable to restore a

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

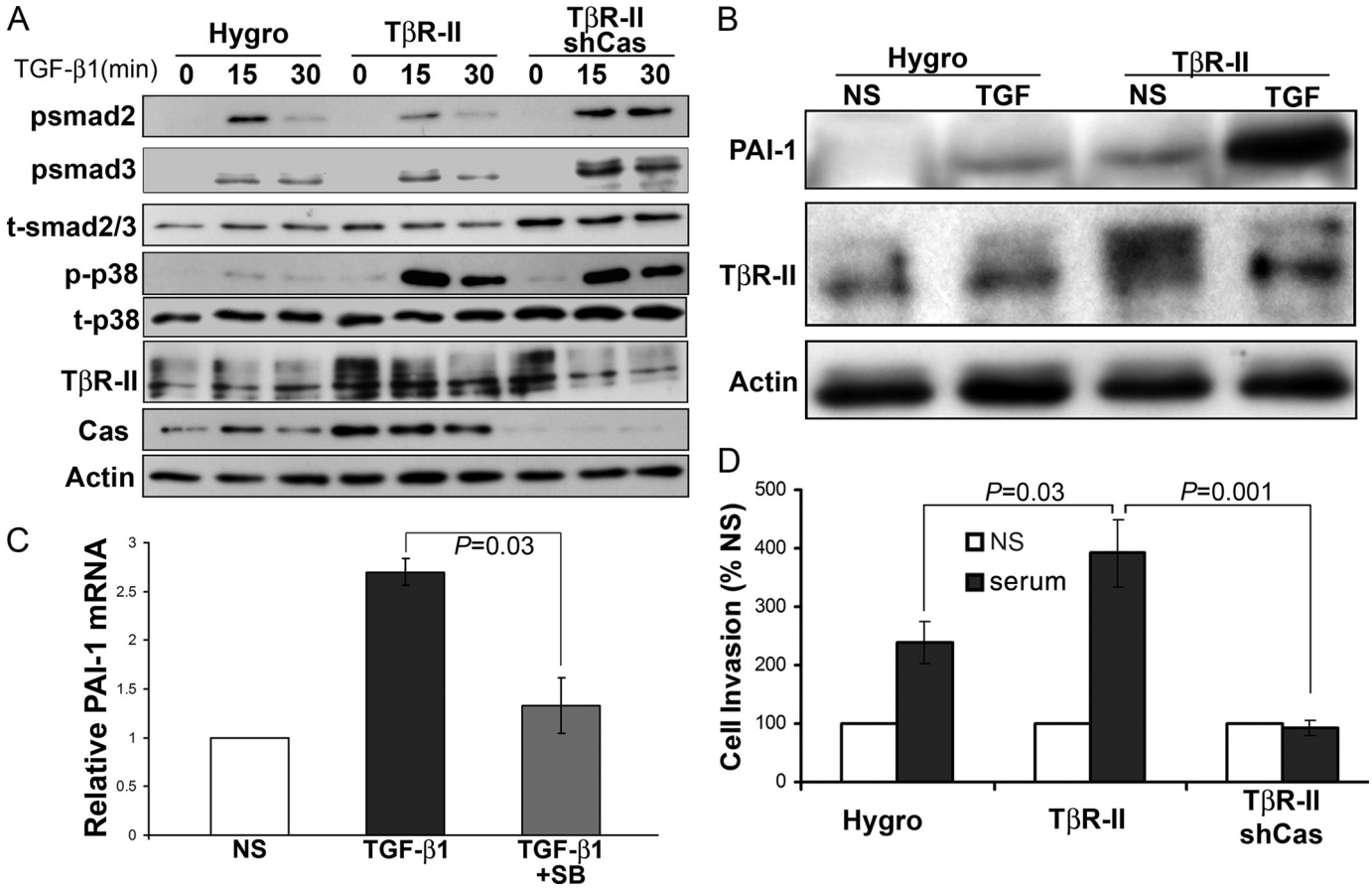


FIGURE 6. **p130Cas balances canonical and noncanonical TGF-β signaling.** *A,* control (*Hygro*), TβR-II- (*TβR-II*), or TβR-II-expressing 4T1 cells deficient in p130Cas expression (*TβR-II/shCas*) were stimulated with TGF-β1 (5 ng/ml) for varying times, and immunoblotted with phospho-specific antibodies against Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*) as indicated. Membranes were stripped and reprobed with antibodies against total Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), TβR-II, p130Cas (*Cas*), and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed at least three times with similar results. *B,* quiescent control (*Hygro*) and TβR-II-expressing 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h. *NS,* no stimulation. The resulting conditioned medium was collected, precipitated, and immunoblotted for PAI-1. The corresponding cell lysates were probed for TβR-II and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed three times with similar results. *C,* 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor, SB208530 (10 μM), and analyzed by semi-quantitative reverse transcription-PCR for PAI-1 mRNA. Data are the mean ± S.E. induction of PAI-1 relative to unstimulated MECs (*NS*) observed in three independent experiments. *D,* 4T1-TβR-II cell variants described *A* were induced to invade synthetic basement membranes by 2% fetal bovine serum (*serum*). Data are the mean ± S.E. invasion relative to unstimulated (*NS*) controls of three independent experiments completed in triplicate.

strong cytostatic response in 4T1 cells upon TGF-β administration (see supplemental Fig. S2*A*). However, it is known that culturing cells on plastic can mask several cell signaling events, most notably those of TGF-β (31, 36, 37). As such, we propagated control and p130Cas-depleted 4T1 cells in three-dimensional organotypic cultures in the absence or presence of TGF-β1 (Fig. 4*D*). In addition to restoring a more rounded, normal acinar structure (Fig. 4*D*), depletion of p130Cas also significantly increased the growth inhibitory effects of TGF-β1 as compared their p130Cas-expressing counterparts (Fig. 4*E*). Taken together, these findings suggest that p130Cas functions to sequester Smad2/3 in the cytoplasm, thereby inhibiting their activity and the cytostatic function of TGF-β. Our findings also suggest that aberrant expression of p130Cas may elicit profound effects on mammary tumor growth regulated by TGF-β.

p130Cas Deficiency Inhibits Primary Mammary Tumor Growth and Cell Invasion—To further assess the role of p130Cas in TGF-β-mediated tumor progression, we engrafted parental and p130Cas-deficient 4T1 cells onto the mammary fat pads of syngeneic Balb/c mice. Indeed, orthotopic tumors lacking p130Cas clearly grew more slowly as compared with their parental counterparts (Fig. 5*A*). Consistent with a reduction in 4T1 tumor weights, we also observed p130Cas deficiency to elicit significantly impaired proliferative indices as determined by Ki67 immunohistochemistry of primary tumor sections (Fig. 5*B*). These data support our *in vitro* findings (Figs. 2*D* and 4*E*) and suggest that measures capable of inactivating p130Cas expression and/or function may provide a novel means to partially restore the tumor suppressive activity of TGF-β.

Through its inclusion in focal adhesion complexes, p130Cas has also been proposed to play a critical role in mediating cell migration and invasion (15). Indeed, p130Cas deficiency abrogated 4T1 cell invasion induced by TGF-β1 (Fig. 5*C*); however, this same cellular condition failed to impact the pulmonary metastasis of 4T1 cells engrafted onto the mammary fat pad of Balb/c mice (Fig. 5*D*). These findings underscore the complexities of carcinoma metastasis *in vivo* and point to the existence of alternative and TGF-β-independent pathways that can compensate for the loss of cellular invasion normally regulated by

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

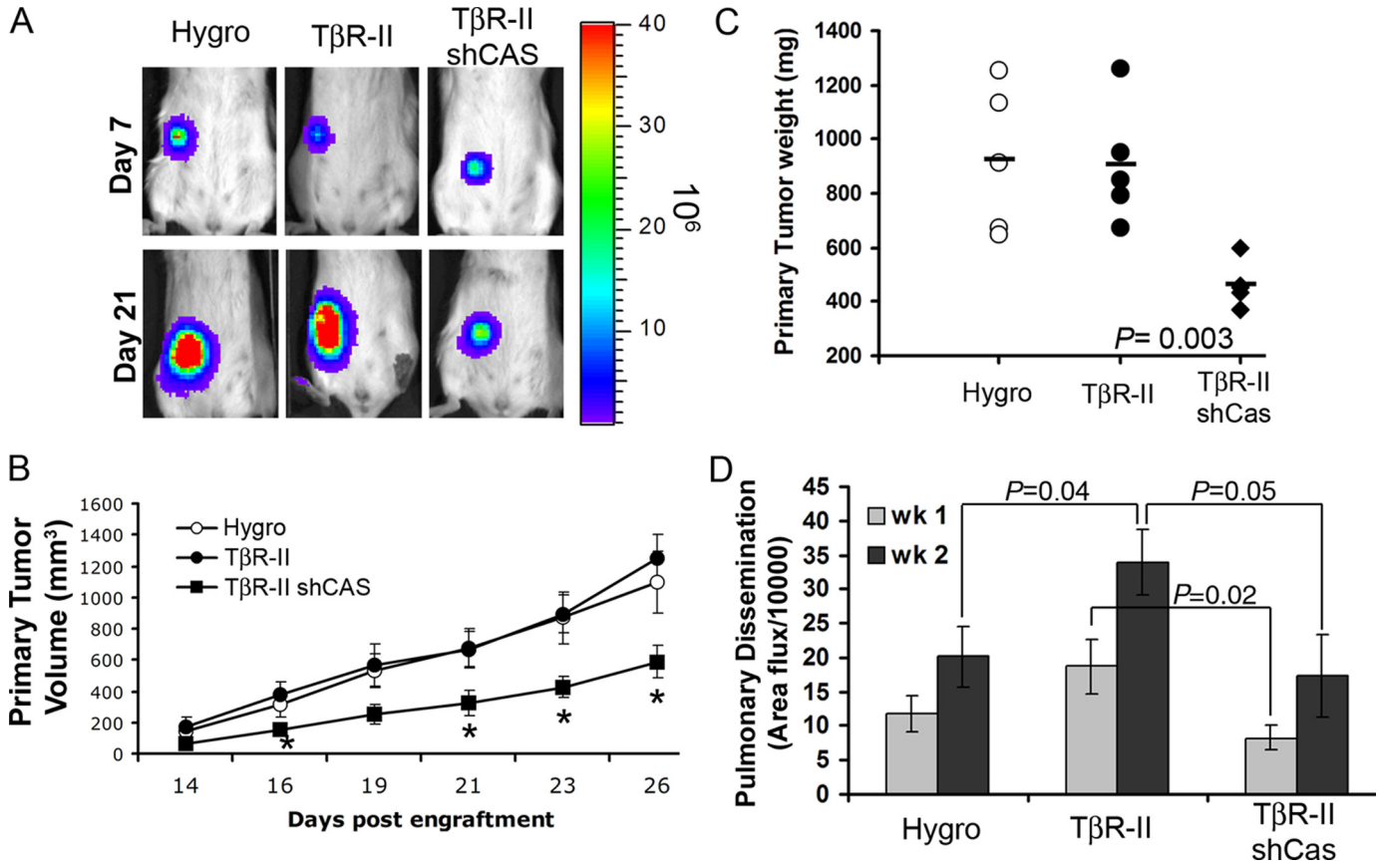


FIGURE 7. **Coupling TβR-II expression with p130Cas deficiency prevents TGF-β-driven breast cancer metastasis.** *A*, control (*Hygro*), TβR-II- (*TβR-II*), and TβR-II-expressing 4T1 cells lacking expression of p130Cas (*TβR-II/shCas*) were engrafted onto the mammary fat pad of Balb/c mice. Bioluminescent visualization of primary 4T1 tumors showed an equal establishment at day 7 post-engraftment, but a significant growth defect at day 26 in TβR-II-expressing 4T1 tumors lacking p130Cas expression. *B*, data are the mean ± S.E. of tumor volumes measured for the indicated 4T1 tumor variants. *, $p < 0.05$, $n = 5$ mice/group. *C*, primary 4T1 tumors were removed surgically 26 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (5 mice/group), $p = 0.003$. *D*, data are the mean ± S.E. ($n = 5$ mice/group) of pulmonary photon flux units measured at 1-week intervals following engraftment of the 4T1 variants onto the mammary fat pads.

p130Cas. Indeed, it is tempting to speculate that pulmonary metastasis of p130Cas-deficient 4T1 cells reflects their maintenance of p38 MAPK activity (Fig. 3*A*), a signaling pathway in which we demonstrated previously to be necessary for TGF-β stimulation of 4T1 pulmonary metastasis (11).

p130Cas Balances Canonical and Noncanonical TGF-β Signaling—We previously demonstrated that transgenic expression of human TβR-II in 4T1 cells significantly enhances their invasion (12) and pulmonary metastasis (11) in mice. Therefore, we next sought to utilize this model to specifically address the role of p130Cas in mediating TGF-β-driven tumor progression and metastasis. Indeed, transgenic expression of TβR-II dramatically enhanced the coupling of TGF-β to the activation of p38 MAPK, but had little to no affect on Smad2 or Smad3 phosphorylation (Fig. 6*A*). This shift in TGF-β signaling was reflected by the increased basal and TGF-β-induced expression of the prometastatic factor, plasminogen activator inhibitor-1 (PAI-1; Fig. 6*B*). Moreover, pharmacological inhibition of p38 MAPK activity significantly impaired the ability of 4T1 cells to up-regulate PAI-1 in response to TGF-β1 (Fig. 6*C*). Importantly, rendering these "hyperinvasive" 4T1-TβR-II cells deficient in p130Cas had no appreciable effect on their enhanced ability to phosphorylate p38 MAPK in response to TGF-β; however, this same cellular condition did elicit elevated phosphorylation of both Smad2 and Smad3 (Fig. 6*A*). Thus, diminishing p130Cas expression in 4T1-TβR-II cells restores the physiologic balance between canonical and noncanonical TGF-β signaling (Fig. 6*A*). In accord with their increased p38 MAPK activity and PAI-1 secretion, 4T1-TβR-II cells are significantly more invasive compared with parental 4T1 cells (Fig. 6*C*) (11, 12). Importantly, depleting 4T1-TβR-II cells of p130Cas expression abrogated their enhanced invasiveness mediated by TβR-II expression and a serum stimulation (Fig. 6*C*). Taken together, these findings clearly show that elevated TβR-II expression enhances the coupling of TGF-β to its noncanonical effector, p38 MAPK, leading to augmented PAI-1 expression and cellular invasion. Furthermore, we show for the first time that p130Cas deficiency restores a physiologic balance between canonical and noncanonical TGF-β signaling, and as such, prevents breast cancer cell invasion.

p130Cas Deficiency Prevents Early TGF-β-driven Breast Cancer Dissemination—We next sought to utilize the 4T1-TβR-II model to define the specific role of p130Cas in mediating *in vivo* TGF-β-driven breast cancer progression. Bioluminescent imaging of tumor bearing Balb/c mice showed that parental, TβR-II-, and TβR-II-shCas-expressing 4T1 tumors exhibited similar rates of establishment (Fig. 7*A*, *Day 7*). However, their growth rates thereafter diverged rapidly due to the

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

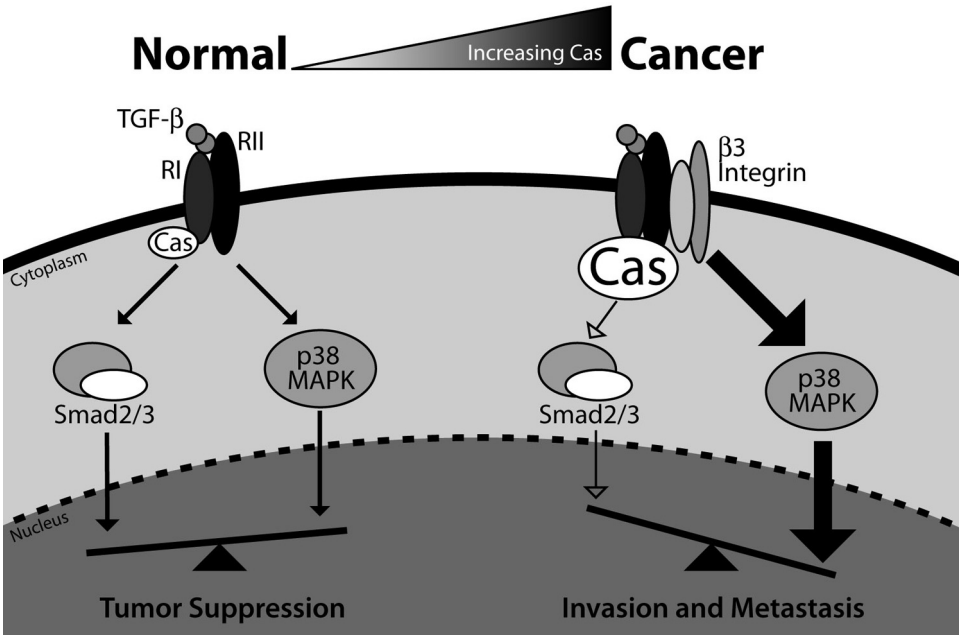


FIGURE 8. **p130Cas functions as a molecular rheostat that maintains the balance between canonical and noncanonical TGF-β signaling.** In normal MECs, physiologic expression levels of integrins and p130Cas maintain the balance between Smad2/3 and p38 MAPK signaling, which collectively support the tumor suppressing and cytostatic functions of TGF-β. During breast cancer progression, aberrant up-regulation of p130Cas expression inhibits Smad2/3 activation in a manner that parallels the inappropriate formation of β3 integrin·TβR-II complexes, which promotes increased coupling of TGF-β to p38 MAPK. Overall, these untoward events shift the balance of TGF-β signaling to favor activation of noncanonical effectors, particularly p38 MAPK, during the acquisition of metastatic phenotypes by breast cancer cells. Importantly, rendering late-stage breast cancer cells deficient in p130Cas enhances the activation of Smad2/3 by TGF-β, which thereby restores its ability to suppress the growth and pulmonary metastasis of breast cancer cells in mice.

inability of TβR-II-shCas tumors to grow out as efficiently as the parental and TβR-II-expressing control cells (Fig. 7, *A* and *B, Day 21*). Importantly, combining TβR-II expression with p130Cas depletion significantly exacerbated (by 10-fold) the growth defects originally observed upon p130Cas depletion in wild-type 4T1 tumors (Fig. 7*C*). Thus, abrogating p130Cas was sufficient in restoring the tumor suppressing activities of TGF-β.

Finally, we found that TβR-II expression elicited a dramatic increase in the early dissemination of 4T1 tumors to lungs as compared with parental cells (Fig. 7*D*). Importantly, this TGF-β/TβR-II-driven metastatic process was specifically inhibited by rendering TβR-II-expressing 4T1 cells deficient in p130Cas (Fig. 7*D*). Taken together, these findings show that p130Cas is critically involved in promoting primary mammary tumor growth, and is specifically required in facilitating early events in TGF-β-driven primary tumor dissemination.

DISCUSSION

TGF-β is a principal player involved in suppressing mammary tumorigenesis, doing so through its ability to maintain the composition of normal MEC microenvironments, and by inhibiting the aberrant proliferation of normal MECs (6, 38). Mammary tumorigenesis has evolved a variety of mechanisms that subvert the tumor suppressing functions of TGF-β, and in doing so, confer oncogenic and metastatic activities upon this multifunctional cytokine (34). Indeed, how TGF-β both suppresses and promotes mammary tumorigenesis remains a fundamental question that directly impacts the ability of science and medicine to effectively target the TGF-β signaling system during the treatment of breast cancer patients. Deciphering this paradox remains the most important question concerning the biological and pathological actions of this multifunctional cytokine (39).

We previously established the importance of aberrant interactions between β3 integrin and TβR-II to promote Src-mediated phosphorylation of TβR-II, which then recruits and binds Grb2. Once bound to phospho-Tyr-284 in TβR-II, Grb2 facilitates TGF-β-mediated activation of noncanonical MAP kinase signaling without affecting the coupling of TGF-β to Smad2/3 (12, 13). Importantly, measures capable of disrupting this signaling axis readily prevent TGF-β from driving breast cancer invasion and metastasis (11, 40). Thus, in addition to establishing the critical importance of p38 MAPK activation in mediating breast cancer metastasis stimulated by TGF-β, these studies also suggested that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems may in fact underlie its prometastatic activities in breast cancer cells. Our findings herein provide the first definitive evidence that (i) canonical and noncanonical signaling imbalances do indeed dictate MEC response to TGF-β, and (ii) p130Cas functions as a novel molecular rheostat that governs the delicate balance between canonical and noncanonical TGF-β effectors. Indeed, overexpression of either full-length or the carboxyl terminus of p130Cas was sufficient to decrease TGF-β-induced Smad2/3 phosphorylation while simultaneously increasing that of p38 MAPK. Moreover, depleting p130Cas significantly increased the activity of Smad2/3 and concomitantly decreased that of p38 MAPK induced by TGF-β, and finally, elevating TβR-II expression amplified the activation of p38 MAPK by TGF-β, which significantly enhanced early metastatic progression of mammary tumors in mice (Fig. 7) (11). In "hypermetastatic" TβR-II-expressing cells, p130Cas deficiency similarly increased the coupling of TGF-β to Smad2/3, an event that negated the proinvasive and prometastatic activities of p38 MAPK in developing 4T1 tumors. Thus, p130Cas functions in balancing the activation status of canonical and noncanonical effectors targeted by TGF-β, findings that are clinically and medically relevant to the development and progression of mammary tumors regulated by TGF-β.

A schematic depicting the function of p130Cas in TGF-β signaling is presented in Fig. 8. Indeed, in normal MECs, TGF-β receptors fail to interact significantly with integrins, which lim-

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

its TGF-β stimulation of p38 MAPK and the initiation of oncogenic signaling by TGF-β (13, 40–42). The net effect of these signaling events results in tumor suppression by TGF-β. However, during mammary tumorigenesis, p130Cas expression is up-regulated dramatically, as is the aberrant formation of integrin and TGF-β receptor complexes (11–13, 40), which collectively decrease the activity of Smad2/3 and increase that of p38 MAPK and other noncanonical effectors that promote breast cancer metastasis stimulated by TGF-β (11, 40). This signaling imbalance can be potentiated by elevated TβR-II expression and its consequential enhancement of p38 MAPK activation and metastasis (11, 34, 40, 43). In all cases, these various signaling inputs are critically balanced and influenced by the level of p130Cas expression. Indeed, we (see Fig. 1) and others (20) find mammary tumorigenesis to dramatically increase the expression of p130Cas. Based on our findings presented herein, we suggest that this event limits TGF-β stimulation of Smad2/3, which (i) diminishes MEC responsiveness to the cytostatic activities of TGF-β (44); and (ii) promotes amplified coupling of TGF-β to its noncanonical effectors, leading to breast cancer invasion and metastasis. In fact, our findings strongly support the progressive hypothesis that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems underlies the acquisition of metastatic phenotypes in mammary carcinomas, as well as facilitates the oncogenic switch of TGF-β from a tumor suppressor to a prometastatic molecule.

Along these lines, a recent report suggests that murine mammary tumor virus-driven p130Cas expression in mice is sufficient to induce mammary gland hyperplasia (20). However, it was necessary to combine transgenic p130Cas expression with that of HER2 to enhance formation of mammary tumors (20). Although specific effects on TGF-β activity and signaling were not examined in this mouse model, these findings do suggest that the tumor promoting properties of p130Cas only manifest in the face of additional oncogenic signaling inputs (*i.e.* elevated HER2 expression), which mirrors our own results showing that heightened TGF-β signaling (TβR-II expression) requires p130Cas to induce pulmonary dissemination. Moreover, we show that transgenic TβR-II expression led to increased basal and TGF-β-induced production of the prometastatic protein, PAI1, without impacting the phosphorylation of Smad2/3. These findings suggest that (i) p130Cas specifically regulates the activity of Smad2/3 as opposed to that of the TGF-β receptors, and (ii) Smad2/3 expression levels, not those of TGF-β receptors, are rate-limiting during the activation of canonical TGF-β signaling. Thus, p130Cas acts as a molecular rheostat of canonical Smad2/3 and noncanonical p38 MAPK signaling stimulated by TGF-β, and disruption of the balance between these two pathways has dramatic affects on breast cancer growth and progression.

In summary, we demonstrated that p130Cas functions to regulate the balance between TGF-β-mediated activation of Smad2/3 and p38 MAPK in normal and metastatic MECs. Moreover, we provide compelling evidence that p130Cas is both necessary and sufficient to drive the oncogenic activities of TGF-β, including its regulation of mammary tumor growth and the initiation of early steps in the metastatic dissemination of breast cancer cells. Collectively, our findings establish p130Cas as an essential mediator that underlies the oncogenic conversion of TGF-β function, thereby enhancing its ability to promote the progression of mammary carcinomas.

Acknowledgments—We thank members of the Schiemann Laboratory for critical reading of the manuscript. We also thank Dr. Amy Bouton for providing the p130Cas-CT and -FL cDNA constructs.

REFERENCES

1. Yoshida, B. A., Sokoloff, M. M., Welch, D. R., and Rinker-Schaeffer, C. W. (2000) *J. Natl. Cancer Inst.* **92,** 1717–1730
2. Wakefield, L. M., Piek, E., and Böttinger, E. P. (2001) *J. Mammary Gland Biol. Neoplasia* **6,** 67–82
3. Buck, M. B., and Knabbe, C. (2006) *Ann. N. Y. Acad. Sci.* **1089,** 119–126
4. Benson, J. R. (2004) *Lancet Oncol.* **5,** 229–239
5. Chen, R. H., and Chang, T. Y. (1997) *Cell Growth Differ.* **8,** 821–827
6. Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000) *N. Engl. J. Med.* **342,** 1350–1358
7. Galliher, A. J., Neil, J. R., and Schiemann, W. P. (2006) *Future Oncol.* **2,** 743–763
8. Gomis, R. R., Alarcón, C., Nadal, C., Van Poznak, C., and Massagué, J. (2006) *Cancer Cell* **10,** 203–214
9. Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M. B., Guzzardo, V., Parenti, A. R., Rosato, A., Bicciato, S., Balmain, A., and Piccolo, S. (2009) *Cell* **137,** 87–98
10. Tian, M., and Schiemann, W. P. (2009) *Future Oncol.* **5,** 259–271
11. Galliher-Beckley, A. J., and Schiemann, W. P. (2008) *Carcinogenesis* **29,** 244–251
12. Galliher, A. J., and Schiemann, W. P. (2007) *Cancer Res.* **67,** 3752–3758
13. Galliher, A. J., and Schiemann, W. P. (2006) *Breast Cancer Res.* **8,** R42
14. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) *Prog. Biophys. Mol. Biol.* **71,** 435–478
15. Brábek, J., Constancio, S. S., Shin, N. Y., Pozzi, A., Weaver, A. M., and Hanks, S. K. (2004) *Oncogene* **23,** 7406–7415
16. Geiger, B. (2006) *Cell* **127,** 879–881
17. Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y., and Hirai, H. (1998) *Nat. Genet.* **19,** 361–365
18. van der Flier, S., Chan, C. M., Brinkman, A., Smid, M., Johnston, S. R., Dorssers, L. C., and Dowsett, M. (2000) *Int. J. Cancer* **89,** 465–468
19. Ta, H. Q., Thomas, K. S., Schrecengost, R. S., and Bouton, A. H. (2008) *Cancer Res.* **68,** 8796–8804
20. Cabodi, S., Tinnirello, A., Di Stefano, P., Bisarò, B., Ambrosino, E., Castellano, I., Sapino, A., Arisio, R., Cavallo, F., Forni, G., Glukhova, M., Silengo, L., Altruda, F., Turco, E., Tarone, G., and Defilippi, P. (2006) *Cancer Res.* **66,** 4672–4680
21. Kim, W., Seok Kang, Y., Soo Kim, J., Shin, N. Y., Hanks, S. K., and Song, W. K. (2008) *Mol. Biol. Cell* **19,** 2135–2146
22. Wendt, M. K., Cooper, A. N., and Dwinell, M. B. (2008) *Oncogene* **27,** 1461–1471
23. Neil, J. R., Johnson, K. M., Nemenoff, R. A., and Schiemann, W. P. (2008) *Carcinogenesis* **29,** 2227–2235
24. Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Eliason, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H., and Miller, F. R. (2001) *Breast Cancer Res. Treat.* **65,** 101–110
25. Dawson, P. J., Wolman, S. R., Tait, L., Heppner, G. H., and Miller, F. R. (1996) *Am. J. Pathol.* **148,** 313–319
26. Aslakson, C. J., and Miller, F. R. (1992) *Cancer Res.* **52,** 1399–1405
27. Jin, W., Yun, C., Kwak, M. K., Kim, T. A., and Kim, S. J. (2007) *Oncogene* **26,** 7684–7691
28. Ingman, W. V., and Robertson, S. A. (2008) *Biol. Reprod.* **79,** 711–717
29. Nelson, C. M., Vanduijn, M. M., Inman, J. L., Fletcher, D. A., and Bissell, M. J. (2006) *Science* **314,** 298–300
30. Silberstein, G. B., and Daniel, C. W. (1987) *Science* **237,** 291–293
31. Weaver, V. M., Fischer, A. H., Peterson, O. W., and Bissell, M. J. (1996)

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/10/12/M109.023614.DC1.html

Biochem. Cell Biol. **74,** 833–851
32. Lin, X., Liang, M., and Feng, X. H. (2000) *J. Biol. Chem.* **275,** 36818–36822
33. Brown, K. A., Pietenpol, J. A., and Moses, H. L. (2007) *J. Cell. Biochem.* **101,** 9–33
34. Tang, B., Vu, M., Booker, T., Santner, S. J., Miller, F. R., Anver, M. R., and Wakefield, L. M. (2003) *J. Clin. Invest.* **112,** 1116–1124
35. Nam, J. S., Terabe, M., Mamura, M., Kang, M. J., Chae, H., Stuelten, C., Kohn, E., Tang, B., Sabzevari, H., Anver, M. R., Lawrence, S., Danielpour, D., Lonning, S., Berzofsky, J. A., and Wakefield, L. M. (2008) *Cancer Res.* **68,** 3835–3843
36. Kenny, P. A., Lee, G. Y., Myers, C. A., Neve, R. M., Semeiks, J. R., Spellman, P. T., Lorenz, K., Lee, E. H., Barcellos-Hoff, M. H., Petersen, O. W., Gray, J. W., and Bissell, M. J. (2007) *Mol. Oncol.* **1,** 84–96
37. Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R. A. (2008) *Cell* **133,** 704–715
38. Siegel, P. M., and Massagué, J. (2003) *Nat. Rev. Cancer* **3,** 807–821
39. Schiemann, W. P. (2007) *Expert Rev. Anticancer Ther.* **7,** 609–611
40. Wendt, M. K., and Schiemann, W. P. (2009) *Breast Cancer Res.* **11,** R68
41. Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y. E. (2008) *Mol. Cell* **31,** 918–924
42. Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C. H., and Landström, M. (2008) *Nat. Cell Biol.* **10,** 1199–1207
43. Muraoka, R. S., Dumont, N., Ritter, C. A., Dugger, T. C., Brantley, D. M., Chen, J., Easterly, E., Roebuck, L. R., Ryan, S., Gotwals, P. J., Koteliansky, V., and Arteaga, C. L. (2002) *J. Clin. Invest.* **109,** 1551–1559
44. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10669–10674

Downloaded from www.jbc.org at Univ Colorado - Denison Memorial Library, on December 2, 2009

Supplemental Figures

p130Cas IS REQUIRED FOR MAMMARY TUMOR GROWTH AND TRANSFORMING GROWTH FACTOR-β (TGF-β)-MEDIATED METASTASIS THROUGH REGULATION OF SMAD2/3 ACTIVITY

Michael K. Wendt, Jason A. Smith, and William P. Schiemann

From the Department of Pharmacology, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045

Address Correspondence To: William P. Schiemann, Department of Pharmacology, MS-8303, University of Colorado Denver, Anschutz Medical Campus, RC1 South Tower, Room L18-6110, 12801 East 17th Avenue, PO Box 6511, Aurora, CO 80045. Phone: 303-724-1541. Fax: 303-724-3663. Email: Bill.Schiemann@ucdenver.edu

Supplemental Figures:

Figure S1: M.K. Wendt, J.A. Smith, and W.P. Schiemann

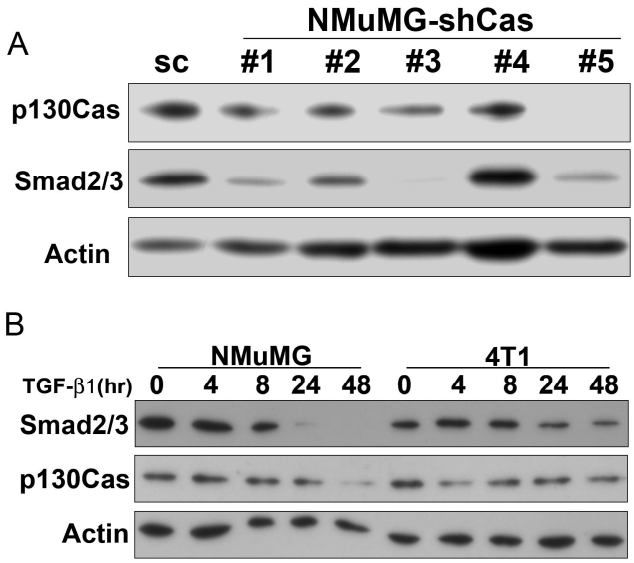


FIGURE S1. **p130Cas-deficiency decreases Smad2 expression in normal MECs.** *A*, Five unique shRNA sequences targeting p130Cas (shCas #1-5) were stably expressed in NMuMG cells, and differences in p130Cas expression were analyzed by immunoblotting with anti-p130Cas antibodies (sc, scrambled shRNA). β-actin immunoreactivity (Actin) is provided as a loading control. *B*, Quiescent NMuMG and 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for varying times, and the resulting whole-cell extracts were immunoblotted with Smad2/3 antibodies, followed by p130Cas and β-actin (Actin) as a loading control. Data are representative of 2 independent experiments and show that Smad2/3 are degraded more readily upon prolonged TGF-β treatment in NMuMG cells as compared to 4T1 cells.

Figure S2: M.K. Wendt, J.A. Smith, and W.P. Schiemann

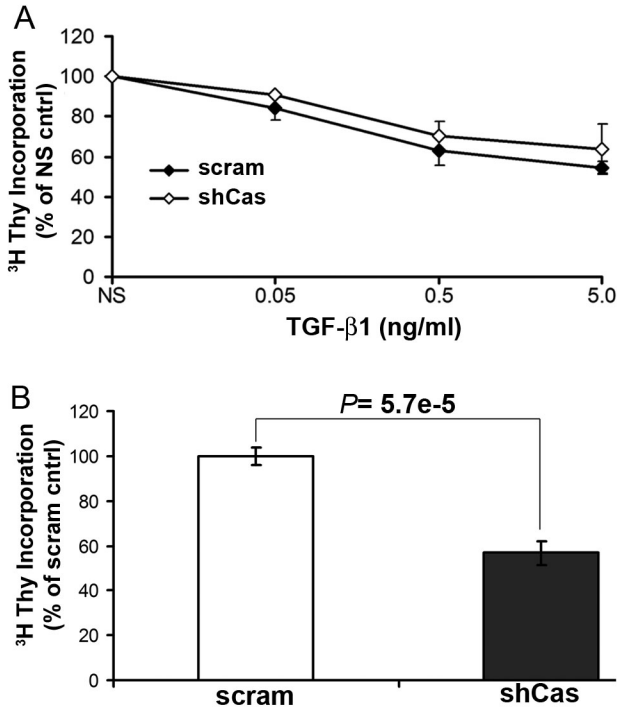


FIGURE S2. **p130Cas-deficiency decreases basal 4T1 proliferation but does not affect TGF-β1-growth inhibition when cultured on plastic**. *A*, Control (scram) and p130Cas-deficient (shCas) cells were stimulated with the increasing TGF-β1 concentrations for 48 h as indicated. Afterward, [^{3}H]thymidine incorporation into cellular DNA was determined. Data are the mean (± SE; n=3) [^{3}H]thymidine incorporation normalized to untreated controls (NS, no stimulation). *B*, Data are the mean (± SE; n=3) basal [^{3}H]thymidine incorporation normalized to control (scram) 4T1 cells.

J Mammary Gland Biol Neoplasia (2010) 15:169–190
DOI 10.1007/s10911-010-9181-1

The Pathophysiology of Epithelial-Mesenchymal Transition Induced by Transforming Growth Factor-β in Normal and Malignant Mammary Epithelial Cells

Molly A. Taylor · Jenny G. Parvani · William P. Schiemann

Received: 20 March 2010 / Accepted: 22 April 2010 / Published online: 15 May 2010
© Springer Science+Business Media, LLC 2010

Abstract Epithelial-mesenchymal transition (EMT) is an essential process that drives polarized, immotile mammary epithelial cells (MECs) to acquire apolar, highly migratory fibroblastoid-like features. EMT is an indispensable process that is associated with normal tissue development and organogenesis, as well as with tissue remodeling and wound healing. In stark contrast, inappropriate reactivation of EMT readily contributes to the development of a variety of human pathologies, particularly those associated with tissue fibrosis and cancer cell invasion and metastasis, including that by breast cancer cells. Although metastasis is unequivocally the most lethal aspect of breast cancer and the most prominent feature associated with disease recurrence, the molecular mechanisms whereby EMT mediates the initiation and resolution of breast cancer metastasis remains poorly understood. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that is intimately involved in regulating numerous physiological processes, including cellular differentiation, homeostasis, and EMT. In addition, TGF-β also functions as a powerful tumor suppressor in MECs, whose neoplastic development ultimately converts TGF-β into an oncogenic cytokine in aggressive late-stage mammary tumors. Recent findings have implicated the process of EMT in mediating the functional conversion of TGF-β during breast cancer progression, suggesting that the chemotherapeutic targeting of EMT induced by TGF-β may offer new inroads in ameliorating metastatic disease in breast cancer patients. Here we review the molecular, cellular, and microenvironmental factors that contribute to the pathophysiological activities of TGF-β during its regulation of EMT in normal and malignant MECs.

Keywords Epithelial-mesenchymal Transition · Metastasis · Signal transduction · Transforming growth factor-β · Tumor microenvironment

Abbreviations

α-SMA	α-smooth muscle actin
BMP	Bone morphogenic protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
JNK	c-Jun N-terminal kinase
MAP kinase	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
mTOR	Mammalian target of rapamycin
MET	Mesenchymal-epithelial transition
MTA3	Metastasis associated protein 3
NF-κB	Nuclear factor-κB
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide-3-kinase
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptor

Financial Support W.P.S. was supported by grants from the National Institutes of Health (CA114039 and CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651), while M.A.T. was supported by the Department of Defense (BC093128).

M. A. Taylor and J. G. Parvani were equal contributors to this work.

M. A. Taylor · J. G. Parvani · W. P. Schiemann (✉)
Case Comprehensive Cancer Center,
Case Western Reserve University,
Wolstein Research Building, Room 2131, 2103 Cornell Road,
Cleveland, OH 44106, USA
e-mail: William.Schiemann@case.edu

TGF-β	Transforming growth factor-β
uPA	Urokinase plasminogen activator
uPAR	uPA receptor
ZO-1	Zonula occluden-1

Introduction

Epithelial-mesenchymal transition (EMT) is a complex process whereby polarized epithelial cells transition into apolar fibroblastoid-like cells, a phenomenon that underlies tissue morphogenesis and organogenesis in the embryo, as well as tissue remodeling and repair in adults [1–3]. Moreover, the inappropriate reactivation of developmental EMT programs plays a significant role in the pathology of fibrotic diseases and cancer, including those of the breast. Epithelial cell sheets manifest as tightly packed cell monolayers that compose the skin and line the internal cavities (e.g., airways and gastrointestinal tract), and in doing so, form a barrier that protects the host from environmental insults. In a similar fashion, mammary epithelial cells (MECs) exhibit a cobblestone appearance and are linked through the actions of numerous cell–cell complexes, including desmosomes, adherens, gap, and tight junctions [4]. Collectively, these junctional structures provide MECs with their characteristic apical-basolateral polarity and cortical actin architecture. In stark contrast, mesenchymal cells lack cell–cell junctional complexes, leading to their apolar morphologies and enhanced migratory activities through the extracellular matrix (ECM). The plasticity of MECs enables them to dedifferentiate during EMT, and in doing so, transitioning MECs forego their cobblestone morphologies and instead acquire a spindle-shaped appearance characteristic of mesenchymal cells. In undertaking this phenotypic and morphologic transition, MECs first experience a disruption and delocalization of tight junction complexes (e.g., zonula occluden-1 (ZO-1), claudin, and occludin), which is succeeded by the loss of E-cadherin expression and activity that results in the stabilization and nuclear accumulation of β-catenin. This process is also characterized by the dramatic remodeling of the cytoskeleton and its formation of actin stress fibers as transitioning cells acquire migratory mesenchymal phenotypes [1–3]. Thus, EMT reflects the initiation of a complex cascade of genetic and epigenetic events that culminate in MECs discarding their expression of epithelial gene signatures (e.g., E-cadherin, β4 integrin, and ZO-1) and acquiring those of mesenchymal cells [e.g., N-cadherin, vimentin, α-smooth muscle actin (α-SMA)]. Moreover, the process of EMT is highly metastable and is readily subject to phenotypic and morphologic reversion by mesenchymal-epithelial transition (MET), the molecular mechanisms of which are poorly understood and will not be discussed further herein (*see* [5, 6]). These general steps exhibited by transitioning MECs underlie both the biological and pathological episodes of EMT, which recently have been categorized into three distinct subtypes—i.e., *a*) embryonic and developmental EMT, which is referred to as type 1 EMT; *b*) tissue regeneration and fibrotic EMT, which is referred to as type 2 EMT; and *c*) cancer progression and metastatic EMT, which is known as type 3 EMT [2].

Here we review recent findings that directly impact our understanding of the role transforming growth factor-β (TGF-β) plays in regulating the initiation and resolution of individual subtypes of EMT. In addition, we also discuss the clinical implications afforded by chemotherapeutic targeting of TGF-β effectors coupled to type 3 EMT and their potential to suppress breast cancer progression and the oncogenic activities of TGF-β, particularly its induction of EMT and metastasis in developing mammary carcinomas.

TGF-β and EMT Subtypes

TGF-β Signaling and Epithelial Plasticity

TGF-β is a multifunctional cytokine and a powerful tumor suppressor that governs essentially every aspect of the physiology and homeostasis of MECs, including their ability to proliferate, migrate, differentiate, and survive [7–9]. During mammary tumorigenesis, a variety of genetic and epigenetic events conspire to circumvent the cytostatic and tumor suppressing activities of TGF-β, thereby enhancing the development and progression of evolving mammary neoplasms [1, 7, 8]. Even more remarkably, neoplastic MECs that have acquired resistance to the cytostatic activities of TGF-β often exhibit oncogenic behaviors when stimulated by TGF-β. This phenotypic switch in TGF-β function during tumorigenesis is known as the "TGF-β Paradox," which represents the most important and unanswered question concerning the pathophysiological actions of this pleiotropic cytokine [10]. Interestingly, the differentiation and migration of mammary stem cells results in the production of both the outer myoepithelial and inner luminal layers that ultimately give rise to mature mammary glands [11–13], suggesting that the process of EMT is in someway linked to the generation and maintenance of stem cell populations. Numerous studies have established TGF-β as a master regulator of EMT in normal and malignant MECs [1, 14, 15], while more recent findings have associated TGF-β stimulation of EMT with the acquisition of "stemness" in transitioning MECs [16, 17], and with the selection and expansion of breast cancer stem cells [18, 19]. Along these lines, we and others have established EMT as being a vital component underlying the

initiation of oncogenic TGF-β signaling in normal and malignant MECs [1, 14, 20]. Thus, identifying the molecular mechanisms whereby EMT is induced by TGF-β is paramount to maintaining mammary gland homeostasis, and to suppressing the development and progression of mammary tumors.

The mechanisms through which TGF-β initiates its pathophysiological activities and initiation of EMT are shown schematically in Fig. 1. Indeed, transmembrane signaling stimulated by TGF-β commences by its binding to the high-affinity transmembrane Ser/Thr receptor protein kinases, TGF-β type I (TβR-I) and type II (TβR-II). Mammals express three distinct TGF-β isoforms termed TGF-β1, TGF-β2, and TGF-β3, which function analogously in vitro, but give rise to more than 30 distinct phenotypes upon their genetic deletion in mice [21]. In addition, whereas TGF-βs 1 and 3 can bind directly to TβR-II, TGF-β2 requires assistance from the accessory TGF-β receptor, TGF-β type III (TβR-III). In all of these scenarios, the ligation of TGF-β to TβR-II facilitates its transphosphorylation and activation of TβR-I, which subsequently phosphorylates and activates the latent transcription factors, Smads 2 and 3. Once phosphorylated, receptor-activated Smads 2 and 3 associate with the common Smad, Smad4, at which point these heteromeric complexes translocate en masse into the nucleus to regulate the expression of TGF-β-responsive genes. In addition, the amplitude and duration of Smad2/3-based signaling transpires through their physical interaction with a plethora of transcription factors, and with a variety of transcriptional activators and co-repressors in a gene- and cell-specific manner (*see* [22, 23]). The capacity of Smad2/3 to impact MEC behavior is also governed by their association with a number of adapter molecules, including SARA [24], Hgs [25], and Dab2 [26–28]. Likewise, upregulated expression of the inhibitory Smad, Smad7, also limits the extent of Smad2/3 signaling by competitively inhibiting their phosphorylation by TβR-I [29–31], and by promoting the internalization and degradation of TβR-I [32, 33]. Moreover, the anti-TGF-β activity of Smad7 is augmented by its interaction with the adaptor protein STRAP [34], and conversely, is attenuated by its association with either AMSH2 [35] or Arkadia [36–38]. Collectively, TGF-β signals propagated through Smad2/3 are referred to as "canonical" TGF-β signaling, and their specific role in regulating EMT induced by TGF-β is discussed below.

Besides its ability to activate canonical Smad2/3-dependent pathways, TGF-β also regulates MEC behavior and the induction of EMT via the stimulation of numerous "non-canonical" effector systems, including (*a*) small GTP-binding proteins (e.g., Ras, Rho, and Rac1); (*b*) phosphoinositide-3-kinase (PI3K), AKT, and mTOR; (*c*) MAP kinases (e.g., p38 MAPK, ERK1/2, and JNK); and (*d*) NF-κB and Cox-2 [39–51]. In addition, although inactivating mutations and decreased expression of TβR-I and TβR-II have been identified and characterized in human cancers, the occurrence of these mutagenic events is in fact quite rare. However, the loss of TβR-III expression does correlate with increased breast cancer tumorigenicity and decreased patient survival [52, 53], suggesting an important tumor suppressing function for this accessory receptor. Indeed, the functional loss of TβR-III coincides with EMT and enhances cell migration and invasion [54, 55]. The coupling of TGF-β to these receptors and their noncanonical effectors is depicted schematically in Fig. 1, as is our understanding of how integrins and proteins associated with focal adhesion complexes cooperate with TGF-β in regulating the behaviors and EMT status of normal and malignant MECs [45, 46, 56–60]. The specific role of these noncanonical TGF-β effectors in regulating EMT in normal and malignant MECs is presented in the succeeding sections, while future studies clearly need to address the relative contribution of TβR-I, TβR-II, and TβR-III in mediating activation of canonical and non-canonical TGF-β signaling systems.

EMT Subtypes Coupled to TGF-β

The process of EMT occurs in a variety of distinct biological and pathological settings, including during normal embryogenesis and tissue morphogenesis, during tissue remodeling and repair, and during fibrosis and cancer progression. Although the underlying molecular mechanisms that define the pathophysiological activities of EMT in distinct cellular contexts are likely to be overlapping and redundant, the diversity of biological outcomes engendered by EMT is nonetheless highly specialized and has resulted in the classification of three distinct subtypes of EMT [2]. For instance, type 1 EMT is activated during embryogenesis and tissue morphogenesis, leading to the generation of mesenchymal cells that ultimately give rise to secondary epithelial structures. In contrast, type 2 EMT is normally activated during tissue regeneration and repair, and abnormally during tissue fibrosis resulting from dysregulated inflammatory reactions. Finally, type 3 EMT is activated by cancer cells, including those of the breast [1], to facilitate their acquisition of invasive and metastatic phenotypes, and ultimately to establish secondary sites of lethal tumor outgrowth. In the succeeding sections, we highlight the molecular mechanisms and biological settings whereby TGF-β participates in regulating individual EMT subtypes, particularly type 3 EMT during mammary tumorigenesis.

Type 1 EMT

Developmental or type 1 EMT is associated with embryogenesis and its accompanying organogenesis and tissue morphogenesis, both of which require epithelial-derived

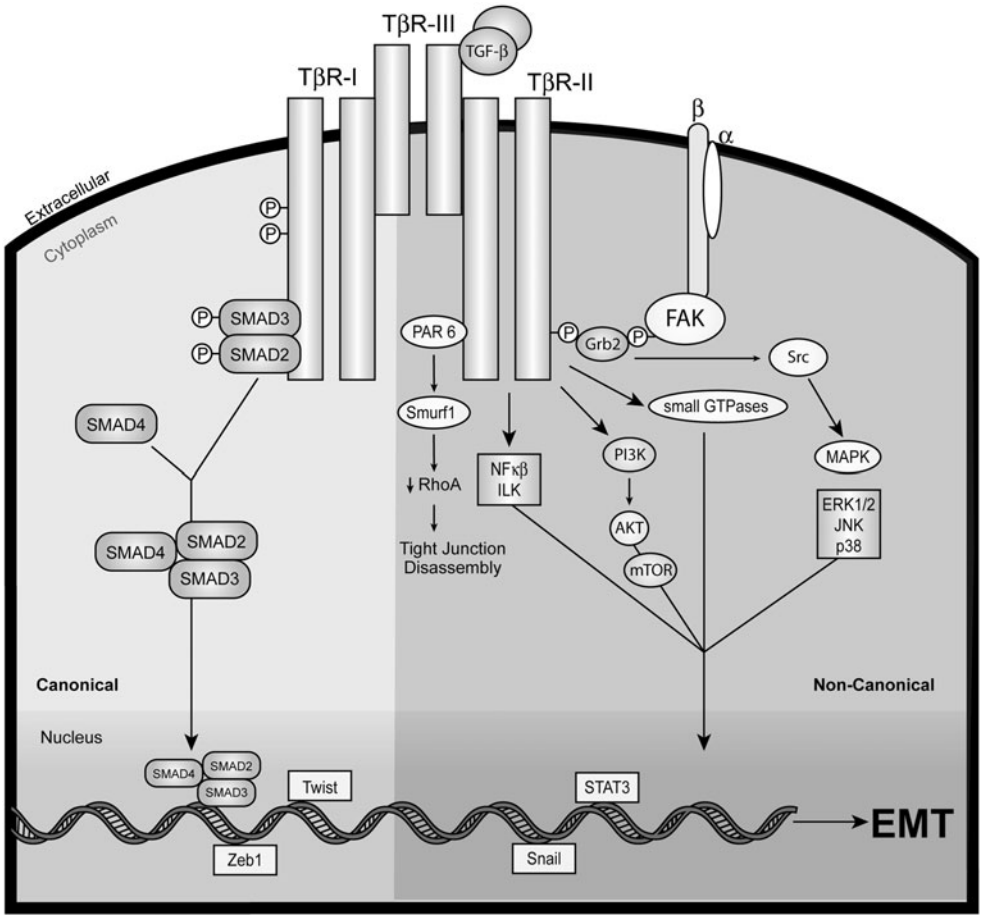


Figure 1 Schematic depicting the canonical and noncanonical TGF-β signaling systems coupled to EMT in MECs. Transmembrane signaling by TGF-β ensues through its binding and activation of the Ser/Thr protein kinase receptors, TβR-I and TβR-II. Indeed, TGF-β binding to either TβR-III or TβR-II enables the recruitment and transphosphorylation of TβR-I, resulting in its activation and subsequent phosphorylation of the receptor-activated Smads, Smad2 and Smad3. Once activated, Smad2/3 form heterocomplexes with the common Smad, Smad4, which collectively translocate en masse to the nucleus to regulate the expression of TGF-β-responsive genes in concert with an ever expanding list of transcriptional coactivators and repressors. This branch of the bifurcated TGF-β signaling system represents the "canonical" or "Smad2/3-dependent" TGF-β pathway (*left*). Alternatively, TGF-β also activates a variety of "noncanonical" or "Smad2/3-independent" effectors, including Par6, NF-κB, ILK, FAK, Src, a variety of small GTPases, members of the MAP kinase family (e.g., ERK1/2, JNK, and p38 MAPK), and the PI3K:AKT:mTOR signaling axis (*right*). Activation of members of the Snail, ZEB, or bHLH family of transcription factors promote EMT by transcriptionally repressing the production of E-cadherin transcripts (*bottom*). Imbalances between canonical and noncanonical TGF-β signaling pathways have recently been identified and established as casual aberrancies underlying the initiation of oncogenic TGF-β signaling and its initiation of EMT in normal and malignant MECs. See text for details.

mesenchymal cells to undergo MET during the formation of new epithelial structures (Fig. 2). In stark contrast to types 2 and 3 EMT, this developmental mode of EMT results in the production of multipotent mesenchymal cells and is not associated with tissue fibrosis and inflammation, or with the aberrant migration of cancer cells. Interestingly, members of the TGF-β superfamily play important regulatory roles during type 1 EMT, particularly during the processes of gastrulation, palate fusion, and neural crest and endocardial cushion formation [61]. Indeed, type 1 EMT is first initiated during embryogenesis to promote gastrulation, which results in the formation of the ectoderm, mesoderm, and endoderm from the invaginating primitive streak [2, 4]. This process requires Wnt signaling inputs to render primitive streak cells competent to undergo EMT in response to the TGF-β superfamily members, Nodal and Vg1 [62–65], both of which are essential for primitive streak and mesoderm formation in gastrulating embryos [66–70].

Type 1 EMT is also initiated during neurulation and occurs in the neural plate to facilitate the formation of the neural tube, which ultimately gives rise to the spine and brain. As the neural tube fuses, EMT also occurs in neural crest cells and facilitates their migration and dissemination throughout the embryo where they ultimately contribute to the generation of numerous specialized tissues, including

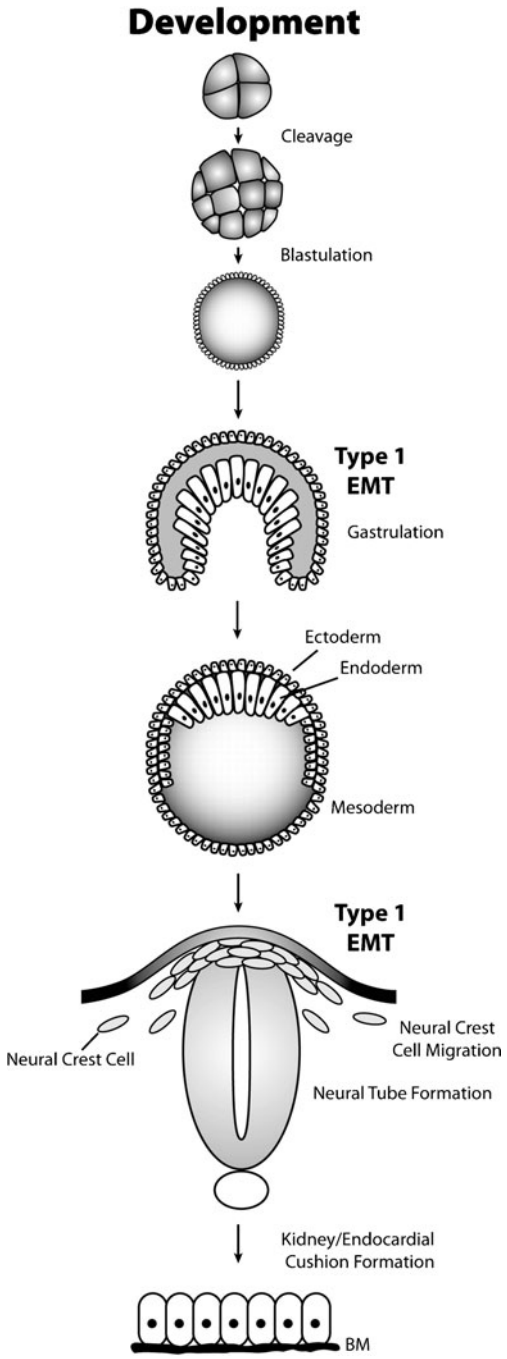


Figure 2 Type 1 EMT in development and embryogenesis. Following fertilization, the zygote undergoes several rounds of cleavage to form a dense spheroid structure, which then undergoes blastulation to form a hollowed blastula. During gastrulation, TGF-β provides inductive EMT signals that elicits the formation of an invaginating structure that generates the three multipotent germ layers, namely the mesoderm, endoderm, and ectoderm. The mesoderm gives rise to muscle, skeleton, and connective tissue, while the endoderm gives rise to internal organs, such as the liver, colon, and stomach. The outer ectodermal layer generates the epidermis and ocular lens, as well as produces the mammary gland. The ectoderm also gives rise to the primordial nervous system via neurulation and neural crest formation, a process that is dependent upon EMT induced by TGF-β. Finally, TGF-β stimulation of EMT during organogenesis is essential for the faithful development of the kidney, and of the endocardial cushion and subsequent atrioventricular valve formation. The role of TGF-β in regulating type 1 EMT during mammary gland branching and invasion is discussed in the text.

the adrenal medulla and the peripheral nervous and skeletal systems [4, 71]. Bone morphogenic proteins (BMPs) belong to the TGF-β superfamily and are essential to the induction of EMT during neurulation [72, 73]. Similarly, TGF-β signaling also oversees the latter stages of heart and secondary palate formation [4, 74]. For instance, myocardial cells actively secrete an ECM that separates the endocardium from the myocardium, which also induces endocardial cells to undergo EMT and migrate into the endocardial cushion to facilitate atrioventricular valve formation [4]. Experiments performed in chicks and mice have identified important roles during development for all three TGF-β isoforms, of which TGF-β2 appears to play a dominant role in stimulating EMT in endocardial cells [75–77]. Similarly, TGF-β3 is essential in mediating secondary palate formation, which requires midline epithelial seam cells to undergo EMT to complete oral palate fusion and closure [78, 79]. Importantly, TβR-III expression is essential for EMT induced during the formation of both the heart and secondary palate [80, 81].

At present, a role for type 1 EMT in mediating mammary gland development has yet to be described; however, branching morphogenesis of normal MEC organoids in 3D-organotypic cultures showed that these structures exhibit a loss in polarity and acquire mesenchymal characteristics at invading branch tips, findings that point to a role of type 1 EMT in mammary gland development [82, 83]. In addition, inappropriate reactivation of embryonic and type 1 EMT programs have been associated with the development and progression of mammary tumors [61]. Indeed, aberrantly elevated expression of the Six1 homeoprotein not only elicits the formation of mammary tumors [84], but also stimulates breast cancer EMT and metastasis in part via a TGF-β-dependent mechanism [85]. Thus, thoroughly defining the role of TGF-β during type 1 EMT will be critical to the development of novel chemotherapeutics capable of circumventing these activities during the inappropriate reactivation of type 1 EMT programs in mammary tumors.

Type 2 EMT

The initiation of type 2 EMT is essential in maintaining tissue homeostasis through its ability to induce wound healing and tissue remodeling in response to noxious insults. An important distinction between type 1 and type 2 EMT is that the latter is governed by inflammatory reactions, whose cessation resolves the EMT phenotype following wound repair [2]. A

corollary states that chronic inflammation underlies the initiation of tissue and organ fibrosis, which eventually elicits organ dysfunction and destruction if left unabated. As depicted in Fig. 3, the process of wound healing involves the orchestrated activities of numerous cell types to facilitate the re-epithelialization of denuded areas [86]. In fact, signaling by TGF-β and the ECM are essential in promoting the activation and differentiation of myofibroblasts, which are the key cells involved in the repair of damaged epithelium and scar formation [86]. Working in concert with epithelial and endothelial cells, activated myofibroblasts secrete matrix metalloproteinases (MMPs) that digest injured tissues and facilitate the synthesis of a provisional ECM [87, 88]. Exposure of platelets and infiltrating immune cells to provisional ECM components elicits platelet degranulation, angiogenesis, and wound contraction, of which the latter response is mediated by myofibroblasts during the final stages of re-epithelialization [86, 87, 89]. Under normal circumstances, the inflammatory reactions within healed wounds resolve, thereby terminating type 2 EMT and stimulating the elimination of myofibroblasts via apoptosis [88]. However, sustained myofibroblast activation in conjunction with chronic inflammation underlies the initiation of fibrotic disorders due to unresolved type 2 EMT reactions [86, 87]. Myofibroblasts represent a specialized cell type that exhibit traits reminiscent of smooth muscle cells, particularly the expression of α-smooth muscle actin (α-SMA; [90, 91]). As a group, myofibroblasts derive from fibroblasts, from circulating progenitor cells, and from epithelial cells following their completion of EMT, which is typically assessed by monitoring the extent of α-SMA expression in fully transitioned cells [1]. Exacerbated α-SMA expression is also indicative of fibrotic states and fibroproliferative disorders [92–94], as well as correlates with increased tumor invasion and decreased survival rates in cancer patients [95]. Not surprisingly, α-SMA expression is induced by TGF-β via the concerted signaling inputs of Smad2/3, RhoA/Rock, and ERK1/2 [96–99]. Furthermore, integrin activation by laminin, fibronectin, and collagen also cooperates with TGF-β to induce EMT and myofibroblasts activation, an event coupled

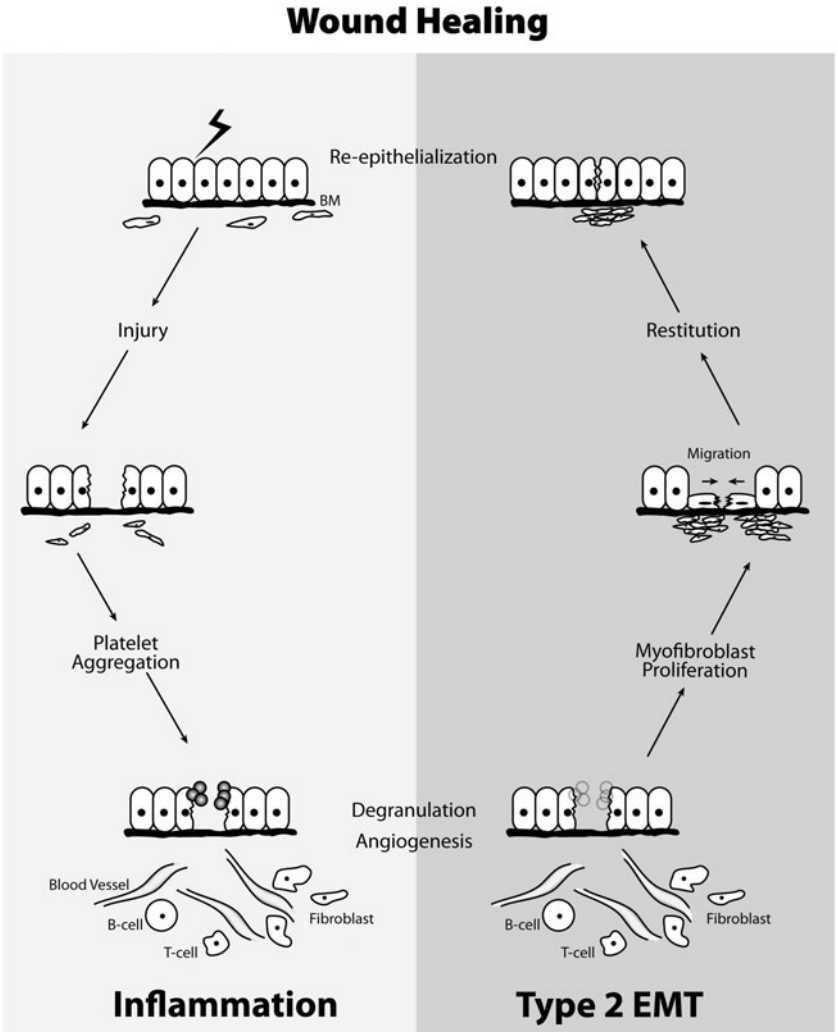


Figure 3 Type 2 EMT in wound healing and tissue regeneration. Normal epithelia that arose from type 1 EMT during development experience a noxious event or injury that triggers endothelial and epithelial cells to produce factors that mediate coagulation and clot formation. These events, coupled with enhanced MMP production facilitate the recruitment of immune cells and platelets to denuded wounds, leading to the production of a provisional ECM and activation of angiogenesis. TGF-β enhances the healing process by inducing EMT in myofibroblasts, which drives their differentiation, activation, and migration into denuded wounds to facilitate wound restitution, closure, and re-epithelialization. The role of TGF-β in regulating type 2 EMT during mammary gland fibrosis is discussed in the text.

to the formation of β-catenin:Smad2 signaling complexes [100–102]. Thus, in addition to its role in promoting type 2 EMT, aberrant TGF-β signaling also supports chronic inflammatory reactions that promote the establishment of fibroproliferative disorders in humans (Fig. 3).

At present, the overall importance of fibrotic reactions in promoting mammary tumorigenesis remains to be determined definitively. However, mammographically dense and fibrotic breast tissue have been linked to the increased incidence of mammary tumorigenesis [103, 104]. Along these lines, radiation therapy of breast cancers is associated with the development of fibrosis [105], and with the initiation of EMT via a TGF-β-dependent mechanism [106]. Moreover, mammary tumorigenesis is often accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that (*a*) enhance the selection and expansion of developing neoplasms, particularly that of late-stage metastatic tumors, and (*b*) predict for poor clinical outcomes in patients with breast cancer [107–109]. Interestingly, these aberrant cellular activities are highly reminiscent of those attributed to TGF-β [110], suggesting that TGF-β stimulation of EMT and fibrosis may promote the development and progression of mammary tumors. This supposition is bolstered by a recent study showing that inhibiting the cross-linking of collagen during mammary fibrosis reduces breast cancer progression in mice [111]. Collectively, these findings suggest that chemotherapeutic targeting of the EMT inducing activities of TGF-β may offer a novel two-pronged approach to alleviate breast cancer progression—namely, the inactivation of pathologic type 2 (i.e., fibrotic) and type 3 EMT (*see below*).

Mechanisms of Type 3 EMT Induced by TGF-β

The initiation of type 3 EMT is essential in facilitating cancer progression and metastasis, including that by mammary tumors [1, 2]. In addition, type 3 EMT is primarily distinguished from its type 1 and 2 counterparts by the cellular context in which this phenotypic transition transpires—namely, type 3 EMT occurs in oncogenically transformed cells that house a variety of genetic and epigenetic abnormalities that conspire with the molecular cascade that underlies EMT to elicit metastatic dissemination. Even more remarkably, TGF-β stimulation of EMT has been associated with the selection and expansion of breast cancer stem cells [16–19], which by their nature exhibit robust resistance to traditional cancer chemotherapies [112]. In the succeeding sections, we present our understanding of the molecular, cellular, and microenvironmental factors that contribute to the initiation of type 3 EMT by TGF-β in breast cancer cells (Fig. 4).

Canonical TGF-β Signaling During EMT

Although the activities attributed to Smad2 and Smad3 during TGF-β signaling are commonly conjoined in the scientific literature, recent findings indicate that these latent transcription factors do in fact mediate distinct biological activities in response to TGF-β [113]. These disparate functions mediated by Smads 2 and 3 also extend to EMT, which is inhibited by Smad2 and stimulated by Smad3. For instance, Smad2-deficiency enhances the development and progression of squamous cell carcinomas by elevating Snail expression and its induction of EMT [114], as well as elicits the acquisition of mesenchymal and fibrotic morphologies in hepatocytes [115]. Interestingly, oncogenic Ras cooperates with Smads 2 and 3 to drive the formation of spindle cells and their subsequent acquisition of EMT and metastatic phenotypes [116]. TGF-β stimulation of EMT in kidney cells results in the attenuated expression of SARA, an adapter molecule that facilitates the presentation of Smad2/3 to TβR-I [24]. Moreover, depleting cells of SARA increased the ubiquitination and degradation of Smad2, leading to the acquisition of EMT phenotypes [117]. In stark contrast, Smad3-deficiency (*a*) prevented TGF-β stimulation of EMT in lens [118] and renal [119] epithelial cells; (*b*) reduced the EMT and migratory abilities of keratinocytes to TGF-β [120, 121]; and (*c*) preserved an epithelial phenotype in hepatocytes stimulated by TGF-β [115]. Thus, these studies implicate a pro-EMT function associated with Smad3 activation, which is mirrored by the activation of Smad4 in pancreatic cells undergoing EMT induced by TGF-β [122]. Consistent with its designation as an inhibitory Smad, over expression of Smad7 is sufficient to abrogate the ability of TGF-β to induce EMT in epithelial cells of the breast [123], liver [124], and lens [125]. Collectively, these studies demonstrate that the expression and activities of TGF-β-regulated Smads are critical to the initiation and resolution of EMT in diverse epithelial cell lineages.

Smad2/3 activation also figures prominently in mediating TGF-β stimulation of EMT in MECs. Indeed, TGF-β signaling through Smads 2, 3, and 4 induce an EMT transcriptional program in normal MECs, a physiological reaction that is blocked by overexpression of Smad7 [123]. Interestingly, chronic TGF-β signaling elicits a metastable EMT phenotype in normal MECs that is characterized by reduced Smad2/3 signaling, leading to MEC resistance to the cytostatic and apoptotic activities of TGF-β [126]. Thus, EMT may underlie the conversion of TGF-β function from that of a tumor suppressor to a tumor promoter in mammary tumors (*see below*). Along these lines, Smad4-deficiency not only prevented TGF-β stimulation of EMT in normal and malignant MECs, but also alleviated its induction of bone metastasis by breast cancer cells in mice [127]. Furthermore, targeting and inactivating Smad2/3 signaling using aptamer

technology was observed to neutralize the ability of TGF-β to induce EMT in normal MECs [128]. Interestingly, Smad2 signaling has recently been shown to promote EMT in MECs by enhancing the DNA binding activity of the DNA methyltransferase, DNMT1, leading to chronic epigenetic silencing of epithelial-associated genes [129]. Finally, a recent study established that the aberrant coupling of TGF-β to BMP-regulated Smads (e.g., Smads 1 and 5) during mammary tumorigenesis confers a pro-migratory phenotype in breast cancer cells [130]. Although a role for this unusual coupling event in mediating EMT by TGF-β was not investigated, it is nonetheless tempting to speculate that inappropriate cross-talk between TGF-β superfamily members may contribute to the pathophysiological outcomes of EMT initiated by TGF-β. Future studies will need to address this question, as well as define the underlying relationship between Smad-dependent and -independent signaling during TGF-β stimulation of EMT in MECs (*see below*).

Noncanonical TGF-β Signaling During EMT

The aberrant amplification of noncanonical TGF-β signaling systems plays a salient role in mediating the ability of TGF-β to induce EMT in normal and malignant MECs, and in underlying the initiation of the "TGF-β Paradox." The function of noncanonical TGF-β effectors in coupling this cytokine to EMT and metastasis are discussed in the succeeding sections.

Rho-family GTPases

Oncogenic TGF-β signaling is often associated with the dysregulated activity of the Rho GTPase family, which includes RhoA/B/C, Rac1, and Cdc42 [1, 14, 20]. This family of small GTP-binding proteins are anchored to the plasma membrane where they regulate dynamic changes in cell adhesion, morphology, and motility in part by modulating the formation of filopodia (e.g., Cdc42), lamellipodia (e.g., Rac1), and actin stress fibers (e.g., RhoA) [131, 132]. For instance, RhoA activation mediates the dissolution of adhesion complexes at cell–cell junctions, while that of Rac1 actually promotes the formation of these same complexes [133, 134]. Additionally, constitutive activation of Cdc42 by TβR-III inhibits directional migration induced by TGF-β [135]. Interestingly, the expression and activation of RhoC enhances the invasion and EMT of breast [136], prostate [137], and colon [138] cancer cells. The ability of TGF-β to induce EMT in MECs requires the activation of RhoA and its downstream effector, $p160^{ROCK}$ [41]. In addition, the

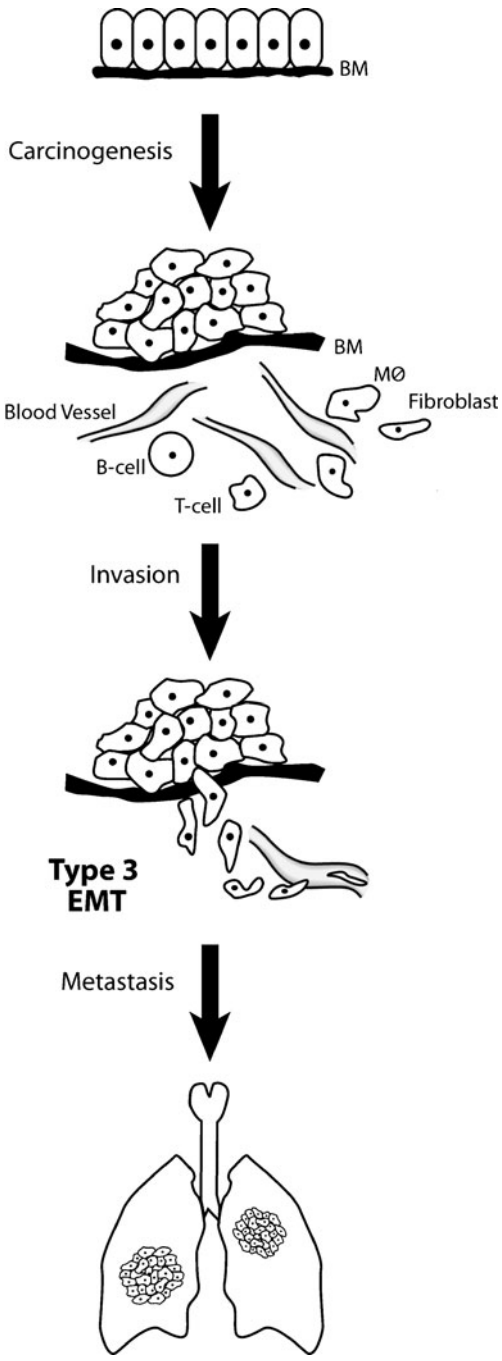


Figure 4 Type 3 EMT in cancer metastasis. Normal epithelia that arose from type I EMT during development experience a carcinogenic event that ultimately results in their oncogenic transformation and tumor formation. The development and progression of mammary tumors is accompanied by their acquisition of dysplastic and abnormal morphologies, and by their evolution in chronically inflamed tumor microenvironments, which further enhances their acquisition of EMT and fibrotic phenotypes. Through its ability to stimulate oncogenic EMT, TGF-β enables transitioned mammary carcinoma cells to invade the underlying basement membrane (BM) and escape the confines of the primary tumor. Once liberated, metastatic MECs undergo intravasation and traverse the blood stream prior to taking up residence at distant locales, an event that ultimately leads to disease recurrence and poor clinical outcomes in breast cancer patients. See text for specific details on the molecular mechanisms whereby TGF-β promotes type 3 EMT and metastasis in breast cancer cells.

coupling of TGF-β to RhoA and RhoC also correlates with altered expression of E-cadherin and α-SMA during EMT [96, 139]. Recently, TβR-II-mediated phosphorylation of Par6 was shown to underlie the ubiquitination and degradation of RhoA, leading to the dissolution of tight junctions during the acquisition of EMT and metastatic phenotypes in breast cancer cells [140, 141]. Along these lines, upregulation of miR-155 by canonical TGF-β signaling was observed to promote EMT in MECs by targeting the destruction of RhoA mRNA [142]. In stark contrast, reduced RhoA expression mediated by miR-31 was found to suppress, not promote, breast cancer metastasis [143, 144], suggesting an intricate and complex role for RhoA and its relatives in regulating the initiation of EMT and metastasis.

PI3K, AKT, and mTOR

Oncogenic TGF-β signaling and its stimulation of EMT also requires the activities of phosphoinositide 3-kinase (PI3K) and Akt, both of which confer proliferative and survival advantages to developing carcinomas [145]. In addition, PI3K and Akt also mediate TGF-β stimulation of EMT in MECs [40], an event that arises directly from TGF-β receptor signaling inputs, or indirectly through their transactivation of the receptors for EGF [146] and PDGF [147]. Interestingly, co-administration of TGF-β and EGF elicits an exaggerated EMT through the activation of ERK1/2 and PI3K/Akt [148]. Somewhat surprisingly, pharmacological inhibition of PI3K/Akt had no effect on the morphologic and phenotypic characteristics of EMT; however, this same treatment regimen did alleviate the ability of TGF-β and EGF to induce cell migration and invasion [148], suggesting that the morphologic and motile features of EMT may in fact be distinct physiological entities. Along these lines, inactivating mTOR pharmacologically with rapamycin prevents TGF-β from increasing the physical size of MECs, as well as from stimulating their migration and invasion [42]. As above, mTOR antagonism failed to impact the morphological features of EMT, which suggests that mTOR may facilitate the synergistic effects of TGF-β and EGF on EMT. Collectively, these studies highlight the essential function of the PI3K/Akt/mTOR pathway in promoting EMT and metastasis stimulated by TGF-β, while future studies need to clarify the underlying dissociation between "fibroblastoid-like" phenotypes and cell motility.

Nuclear Factor-κB

Nuclear factor-κB (NF-κB) plays a prominent role in regulating the initiation and resolution of inflammatory reactions, and in promoting the growth, angiogenesis, invasion, and survival of developing carcinomas [149]. In nontransformed tissues, TGF-β typically inhibits the activation of NF-κB [49, 50, 150], presumably by inducing the expression of IκBα [151], and by promoting the formation of TβR-III:β-arrestin2 complexes that prevent IκBα degradation [152]. In stark contrast, mammary tumorigenesis converts TGF-β from an inhibitor to a stimulator of NF-κB activity by inducing the formation of TβR-I:xIAP:TAB1:TAK1:IKKβ complexes [49, 50, 153]. Furthermore, activation of this noncanonical effector system by TGF-β was found to be essential for its induction of EMT in normal and malignant MECs [49, 50, 153, 154], and for its stimulation of mammary tumor development via activation of the innate immune system [49]. Moreover, the coupling of TGF-β to NF-κB activation underlies the ability for Ras-transformed breast cancer cells to colonize the lung [47, 155], and elicits the initiation of an autocrine Cox-2:PGE2:EP2 receptor signaling cascade that not only induces EMT in normal and malignant MECs, but also stimulates breast cancer metastasis [50, 51]. Finally, TGF-β stimulation of NF-κB in post-EMT cells stimulates their migration by establishing a SDF-1/CXCR4 signaling axis [156]. Collectively, these studies highlight the importance of NF-κB to the induction of EMT by TGF-β, while future studies need to assess the relative contribution of these events to enhanced survival and chemoresistance exhibited by post-EMT breast cancer cells.

MAP Kinases

The propensity of TGF-β to induce pathological EMT and metastasis is also associated with its stimulation of members of the MAP kinase family of protein kinases, including ERKs, JNKs, and p38 MAPKs [39, 157–159]. For instance, pharmacological inhibition of MEK1/2 prevents TGF-β from stimulating EMT and its characteristic formation of actin stress fibers and delocalization of E-cadherin and ZO-1 from the cell surface in MECs [158]. Similarly, methods that disrupt the coupling of TGF-β to JNK activation prevent the morphological and transcriptional changes associated with EMT [160, 161]. Along these lines, there exists a dynamic interplay between TGF-β and its production of various ECM components, which subsequently potentiate the activation of MAP kinase pathways during EMT. For instance, the expression of type I collagen activates PI3K, Akt, and JNK to induce EMT [162, 163]. Moreover, TGF-β in conjunction with vitronectin signaling is necessary in mediating TβR-II phosphorylation by Src, a phosphotransferase reaction operant in activating p38 MAPK and EMT in MECs [23, 45]. Likewise, the coupling of TGF-β to ERK1/2 and p38 MAPK activation is dependent upon the localization of TGF-β receptors to lipid rafts, not clathrin-coated pits, and as such, cholesterol-depleting methodologies are sufficient to block cell migration and EMT stimulated by TGF-β

[164]. Collectively, these studies implicate MAP kinases as crucial mediators linking the ability of TGF-β and ECM components to promote EMT.

Integrin-linked Kinase

The coupling of TGF-β to its noncanonical effectors during type 3 EMT is further exacerbated upon the activation of integrin-linked kinase (ILK), which is a Ser/Thr protein kinase coupled to the activation of small GTPases, PI3K/Akt, and MAP kinases [165–167]. ILK activation also leads to the inhibition of GSK-3β activity, which stabilizes β-catenin and facilitates its nuclear accumulation during EMT [166]. Indeed, elevated expression of ILK in MECs is associated with their decreased expression of E-cadherin and increased invasion [168], and with their oncogenic transformation by hyperactive ERK1/2 and Akt [169]. Along these lines, ILK-deficiency prevented TGF-β from stimulating cell migration and invasion in part by uncoupling this cytokine from regulation of the uPA/PAI-1 system [170]. Finally, TGF-β stimulation of Smad2/3 induces PINCH-1 expression, which interacts physically with ILK during the initiation of EMT and its consequential loss of E-cadherin and ZO-1 expression [171]. Collectively, these findings suggest that ILK interfaces integrin signaling with that of TGF-β, resulting in aberrant protease activation that drives the acquisition of EMT, invasive, and metastatic phenotypes.

Integrins and Focal Adhesion Signaling During EMT

Integrins and EMT Stimulated by TGF-β

Communication within cell microenvironments is controlled in part by integrins, which govern cell adhesion, migration, and invasion, as well as cell proliferation and survival [172, 173]. Cells undergoing neoplastic transformation exhibit dramatically altered integrin expression profiles, as well as altered integrin affinities for ECM substrates, both of which enhance cancer cell invasion and metastasis [174]. As a receptor family, integrins are unique in their capacity to physically link the ECM to cytoskeletal system within cells, thus enabling the efficient propagation of mechanotransduction in a bidirectional manner [111, 175]. In addition, focal adhesion kinase (FAK) serves as a molecular bridge that links integrins to the receptors to EGF and PDGF, thereby conferring cell migration activities to these growth factors [176, 177]. Integrins also play an important role in eliciting EMT and cell migration stimulated by TGF-β. For instance, αvβ6 and αvβ8 integrins bind latent TGF-β complexes and elicit the presentation of TGF-β to its cell surface receptors [178], presumably by promoting matrix metalloproteinase (MMP)-14 activation [27, 179]. Along these lines, TGF-β induces the expression of α3β1 and αvβ3 integrins, which confer migration and invasion properties to MECs [45, 46, 56, 157]. Administering neutralizing β1 integrin antibodies to MECs uncoupled TGF-β from the activation of p38 MAPK and the induction of EMT [157]. In addition, genetic or pharmacologic inactivation of αvβ3 integrin in normal and malignant MECs prevented TGF-β from inducing EMT and pulmonary metastasis [45, 46, 56]. Mechanistically, upregulated β3 integrin expression stimulated by TGF-β results in the FAK-dependent formation of β3 integrin:TβR-II complexes that promote the activation of Src and its phosphorylation of TβR-II at Y284 [56, 180]. Upon its phosphorylation, Y284 coordinates the recruitment and binding of the SH2-domain proteins, Grb2 and ShcA, which promote p38 MAPK activation and the initiation of EMT [45, 46, 56]. Importantly, measures that disrupt this oncogenic TGF-β signaling axis completely abrogate the ability of TGF-β to induce EMT, and to promote the metastasis of breast cancer cells to the lung [46, 180, 181] and bone [182]. Recently, we observed an interesting interplay between β1 and β3 integrins in regulating MEC response to TGF-β, such that genetic inactivation of β1 integrin in MECs elicits a compensatory upregulation of β3 integrin expression that impacts the coupling of TGF-β to EMT and cell motility (J.G. Parvani and W.P. Schiemann, *unpublished observation*). These findings implicate "integrin switching" as a potentially dangerous mechanism that may enable metastatic breast cancers to escape integrin-based chemotherapies. Future studies clearly need to investigate the validity of this hypothesis, as well as to identify the repertoire of integrins capable of regulating the diverse pathophysiological activities of TGF-β in normal and malignant MECs.

Nonreceptor Protein Tyrosine Kinases and Adapter Molecules of Focal Adhesion Complexes

As mentioned above, EMT and oncogenic TGF-β signaling transpires through a β3 integrin:FAK:Src:phospho-Y284-TβR-II:Grb2:p38 MAPK signaling axis that forms constitutively in basal-like breast cancer cells [45, 46, 56, 180, 182]. The importance of this αvβ3 integrin-based signaling axis in promoting the oncogenic activities of TGF-β in other genetically distinct breast cancer subtypes remains unexplored; however, a number of recent studies have identified essential functions for a variety of focal adhesion complex proteins in mediating the coupling of TGF-β to EMT and metastasis. Indeed, in addition to its ability to coordinate the formation of β3 integrin:TβR-II complexes, FAK expression and activity are essential in coupling TGF-β to EMT and p38 MAPK activation, and to inducing pulmonary metastasis of breast cancer cells [180]. Adjuvant FAK chemotherapy also inhibited breast cancer growth by suppressing the ability of macrophages to infiltrate the

primary mammary tumor [180], suggesting that the tumor promoting activities of FAK manifest in carcinoma cells and their associated stromal compartment. Along these lines, FAK is essential in mediating TGF-β stimulation of E-cadherin redistribution and α-SMA expression during EMT [183, 184]. In addition, the phosphorylation and activation of p130Cas functions as a molecular rheostat that governs the balance between canonical and noncanonical TGF-β signaling inputs. Indeed, activated p130Cas forms a heteromeric complex with Smad3 and TβR-I, which diminishes the phosphorylation of Smad3 and uncouples TGF-β from regulation of cell cycle progression [185]. Interestingly, breast cancer patients whose tumors express abnormally high levels of p130Cas exhibit tamoxifen- and adriamycin-resistance, as well as diminished time to disease recurrence [186, 187]. Likewise, elevated p130Cas expression significantly reduced the latency of mammary tumor formation driven by transgenic expression of Her2/Neu [188]. We recently observed elevated p130Cas expression to mark the development of metastasis in breast cancers, and to skew the balance of TGF-β signaling from canonical to noncanonical effectors in metastatic MECs [189].

Similar to p130Cas, the adapter molecules Hic5 and Dab2 also promote the coupling of TGF-β to its noncanonical effectors during EMT. Indeed, Hic5 is a member of the paxillin superfamily that functions in the cytoplasm as a component of focal adhesion complexes [190], and in the nucleus as a transcriptional co-activator of the androgen receptor [191]. Polarized MECs express low levels of Hic5, which are increased rapidly during EMT via a RhoA/ROCK-dependent pathway [190, 192]. Interestingly, the LIM domain of Hic5 binds and inactivates Smad3 and Smad7 in prostate cancer cells [193, 194], which collectively diminishes Smad-dependent gene expression (i.e., targeting Smad3) in the context of enhanced TGF-β receptor signaling (i.e., targeting Smad7). Whether Hic-5 possesses similar anti-Smad activity in breast cancers remains unknown; however, it is tempting to speculate that Hic5 may cooperate with p130Cas in amplifying the coupling of TGF-β to its noncanonical effectors.

Finally, the adaptor molecule, Dab2 (Disabled-2) regulates the dynamics associated with the remodeling of the actin cytoskeleton during MEC adhesion and migration [195]. In contrast to p130Cas and Hic5, Dab2 associates with TGF-β receptors and facilitates their activation of Smad2/3 [26], as well as that of TAK1 and JNK, which stimulate fibronectin expression and cell migration during EMT [160, 196]. In addition, TGF-β stimulation of EMT assembles Dab2:β1 integrin complexes that induce FAK activation. Mechanistically, translation of Dab2 mRNA is strongly repressed in polarized MECs by the actions of hnRNP E1, which binds structural elements in the 3′UTR of Dab2 transcripts. When activated by TGF-β, Akt2 phosphorylates and releases hnRNP E1 from Dab2 mRNA, thereby enabling the production of Dab2 and its initiation of EMT in MECs [197]. Future studies need to identify other genes targeted by this novel post-transcriptional regulon, as well as to define their role in mediating the pathophysiological outcomes of EMT stimulated by TGF-β.

Gene Regulation-Coupled to EMT Induced by TGF-β

The ultimate phenotypic change associated with the activation of canonical and noncanonical TGF-β signaling inputs derives from altered patterns of gene expression and repression that transpire in a cell- and context-specific manner. In the succeeding sections, we highlight the important transcriptional mediators operant in driving and fine-tuning the EMT transcriptome targeted by TGF-β in normal and malignant MECs.

Nuclear Transcription Factors Targeted by TGF-β During EMT

Members of the Snail (SNAI1 and SNAI2/Slug), ZEB (ZEB1 and ZEB2/SIP1), basic helix-loop-helix (Twist), and Six family of homeobox (Six1) transcription factors are considered to be master regulators of EMT, including that stimulated by TGF-β [198, 199]. As a group, these transcription factors play essential roles in mediating type I EMT during embryogenesis and tissue morphogenesis; however, their inappropriate reactivation of developmental EMT programs during tumorigenesis is considered a hallmark of disease progression and metastasis initiation [61]. Indeed, activated Snail molecules readily form complexes with Smads 3 and 4 that collectively target conserved E-box sequences in the promoters for E-cadherin, occludin, and claudin, which strongly represses their expression and inactivates adherens (i.e., E-cadherin) and tight junction (i.e., occludins and claudin) complexes during EMT [200, 201]. Similar targeting and inactivation of E-cadherin is associated with all of the aforementioned transcription factors, whose underlying roles in mediating the pathophysiology of EMT has been the subject of several recent reviews (*see* [198, 199]). Interestingly, dysregulated Myc expression has been observed to function cooperatively with Smad4 to induce an EMT-related transcriptional profile in normal and malignant MECs [202]. Likewise, the reactivation of fibulin-5 expression in transitioning MECs initiated a positive-feedback loop that sensitized MECs to the EMT-promoting activities of TGF-β, an event dependent upon the synergistic induction of Twist expression by fibulin-5 and TGF-β [203]. In addition, TGF-β stimulation of Smad3 results in upregulated Mdm2 expression, which destabilizes p53 during the initiation of breast cancer EMT and metastasis [204]. Smad3/4 signaling

also promotes the expression of HMGA2, which stimulates the EMT transcriptional program by inducing the expression of Snail, Slug, and Twist, while simultaneously repressing that of Id2 [205]. Finally, members of the homeobox family of transcription factors have also been implicated in mediating EMT induced by TGF-β. For instance, inappropriate LBX1 (**l**ady**b**ird homeobo**x 1**) expression drives EMT and its expansion of breast cancer stem cells by stimulating the expression of TGF-β2, Snail, and ZEBs 1 and 2 [206]. Similarly, aberrant reactivation of the homeoprotein Six1 promotes the acquisition of EMT and metastatic phenotypes in breast cancer cells in part by upregulating the messages transduced through the TGF-β signaling system [85]. Collectively, these findings highlight the role of developmental EMT pathways that enhance the oncogenic activities of TGF-β during mammary tumorigenesis.

Estrogen Receptor-α

Estrogen receptor (ER) status has long been recognized as an important prognostic marker in developing breast cancers, particularly in terms of its diagnostic and therapeutic value. Indeed, the loss of ER-α expression during mammary tumorigenesis is associated with poor clinical outcomes, and with increased likelihood of breast cancer metastasis and disease recurrence [207]. More recently, ER-α has been linked to the initiation of EMT through its ability to activate metastasis associated protein 3 (MTA3) in MECs [208, 209]. Mechanistically, MTA3 serves as a subunit of the Mi-2/NuRD chromatin remodeling complex, which represses the expression of Snail [208]. Thus, mammary tumors that have lost expression of ER-α exhibit reduced MTA3 activity that results in the inappropriate expression of Snail and its subsequent stimulation of EMT [208, 209]. This process is further enhanced by the ability of Snail to repress ER-α expression, and to induce the expression of components of the TGF-β signaling system (e.g., TGF-β2 and TβR-II; [208]), thereby creating a powerful positive feedback loop to further potentiate the acquisition and stabilization of EMT phenotypes. Moreover, ER-α interacts physically with Smad3 and inhibits its ability to regulate gene expression [210]. Thus, the loss of ER-α expression and activity may play a prominent role in engendering the initiation of oncogenic TGF-β signaling in normal and malignant MECs [8]. In fact, aberrant cytoplasmic localization of ER-α has recently been proposed as a novel histopathological marker to identify sarcomatoid breast cancers in vivo [211]. Future studies clearly need to elucidate molecular mechanisms that underlie the dynamic relationship between ER-α and TGF-β in regulating EMT, and to identify novel biomarkers capable of staging and stratifying breast cancer patients on the basis of their EMT, ER-α, and TGF-β status.

TGF-β and microRNAs

Recent studies have implicated the aberrant expression of numerous microRNAs (miRs) in the initiation of EMT, and in the development and progression of mammary tumors [212–214]. Indeed, members of the miR-200 family of microRNAs maintain polarized epithelial phenotypes by repressing the expression of the EMT-inducing transcription factors ZEB1 and ZEB2/SIP-1. Accordingly, monitoring the expression of miR-200 family members can be used to distinguish well-differentiated and immotile tumors that express E-cadherin from their poorly-differentiated and highly motile counterparts that express vimentin [215]. In addition, TGF-β abrogates the expression of miR-200 family members, leading to the expression of ZEB1 and ZEB2 and their consequential downregulation of E-cadherin expression to initiate EMT [216, 217]. Not surprisingly, miR-200 family member expression is frequently downregulated in invasive and metastatic mammary tumors, particularly those possessing mesenchymal-type breast cancer cells [216]. Once expressed, ZEB1 can further repress the expression of miR-200 family members, thereby stabilizing the EMT phenotype in transitioning MECs [218].

The stimulation of EMT by TGF-β also transpires through its regulation of additional miRs. Indeed, TGF-β stimulation of normal MECs elicits their upregulated expression of miR-155 via a Smad4-dependent pathway. Once expressed, miR-155 participates in EMT by downregulating RhoA expression, leading to the dissolution of tight junctions [142]. Along these lines, upregulated expression of miR-21 induced by TGF-β abrogates the expression of several tumor suppressors, including *a*) peroxisome proliferator-activated receptor; *b*) tissue inhibitor of metalloproteinase-3; *c*) programmed cell death 4; and *d*) AT-rich interactive domain 1A [219]. Additionally, miR-21 expression also participates in the initiation of EMT by downregulating the expression of tropomyosin, leading to enhanced breast cancer motility, invasion, and anchorage-independent growth [220, 221]. Thus, the coupling of TGF-β to the regulation of miR expression and activity affords new avenues to potentially manipulate the pathophysiology associated with type 3 EMT in mammary tumors. As such, future studies need to rapidly and accurately define the cellular targets of EMT-associated miRs, and to determine the molecular mechanisms operant in regulating their expression in transitioning MECs.

DNA Hypermethylation

Hypermethylation and silencing of the E-cadherin promoter has been linked to the initiation of EMT, migration, and invasion in breast cancer cells [222–224]. More recently, TGF-β signaling was observed to maintain DNA methylation patterns during EMT, resulting in the silencing of E-

cadherin (*CDH1*), the tight junction genes *CGN* and *CLDN4*, and the protease *KLK10/NES1*. Mechanistically, overexpressing Smad7 in MECs or rendering them deficient in Smad2 inhibited the activity of the DNA methyltransferase, DNMT1, which suppressed EMT and cell motility by restoring E-cadherin expression [129]. Along these lines, miR-200c and its relative, miR-141, normally inhibit the initiation of EMT and metastasis in MECs by suppressing the expression of ZEB1/2 [216, 217]. However, during mammary tumorigenesis, aberrant DNA methylation inactivates expression of these miR-200 family members, leading to the acquisition of EMT and metastatic phenotypes in developing and progressing mammary tumors [225]. Moreover, E-cadherin-deficiency that arises during EMT may in fact function as an initiating signal coupled to the expanded and directed hypermethylation of genes normally operant in suppressing mammary tumorigenesis [226]. For instance, hypermethylation of the E-cadherin promoter marks Ras-transformed MECs that have undergone a stable EMT induced by serum versus a transient EMT induced by TGF-β [226]. Collectively, these studies establish an essential role for DNA methylation in facilitating type 3 EMT stimulated by TGF-β, and in differentially stabilizing the EMT phenotype in response to varying stimuli.

Microenvironmental Inputs During EMT Stimulated by TGF-β

Tissue homeostasis in the breast is maintained by the balanced integration of signaling inputs derived from various tissue and cell architectures, and from their supporting microenvironment and ECM. Indeed, whereas normal mammary tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing mammary carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts, endothelial cells, and a variety of infiltrating immune and progenitor cells [227–229]. Along these lines, desmoplasia and fibrosis during mammary tumorigenesis can drive disease progression in a manner that mimics the oncogenic activities of TGF-β [110, 230], suggesting that the interactions between MECs and their supporting stromal constituents play pivotal roles in regulating EMT and metastasis stimulated by TGF-β. Recent findings pertaining to the connections between the microenvironment and EMT are summarized in the following sections.

Adherens Junctions and EMT

A hallmark of EMT is the dissolution of cell–cell junctions, particularly adherens junctions which derive from the homotypic interactions between adjacent E-cadherin molecules housed on neighboring MECs. Similar to integrins, the cytoplasmic domain of E-cadherin is tethered to the cytoskeleton through a heteromeric complex consisting of the α-, β-, and γ-catenins, which collectively serve in marking differentiated MECs and suppressing their tumorigenesis [231, 232]. Interestingly, the process of EMT is often characterized by "cadherin switching," a term referring to the ability of E-cadherin expression and activity to give way to that of N-cadherin as MECs acquire mesenchymal phenotypes [233]. Functional inactivation of E-cadherin transpires through a variety of mechanisms, including hypermethylation and epigenetic silencing of its promoter [234], as well as protease-mediated cleavage and shedding of its ectodomain [231]. In rare cases, mutational inactivation of the E-cadherin gene, *CDH1*, has been observed and linked to increased risk for cancer development in affected individuals [235]. However, transcriptional repression is by far the most common mechanism employed by transitioning MECs to downregulate their expression of E-cadherin. Indeed, TGF-β stimulation of EMT represses E-cadherin expression primarily by targeting the expression of Snail, ZEB, and bHLH family members (*see above*). Moreover, upregulated expression of the mesenchymal cadherins, N-cadherin and cadherin-11, occur concomitantly with the loss of E-cadherin expression and correlate with increased tumor invasiveness and poor clinical outcomes [236–239]. At present, the dynamic relationship between E- and N-cadherin in mediating TGF-β stimulation of EMT and metastasis remains unresolved, as does the manner through which bifurcated TGF-β signals coupled selectively to epithelial versus mesenchymal transcriptional programs influence the pathophysiological outcomes of EMT induced by TGF-β [50, 180].

Neuronal Cell Adhesion Molecule and EMT

Neuronal cell adhesion molecule (NCAM) belongs to the immunoglobulin superfamily and mediates calcium-independent cell–cell adhesion [240]. Inappropriate NCAM expression has been associated with cancer progression and poor prognosis in cancers of the pancreas and colorectal system [233, 240]. In addition, TGF-β stimulation of EMT in MECs significantly upregulates their expression of NCAM, a reaction that requires the activation of Smad4 and inactivation of E-cadherin. Mechanistically, NCAM translocates to lipid rafts and activates a $p59^{fyn}$:FAK:β1 integrin signaling axis that promotes EMT and cell invasion [241]. Interestingly, EMT induced by TGF-β also activates matrix metalloproteinase (MMP)-28, which cleaves NCAM and latent TGF-β complexes [242]. Thus, it remains unclear how upregulated NCAM expression and cleavage ultimately impact the ability of TGF-β to stimulate EMT in normal and malignant cells.

Protease Activation During EMT Induced by TGF-β

Matrix Metalloproteinases

One of the hallmarks of EMT is its propensity to bestow motile phenotypes in previously immotile cells [1–3]. Matrix metalloproteinases (MMPs) are a superfamily of transmembrane and secreted endopeptidases that function in degrading a variety of ECM components, cytokines, and cell surface proteins and receptors. The net effect of these various MMP activities results in dramatic effects on cell differentiation, invasion, and EMT [243]. Members of the MMP superfamily (e.g., MMPs 2, 9, 13, and 14; [179, 244–246]) also function in mediating the cleavage of latent TGF-β complexes, which releases mature TGF-β and initiates transmembrane signaling in neighboring MECs, as well as mediates E-cadherin cleavage and breast cancer progression [243, 247, 248]. Along these lines, TGF-β is a potent regulator of the expression of MMPs 2, 9, and 13 [60, 203, 249–252], thereby establishing a positive autocrine TGF-β signaling loop that (*a*) drives breast cancer EMT, invasion, and metastasis, and (*b*) is readily suppressed by constitutive c-Abl activation in normal and malignant MECs [60]. Future studies need to address how aberrant MMP activation contributes to the initiation of type 2 EMT (*see above*), and how these events ultimately impact the initiation of type 3 EMT and metastasis by TGF-β in mammary tumors.

Urokinase Plasminogen Activator and Plasminogen Activator Inhibitor-1

Urokinase plasminogen activator (uPA) is serine protease that plays important roles in regulating the migration and invasion of breast cancer cells in part via its conversion of inactive plasminogen into active plasmin. Elevated expression of uPA correlates with increased tumor aggressiveness and poor clinical outcomes for a variety of cancers, including those of the breast [253–255]. The role of uPA in promoting breast cancer progression and metastasis has been recapitulated in a mouse model of mammary tumorigenesis [255], and in a chick chorioallantoic membrane model of breast cancer metastasis, which also associated upregulated uPA expression with hypoxia-induced EMT [256]. TGF-β induces uPA expression by activating JNK- and ILK-dependent signaling systems that functionally converge to induce EMT and increased cell motility [161, 170]. As noted previously, these findings point to a prominent role of noncanonical TGF-β effectors in mediating the stimulation of EMT by TGF-β. Accordingly, the expression and activity of FAK is essential in stimulating the production of uPA and its initiation of metastasis in 4T1 cells [255], which we [46, 49, 51, 60, 180, 189, 203] and others [257–259] established as a late-stage model of TGF-β-responsive breast cancer. In addition, hypoxia-induced EMT stimulates the expression of the uPA receptor, uPAR, which interacts physically with α3β1 integrin and promotes the activation of Src, Akt, Rac1, and GSK-3β. The end product of these signaling inputs elicits Snail and Slug expression, which drive the acquisition of EMT phenotypes and loss of E-cadherin in transitioning cells [256, 260]. Thus, future studies clearly need to define the connections linking the activation of these noncanonical TGF-β effectors to the formation of uPAR:integrin complexes.

Inappropriate activation of uPA is held in check by the expression of plasminogen activator inhibitor (PAI)-1 and PAI-2, which bind uPA:uPAR complexes and induce their internalization and degradation [253]. Thus, elevated PAI-1/2 expression would be predicted to alleviate the EMT and metastasis promoting properties of uPA, an assumption that has been validated in a panel of breast, ovarian, endometrial, cervical, and osteosarcoma cell lines [261]. Quite surprisingly, PAI-1 polymorphisms or its elevated expression has also been linked to enhanced disease progression and metastasis development, and to decreased survival in breast cancer patients [253, 261, 262]. TGF-β is a master regulator of PAI-1 expression, doing so through its stimulation of canonical and noncanonical effector systems (*see* [263]). In addition to binding and inactivating uPA:uPAR complexes, PAI-1 also interacts with vitronectin and prevents its activation of integrins [261], an event that may influence the coupling of integrin signaling to MEC migration. Thus, future studies need to clarify the tumor suppressing and promoting activities of PAI-1, particularly with respect to its role in mediating EMT and oncogenic TGF-β signaling in normal and malignant MECs.

Collagen

TGF-β has long be recognized for its ability to induce the expression of collagens [264], which function as important structural components of the ECM and serve as prominent ligands for integrins [243]. In addition, activation of p38 MAPK by TGF-β upregulates the expression of MMPs 2 and 9, which cleave collagen to produce biologically active fragments that readily promote MEC migration and invasion [252]. Along these lines, TGF-β stimulates breast cancer cells to upregulate their expression of the collagen receptor Endo180, which internalizes collagen and induces the growth of mammary tumors in mice [265]. More recently, collagen binding to β1 integrins has been shown to activate TGF-β receptor signaling independent of TGF-β ligands, leading to the activation of FAK and Src that culminate in the stimulation of Smad2/3 activity in MECs [266]. Collectively, these findings establish that TGF-β and collagen engage one another in a reciprocal relationship, yet how these events impact the ability of TGF-β to promote the

acquisition of EMT and metastatic phenotypes mammary tumors remains an unresolved and interesting question.

Fibronectin

Fibronectin is an important component of the ECM and its expression is upregulated dramatically by TGF-β during EMT [264, 267]. Functionally, fibronectin acts as a ligand for integrins during cell adhesion and migration, particularly in Ras-transformed MECs which concomitantly upregulate α5β1 integrin [268]. Importantly, administration of neutralizing antibodies against α5 integrin blocked the ability of fibronectin and TGF-β to stimulate EMT and cell motility in MECs [268]. In addition, fibronectin expression has been shown to modulate the response of cells to TGF-β. For instance, the ability of TGF-β to induce anchorage-independent growth in fibroblasts could be recapitulated by administration of fibronectin, whose activation of cell signaling was dependent upon integrin ligation [264]. Besides its ability to enhance TGF-β stimulation of EMT in bronchial epithelial cells [269], fibronectin expression has also been linked to the development of the "premetastatic niche," which serves as a depot to recruit circulating progenitor cells and metastatic carcinoma cells to sites of secondary tumor growth [270, 271]. Future studies need to assess the relative contributions of TGF-β and fibronectin in mediating EMT and its potential involvement regulating the formation of metastatic niches during breast cancer progression.

Conclusions and Future Directions

TGF-β is universally recognized as a master regulator of EMT, including that occurring during embryonic development and tissue morphogenesis (i.e., type 1 EMT), during wound healing and tissue fibrosis (i.e., type 2 EM), and during invasion and metastasis (i.e., type 3 EMT). Equally exciting are recent findings linking EMT stimulated by TGF-β to the acquisition of "*stem-like*" phenotypes in developing and progressing mammary tumors [16–19]. Thus, pharmacological targeting of the TGF-β signaling system to alleviate EMT may elicit chemosensitivity in cancer stem cells previously resistant to standard treatment regimens, a supposition supported by recent findings obtained in a preclinical model of breast cancer progression [19]. A corollary states that the phenomenon underlying selection and expansion of cancer stem cells via EMT may be "*druggable*" in clinical settings. Accordingly, high-throughput chemical screening technologies identified salinomycin as a novel agent capable of targeting breast cancer stem cells, thereby inhibiting mammary tumor growth in part by promoting epithelial differentiation [272]. Future studies need to determine the efficacy of salinomycin and related compounds in antagonizing EMT stimulated by TGF-β in normal and malignant MECs, as well as investigate the relative contribution of cell microenvironments in mediating the various pathophysiological outcomes of EMT induced by TGF-β. Ultimately, these findings will form the foundation necessary to manipulate EMT and its initiation of the "TGF-β Paradox" in mammary tumors, and as such, to one day improve the clinical course of patients with metastatic breast cancer.

Acknowledgements We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA114039 and CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651), while M.A.T. was supported by the Department of Defense (BC093128).

References

1. Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol. 2009;5(8):1145–68.
2. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420–8.
3. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.
4. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell. 2008;14(6):818–29.
5. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, et al. Epithelial-mesenchymal and mesenchymal-epithelial transitions in carcinoma progression. J Cell Physiol. 2007;213(2):374–83.
6. Wells A, Yates C, Shepard CR. E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis. 2008;25(6):621–8.
7. Barcellos-Hoff MH, Akhurst RJ. TGF-β in breast cancer: too much, too late. Breast Cancer Res. 2009;11(1):202.
8. Buck MB, Knabbe C. TGF-β signaling in breast cancer. Ann NY Acad Sci. 2006;1089:119–26.
9. Serra R, Crowley MR. Mouse models of TGF-β impact in breast development and cancer. Endocr Relat Cancer. 2005;12(4):749–60.
10. Schiemann WP. Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? Expert Rev Anticancer Ther. 2007;7(5):609–11.
11. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006;439(7072):84–8.
12. Stingl J, Raouf A, Eirew P, Eaves CJ. Deciphering the mammary epithelial cell hierarchy. Cell Cycle. 2006;5(14):1519–22.
13. Villadsen R, Fridriksdottir AJ, Ronnov-Jessen L, Gudjonsson T, Rank F, LaBarge MA, et al. Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol. 2007;177(1):87–101.
14. Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol. 2009;21(2):166–76.
15. Zavadil J, Bottinger EP. TGF-β and epithelial-to-mesenchymal transitions. Oncogene. 2005;24(37):5764–74.

16. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704–15.
17. Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS ONE. 2008;3(8):e2888.
18. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet. 2008;40(5):499–507.
19. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell. 2007;11(3):259–73.
20. Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res. 2009;19(2):156–72.
21. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian TGF-β superfamily. Endocr Rev. 2002;23(6):787–823.
22. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev. 2005;19(23):2783–810.
23. Galliher AJ, Neil JR, Schiemann WP. Role of TGF-β in cancer progression. Future Oncol. 2006;2(6):743–63.
24. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGF-β receptor. Cell. 1998;95(6):779–91.
25. Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol. 2000;20(24):9346–55.
26. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the TGF-β receptors to the Smad pathway. EMBO J. 2001;20(11):2789–801.
27. Mok SC, Wong KK, Chan RK, Lau CC, Tsao SW, Knapp RC, et al. Molecular cloning of differentially expressed genes in human epithelial ovarian cancer. Gynecol Oncol. 1994;52(2):247–52.
28. Xu XX, Yang W, Jackowski S, Rock CO. Cloning of a novel phosphoprotein regulated by colony-stimulating factor 1 shares a domain with the Drosophila disabled gene product. J Biol Chem. 1995;270(23):14184–91.
29. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, et al. The MAD-related protein Smad7 associates with the TGF-β receptor and functions as an antagonist of TGF-β signaling. Cell. 1997;89:1165–73.
30. Nakao A, Afrakht M, Moren A, Nakayama T, Christian JL, Heuchel R, et al. Identification of Smad7, a TGF-β-inducible antagonist of TGF-β signalling. Nature. 1997;389:631–5.
31. Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL, et al. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and TGF-β receptors. J Biol Chem. 1998;273:25364–70.
32. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurf1 interacts with TGF-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem. 2001;276(16):12477–80.
33. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol Cell. 2000;6(6):1365–75.
34. Datta PK, Moses HL. STRAP and Smad7 synergize in the inhibition of TGF-β signaling. Mol Cell Biol. 2000;20(9):3157–67.
35. Ibarrola N, Kratchmarova I, Nakajima D, Schiemann WP, Moustakas A, Pandey A, et al. Cloning of a novel signaling molecule, AMSH-2, that potentiates TGF-β signaling. BMC Cell Biol. 2004;5:2.
36. Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, et al. Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7. EMBO J. 2003;22(24):6458–70.
37. Liu FY, Li XZ, Peng YM, Liu H, Liu YH. Arkadia-Smad7-mediated positive regulation of TGF-β signaling in a rat model of tubulointerstitial fibrosis. Am J Nephrol. 2007;27(2):176–83.
38. Liu W, Rui H, Wang J, Lin S, He Y, Chen M, et al. Axin is a scaffold protein in TGF-β signaling that promotes degradation of Smad7 by Arkadia. EMBO J. 2006;25(8):1646–58.
39. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGF-β-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci. 2002;115((Pt) 15):3193–206.
40. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for TGF-β-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000;275(47):36803–10.
41. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. TGF-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell. 2001;12(1):27–36.
42. Lamouille S, Derynck R. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J Cell Biol. 2007;178(3):437–51.
43. Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. TGF-β-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat Cell Biol. 2001;3(8):708–14.
44. Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, et al. Genetic programs of epithelial cell plasticity directed by TGF-β. Proc Natl Acad Sci USA. 2001;98(12):6686–91.
45. Galliher AJ, Schiemann WP. β3 integrin and Src facilitate TGF-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res. 2006;8(4):R42.
46. Galliher-Beckley AJ, Schiemann WP. Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis. 2008;29(2):244–51.
47. Arsura M, Panta GR, Bilyeu JD, Cavin LG, Sovak MA, Oliver AA, et al. Transient activation of NF-κB through a TAK1/IKK kinase pathway by TGF-β1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene. 2003;22(3):412–25.
48. Park J-I, Lee M-G, Cho K, Park B-J, Chae K-S, Byun D-S, et al. TGF-β1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF-κB, JNK, and Ras signaling pathways. Oncogene. 2003;22:4314–32.
49. Neil JR, Schiemann WP. Altered TAB1:IκB kinase interaction promotes TGF-β-mediated NF-κB activation during breast cancer progression. Cancer Res. 2008;68(5):1462–70.
50. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis. 2008;29(11):2227–35.
51. Tian M, Schiemann WP. PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J. 2010;24(4):1105–16.
52. Dong M, How T, Kirkbride KC, Gordon KJ, Lee JD, Hempel N, et al. The type III TGF-β receptor suppresses breast cancer progression. J Clin Invest. 2007;117(1):206–17.
53. Sun L, Chen C. Expression of TGF-β type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells. J Biol Chem. 1997;272(40):25367–72.
54. Gordon KJ, Blobe GC. Role of TGF-β superfamily signaling pathways in human disease. Biochim Biophys Acta. 2008;1782(4):197–228.
55. Gordon KJ, Dong M, Chislock EM, Fields TA, Blobe GC. Loss of type III TGF-β receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition

during pancreatic cancer progression. Carcinogenesis. 2008;29 (2):252–62.
56. Galliher AJ, Schiemann WP. Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res. 2007;67(8):3752–8.
57. Park SS, Eom YW, Kim EH, Lee JH, Min DS, Kim S, et al. Involvement of c-Src kinase in the regulation of TGF-β1-induced apoptosis. Oncogene. 2004;23(37):6272–81.
58. Horowitz JC, Rogers DS, Sharma V, Vittal R, White ES, Cui Z, et al. Combinatorial activation of FAK and AKT by TGF-β1 confers an anoikis-resistant phenotype to myofibroblasts. Cell Signal. 2007;19(4):761–71.
59. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, et al. Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J Biol Chem. 2003;278(14):12384–9.
60. Allington TM, Galliher-Beckley AJ, Schiemann WP. Activated Abl kinase inhibits oncogenic TGF-β signaling and tumorigenesis in mammary tumors. FASEB J. 2009;23(12):4231–43.
61. Micalizzi DS, Ford HL. Epithelial-mesenchymal transition in development and cancer. Future Oncol. 2009;5(8):1129–43.
62. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest. 2009;119(6):1438–49.
63. Skromne I, Stern CD. Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. Development. 2001;128(15):2915–27.
64. Popperl H, Schmidt C, Wilson V, Hume CR, Dodd J, Krumlauf R, et al. Misexpression of Cwnt8C in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. Development. 1997;124(15):2997–3005.
65. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. Nat Genet. 1999;22(4):361–5.
66. Chea HK, Wright CV, Swalla BJ. Nodal signaling and the evolution of deuterostome gastrulation. Dev Dyn. 2005;234 (2):269–78.
67. Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR. Nodal is a novel TGF-β-like gene expressed in the mouse node during gastrulation. Nature. 1993;361(6412):543–7.
68. Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, et al. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development. 1994;120(7):1919–28.
69. Birsoy B, Kofron M, Schaible K, Wylie C, Heasman J. Vg1 is an essential signaling molecule in Xenopus development. Development. 2006;133(1):15–20.
70. Shah SB SI, Hume CR, Kessler DS, Lee KJ, Stern CD, Dodd J. Misexpression of chick Vg1 in the marginal zone induces primitive streak formation. Development. 1997;125(24):5127–38.
71. Sauka-Spengler T, Bronner-Fraser M. A gene regulatory network orchestrates neural crest formation. Nat Rev Mol Cell Biol. 2008;9(7):557–68.
72. Raible DW. Development of the neural crest: achieving specificity in regulatory pathways. Curr Opin Cell Biol. 2006;18(6):698–703.
73. Correia AC, Costa M, Moraes F, Bom J, Novoa A, Mallo M. BMP2 is required for migration but not for induction of neural crest cells in the mouse. Dev Dyn. 2007;236(9):2493–501.
74. Wu MY, Hill CS. TGF-β superfamily signaling in embryonic development and homeostasis. Dev Cell. 2009;16(3):329–43.
75. Boyer AS, Ayerinskas II, Vincent EB, McKinney LA, Weeks DL, Runyan RB. TGF-β2 and TGF-β3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. Dev Biol. 1999;208(2):530–45.
76. Mercado-Pimentel ME, Hubbard AD, Runyan RB. Endoglin and Alk5 regulate epithelial-mesenchymal transformation during cardiac valve formation. Dev Biol. 2007;304(1):420–32.
77. Mercado-Pimentel ME, Runyan RB. Multiple TGF-β isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. Cells Tissues Organs. 2007;185(1–3):146–56.
78. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, et al. Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nat Genet. 1995;11(4):415–21.
79. Ahmed S, Liu CC, Nawshad A. Mechanisms of palatal epithelial seam disintegration by TGF-β3. Dev Biol. 2007;309(2):193–207.
80. Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-β receptor for endocardial cell transformation in the heart. Science. 1999;283(5410):2080–2.
81. Nakajima A, Ito Y, Asano M, Maeno M, Iwata K, Mitsui N, et al. Functional role of TGF-β type III receptor during palatal fusion. Dev Dyn. 2007;236(3):791–801.
82. Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. Science. 2006;314 (5797):298–300.
83. Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. Dev Cell. 2008;14(4):570–81.
84. McCoy EL, Iwanaga R, Jedlicka P, Abbey NS, Chodosh LA, Heichman KA, et al. Six1 expands the mouse mammary epithelial stem/progenitor cell pool and induces mammary tumors that undergo epithelial-mesenchymal transition. J Clin Invest. 2009;119(9):2663–77.
85. Micalizzi DS, Christensen KL, Jedlicka P, Coletta RD, Baron AE, Harrell JC, et al. The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-β signaling. J Clin Invest. 2009;119(9):2678–90.
86. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214(2):199–210.
87. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest. 2007;117 (3):524–9.
88. Radisky DC, Przybylo JA. Matrix metalloproteinase-induced fibrosis and malignancy in breast and lung. Proc Am Thorac Soc. 2008;5(3):316–22.
89. Pohlers D, Brenmoehl J, Loffler I, Muller CK, Leipner C, Schultze-Mosgau S, et al. TGF-β and fibrosis in different organs—molecular pathway imprints. Biochim Biophys Acta. 2009;1792(8):746–56.
90. Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn. 2005;233(3):706–20.
91. Vaughan MB, Howard EW, Tomasek JJ. TGF-β1 promotes the morphological and functional differentiation of the myofibroblast. Exp Cell Res. 2000;257(1):180–9.
92. Wallace K, Burt AD, Wright MC. Liver fibrosis. Biochem J. 2008;411(1):1–18.
93. Grande MT, Lopez-Novoa JM. Fibroblast activation and myofibroblast generation in obstructive nephropathy. Nat Rev Nephrol. 2009;5(6):319–28.
94. Guarino M, Tosoni A, Nebuloni M. Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. Hum Pathol. 2009;40(10):1365–76.
95. Yazhou C, Wenlv S, Weidong Z, Licun W. Clinicopathological significance of stromal myofibroblasts in invasive ductal carcinoma of the breast. Tumour Biol. 2004;25(5–6):290–5.
96. Masszi A, Di Ciano C, Sirokmany G, Arthur WT, Rotstein OD, Wang J, et al. Central role for Rho in TGF-β1-induced α-smooth

muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol. 2003;284(5):F911–24.
97. Akhmetshina A, Dees C, Pileckyte M, Szucs G, Spriewald BM, Zwerina J, et al. Rho-associated kinases are crucial for myofibroblast differentiation and production of extracellular matrix in scleroderma fibroblasts. Arthritis Rheum. 2008;58 (8):2553–64.
98. Vardouli L, Vasilaki E, Papadimitriou E, Kardassis D, Stournaras C. A novel mechanism of TGF-β-induced actin reorganization mediated by Smad proteins and Rho GTPases. FEBS J. 2008;275(16):4074–87.
99. Vasilaki E, Papadimitriou E, Tajadura V, Ridley AJ, Stournaras C, Kardassis D. Transcriptional regulation of the small GTPase RhoB gene by TGF-β-induced signaling pathways. FASEN J. 2010;24(3):891–905.
100. Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, et al. Epithelial cell α3β1 integrin links β-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. J Clin Invest. 2009;119(1):213–24.
101. Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, et al. Integrin α3β1-dependent β-catenin phosphorylation links epithelial Smad signaling to cell contacts. J Cell Biol. 2009;184 (2):309–22.
102. White LR, Blanchette JB, Ren L, Awn A, Trpkov K, Muruve DA. The characterization of α5-integrin expression on tubular epithelium during renal injury. Am J Physiol Renal Physiol. 2007;292(2):F567–76.
103. Boyd NF, Dite GS, Stone J, Gunasekara A, English DR, McCredie MR, et al. Heritability of mammographic density, a risk factor for breast cancer. N Engl J Med. 2002;347(12):886–94.
104. Boyd NF, Rommens JM, Vogt K, Lee V, Hopper JL, Yaffe MJ, et al. Mammographic breast density as an intermediate phenotype for breast cancer. Lancet Oncol. 2005;6(10):798–808.
105. Choi YW, Munden RF, Erasmus JJ, Park KJ, Chung WK, Jeon SC, et al. Effects of radiation therapy on the lung: radiologic appearances and differential diagnosis. Radiographics. 2004;24 (4):985–97.
106. Andarawewa KL, Erickson AC, Chou WS, Costes SV, Gascard P, Mott JD, et al. Ionizing radiation predisposes nonmalignant human mammary epithelial cells to undergo TGF-β induced epithelial to mesenchymal transition. Cancer Res. 2007;67(18):8662–70.
107. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. Nat Rev Cancer. 2009;9(2):108–22.
108. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. Cancer Cell. 2005;8(3):241–54.
109. Turley EA, Veiseh M, Radisky DC, Bissell MJ. Mechanisms of disease: epithelial-mesenchymal transition—does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol. 2008;5 (5):280–90.
110. Tian M, Schiemann WP. The TGF-β paradox in human cancer: an update. Future Oncol. 2009;5(2):259–71.
111. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891–906.
112. Frank NY, Schatton T, Frank MH. The therapeutic promise of the cancer stem cell concept. J Clin Invest. 2010;120(1):41–50.
113. Brown KA, Pietenpol JA, Moses HL. A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-β signaling. J Cell Biochem. 2007;101(1):9–33.
114. Hoot KE, Lighthall J, Han G, Lu SL, Li A, Ju W, et al. Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. J Clin Invest. 2008;118(8):2722–32.
115. Ju W, Ogawa A, Heyer J, Nierhof D, Yu L, Kucherlapati R, et al. Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol Cell Biol. 2006;26 (2):654–67.
116. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-Ras and Smad2 levels. Nat Cell Biol. 2002;4(7):487–94.
117. Runyan CE, Hayashida T, Hubchak S, Curley JF, Schnaper HW. Role of SARA (SMAD anchor for receptor activation) in maintenance of epithelial cell phenotype. J Biol Chem. 2009;284 (37):25181–9.
118. Saika S, Kono-Saika S, Ohnishi Y, Sato M, Muragaki Y, Ooshima A, et al. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am J Pathol. 2004;164(2):651–63.
119. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF-β1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest. 2003;112(10):1486–94.
120. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol. 1999;1(5):260–6.
121. Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-β1 involves MAPK, Smad and AP-1 signalling pathways. J Cell Biochem. 2005;95(5):918–31.
122. Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev. 2006;20(22):3130–46.
123. Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell. 2005;16(4):1987–2002.
124. Dooley S, Hamzavi J, Ciuclan L, Godoy P, Ilkavets I, Ehnert S, et al. Hepatocyte-specific Smad7 expression attenuates TGF-β-mediated fibrogenesis and protects against liver damage. Gastroenterology. 2008;135(2):642–59.
125. Saika S, Ikeda K, Yamanaka O, Sato M, Muragaki Y, Ohnishi Y, et al. Transient adenoviral gene transfer of Smad7 prevents injury-induced epithelial-mesenchymal transition of lens epithelium in mice. Lab Invest. 2004;84(10):1259–70.
126. Gal A, Sjoblom T, Fedorova L, Imreh S, Beug H, Moustakas A. Sustained TGF-β exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. Oncogene. 2008;27 (9):1218–30.
127. Deckers M, van Dinther M, Buijs J, Que I, Lowik C, van der Pluijm G, et al. The tumor suppressor Smad4 is required for TGF-β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res. 2006;66(4):2202–9.
128. Zhao BM, Hoffmann FM. Inhibition of TGF-β1-induced signaling and epithelial-to-mesenchymal transition by the Smad-binding peptide aptamer Trx-SARA. Mol Biol Cell. 2006;17(9):3819–31.
129. Papageorgis P, Lambert AW, Ozturk S, Gao F, Pan H, Manne U, et al. Smad signaling is required to maintain epigenetic silencing during breast cancer progression. Cancer Res. 2010;70(3):968–78.
130. Liu IM, Schilling SH, Knouse KA, Choy L, Derynck R, Wang XF. TGF-β-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGF-β switch. EMBO J. 2009;28(2):88–98.
131. Hall A. Rho GTPases and the control of cell behaviour. Biochem Soc Trans. 2005;33(Pt 5):891–5.
132. Hall A, Nobes CD. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci. 2000;355(1399):965–70.

133. Takaishi K, Sasaki T, Kotani H, Nishioka H, Takai Y. Regulation of cell–cell adhesion by Rac and Rho small G proteins in MDCK cells. J Cell Biol. 1997;139(4):1047–59.
134. Sander EE, ten Klooster JP, van Delft S, van der Kammen RA, Collard JG. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. J Cell Biol. 1999;147(5):1009–22.
135. Mythreye K, Blobe GC. The type III TGF-β receptor regulates epithelial and cancer cell migration through β-arrestin2-mediated activation of Cdc42. Proc Natl Acad Sci USA. 2009;106(20):8221–6.
136. Simpson KJ, Dugan AS, Mercurio AM. Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. Cancer Res. 2004;64(23):8694–701.
137. Sequeira L, Dubyk CW, Riesenberger TA, Cooper CR, van Golen KL. Rho GTPases in PC-3 prostate cancer cell morphology, invasion and tumor cell diapedesis. Clin Exp Metastasis. 2008;25(5):569–79.
138. Bellovin DI, Simpson KJ, Danilov T, Maynard E, Rimm DL, Oettgen P, et al. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. Oncogene. 2006;25(52):6959–67.
139. Hutchison N, Hendry BM, Sharpe CC. Rho isoforms have distinct and specific functions in the process of epithelial to mesenchymal transition in renal proximal tubular cells. Cell Signal. 2009;21(10):1522–31.
140. Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, et al. A role for the TGF-β-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci USA. 2009;106(33):14028–33.
141. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGF-β receptors controls epithelial cell plasticity. Science. 2005;307(5715):1603–9.
142. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, et al. MicroRNA-155 is regulated by the TGF-β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol. 2008;28(22):6773–84.
143. Valastyan S, Benaich N, Chang A, Reinhardt F, Weinberg RA. Concomitant suppression of three target genes can explain the impact of a microRNA on metastasis. Genes Dev. 2009;23(22):2592–7.
144. Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, Wang ZC, et al. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell. 2009;137(6):1032–46.
145. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009;9(8):550–62.
146. Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. Oncogene. 2005;24(28):4580–7.
147. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest. 2006;116(6):1561–70.
148. Uttamsingh S, Bao X, Nguyen KT, Bhanot M, Gong J, Chan JL, et al. Synergistic effect between EGF and TGF-β1 in inducing oncogenic properties of intestinal epithelial cells. Oncogene. 2008;27(18):2626–34.
149. Karin M. NF-κB in cancer development and progression. Nature. 2006;441(7092):431–6.
150. Sovak MA, Arsura M, Zanieski G, Kavanagh KT, Sonenshein GE. The inhibitory effects of TGF-β1 on breast cancer cell proliferation are mediated through regulation of aberrant NF-κB/Rel expression. Cell Growth Differ. 1999;10(8):537–44.
151. Arsura M, Wu M, Sonenshein GE. TGF-β1 inhibits NF-κB/Rel activity inducing apoptosis of B cells: transcriptional activation of IκBα. Immunity. 1996;5(1):31–40.
152. You HJ, How T, Blobe GC. The type III TGF-β receptor negatively regulates NF-κB signaling through its interaction with β-arrestin2. Carcinogenesis. 2009;30(8):1281–7.
153. Neil JR, Tian M, Schiemann WP. xIAP and its E3 ligase activity promote TGF-β-mediated NF-κB activation during breast cancer progression. J Biol Chem. 2009;284(32):21209–17.
154. Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-κB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene. 2007;26(5):711–24.
155. Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, et al. NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest. 2004;114(4):569–81.
156. Bertran E, Caja L, Navarro E, Sancho P, Mainez J, Murillo MM, et al. Role of CXCR4/SDF-1α in the migratory phenotype of hepatoma cells that have undergone epithelial-mesenchymal transition in response to the TGF-β. Cell Signal. 2009;21(11):1595–606.
157. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin β1 signaling is necessary for TGF-β activation of p38MAPK and epithelial plasticity. J Biol Chem. 2001;276(50):46707–13.
158. Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. Neoplasia. 2004;6(5):603–10.
159. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in TGF-β-mediated signaling. J Biol Chem. 1997;272(3):1429–32.
160. Hocevar BA, Prunier C, Howe PH. Disabled-2 (Dab2) mediates TGF-β-stimulated fibronectin synthesis through TGF-β-activated kinase 1 and activation of the JNK pathway. J Biol Chem. 2005;280(27):25920–7.
161. Santibanez JF. JNK mediates TGF-β1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes. FEBS Lett. 2006;580(22):5385–91.
162. Shintani Y, Wheelock MJ, Johnson KR. Phosphoinositide-3 kinase-Rac1-c-Jun NH2-terminal kinase signaling mediates collagen I-induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells. Mol Biol Cell. 2006;17(7):2963–75.
163. Shintani Y, Hollingsworth MA, Wheelock MJ, Johnson KR. Collagen I promotes metastasis in pancreatic cancer by activating c-Jun NH(2)-terminal kinase 1 and up-regulating N-cadherin expression. Cancer Res. 2006;66(24):11745–53.
164. Zuo W, Chen YG. Specific activation of mitogen-activated protein kinase by TGF-β receptors in lipid rafts is required for epithelial cell plasticity. Mol Biol Cell. 2009;20(3):1020–9.
165. Dedhar S, Williams B, Hannigan G. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends Cell Biol. 1999;9(8):319–23.
166. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. Nat Rev Cancer. 2005;5(1):51–63.
167. Hehlgans S, Haase M, Cordes N. Signalling via integrins: implications for cell survival and anticancer strategies. Biochim Biophys Acta. 2007;1775(1):163–80.
168. Somasiri A, Howarth A, Goswami D, Dedhar S, Roskelley CD. Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells. J Cell Sci. 2001;114(Pt 6):1125–36.
169. White DE, Cardiff RD, Dedhar S, Muller WJ. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene. 2001;20(48):7064–72.

170. Lin SW, Ke FC, Hsiao PW, Lee PP, Lee MT, Hwang JJ. Critical involvement of ILK in TGFbeta1-stimulated invasion/migration of human ovarian cancer cells is associated with urokinase plasminogen activator system. Exp Cell Res. 2007;313(3):602–13.
171. Li Y, Dai C, Wu C, Liu Y. PINCH-1 promotes tubular epithelial-to-mesenchymal transition by interacting with integrin-linked kinase. J Am Soc Nephrol. 2007;18(9):2534–43.
172. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer. 2002;2(2):91–100.
173. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med. 1999;222(2):124–38.
174. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer. 2010;10(1):9–22.
175. Legate KR, Wickstrom SA, Fassler R. Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev. 2009;23(4):397–418.
176. Hauck CR, Sieg DJ, Hsia DA, Loftus JC, Gaarde WA, Monia BP, et al. Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. Cancer Res. 2001;61(19):7079–90.
177. Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol. 2000;2(5):249–56.
178. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, et al. The integrin αvβ6 binds and activates latent TGF-β1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 1999;96(3):319–28.
179. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, et al. The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. J Cell Biol. 2002;157(3):493–507.
180. Wendt MK, Schiemann WP. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. Breast Cancer Res. 2009;11(5):R68.
181. Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, et al. Inhibition of pulmonary and skeletal metastasis by a TGF-β type I receptor kinase inhibitor. Cancer Res. 2006;66 (13):6714–21.
182. Sloan EK, Pouliot N, Stanley KL, Chia J, Moseley JM, Hards DK, et al. Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. Breast Cancer Res. 2006;8(2):R20.
183. Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A, et al. TGF-β-induced EMT requires focal adhesion kinase (FAK) signaling. Exp Cell Res. 2008;314(1):143–52.
184. Liu S, Xu SW, Kennedy L, Pala D, Chen Y, Eastwood M, et al. FAK is required for TGF-β-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. Mol Biol Cell. 2007;18(6):2169–78.
185. Kim W, Seok Kang Y, Soo Kim J, Shin NY, Hanks SK, Song WK. The integrin-coupled signaling adaptor p130Cas suppresses Smad3 function in TGF-β signaling. Mol Biol Cell. 2008;19(5):2135–46.
186. van der Flier S, Chan CM, Brinkman A, Smid M, Johnston SR, Dorssers LC, et al. BCAR1/p130Cas expression in untreated and acquired tamoxifen-resistant human breast carcinomas. Int J Cancer. 2000;89(5):465–8.
187. Ta HQ, Thomas KS, Schrecengost RS, Bouton AH. A novel association between p130Cas and resistance to the chemotherapeutic drug adriamycin in human breast cancer cells. Cancer Res. 2008;68(21):8796–804.
188. Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, et al. p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis. Cancer Res. 2006;66(9):4672–80.
189. Wendt MK, Smith JA, Schiemann WP. p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem. 2009;284 (49):34145–56.
190. Tumbarello DA, Brown MC, Hetey SE, Turner CE. Regulation of paxillin family members during epithelial-mesenchymal transformation: a putative role for paxillin delta. J Cell Sci. 2005;118(Pt 20):4849–63.
191. Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, et al. Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J Biol Chem. 1999;274 (12):8316–21.
192. Tumbarello DA, Turner CE. Hic-5 contributes to epithelial-mesenchymal transformation through a RhoA/ROCK-dependent pathway. J Cell Physiol. 2007;211(3):736–47.
193. Wang H, Song K, Krebs TL, Yang J, Danielpour D. Smad7 is inactivated through a direct physical interaction with the LIM protein Hic-5/ARA55. Oncogene. 2008;27(54):6791–805.
194. Wang H, Song K, Sponseller TL, Danielpour D. Novel function of androgen receptor-associated protein 55/Hic-5 as a negative regulator of Smad3 signaling. J Biol Chem. 2005;280(7):5154–62.
195. Prunier C, Hocevar BA, Howe PH. Wnt signaling: physiology and pathology. Growth Factors. 2004;22(3):141–50.
196. Prunier C, Howe PH. Disabled-2 (Dab2) is required for TGF-β-induced epithelial to mesenchymal transition (EMT). J Biol Chem. 2005;280(17):17540–8.
197. Chaudhury A, Hussey GS, Ray PS, Jin G, Fox PL, Howe PH. TGF-β-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. Nat Cell Biol. 2010;12(3):286–93.
198. Moreno-Bueno G, Portillo F, Cano A. Transcriptional regulation of cell polarity in EMT and cancer. Oncogene. 2008;27 (55):6958–69.
199. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer. 2007;7(6):415–28.
200. Ikenouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci. 2003;116(Pt 10):1959–67.
201. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition. Nat Cell Biol. 2009;11(8):943–50.
202. Smith AP, Verrecchia A, Faga G, Doni M, Perna D, Martinato F, et al. A positive role for Myc in TGF-β-induced Snail transcription and epithelial-to-mesenchymal transition. Oncogene. 2009;28(3):422–30.
203. Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP. Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-β in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis. 2008;29 (12):2243–51.
204. Araki S, Eitel JA, Batuello CN, Bijangi-Vishehsaraei K, Xie XJ, Danielpour D, et al. TGF-β1-induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. J Clin Invest. 2010;120(1):290–302.
205. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A. TGF-β employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol. 2006;174(2):175–83.
206. Yu M, Smolen GA, Zhang J, Wittner B, Schott BJ, Brachtel E, et al. A developmentally regulated inducer of EMT, LBX1, contributes to breast cancer progression. Genes Dev. 2009;23(15):1737–42.
207. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer. 2002;2 (2):101–12.

208. Dhasarathy A, Kajita M, Wade PA. The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-alpha. Mol Endocrinol. 2007;21(12):2907–18.
209. Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell. 2003;113 (2):207–19.
210. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. Cross-talk between TGF-β and estrogen receptor signaling through Smad3. J Biol Chem. 2001;276(46):42908–14.
211. Radaelli E, Arnold A, Papanikolaou A, Garcia-Fernandez RA, Mattiello S, Scanziani E, et al. Mammary tumor phenotypes in wild-type aging female FVB/N mice with pituitary prolactinomas. Vet Pathol. 2009;46(4):736–45.
212. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65(16):7065–70.
213. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857–66.
214. Silveri L, Tilly G, Vilotte JL, Le Provost F. MicroRNA involvement in mammary gland development and breast cancer. Reprod Nutr Dev. 2006;46(5):549–56.
215. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008;22 (7):894–907.
216. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
217. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem. 2008;283(22):14910–4.
218. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 2008;9(6):582–9.
219. Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ. TGF-β and microRNA:mRNA regulatory networks in epithelial plasticity. Cells Tissues Organs. 2007;185(1–3):157–61.
220. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem. 2007;282 (19):14328–36.
221. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 2008;18(3):350–9.
222. Lombaerts M, van Wezel T, Philippo K, Dierssen JW, Zimmerman RM, Oosting J, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br J Cancer. 2006;94(5):661–71.
223. Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci USA. 1995;92(16):7416–9.
224. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003;349 (21):2042–54.
225. Vrba L, Jensen TJ, Garbe JC, Heimark RL, Cress AE, Dickinson S, et al. Role for DNA methylation in the regulation of miR-200c and miR-141 expression in normal and cancer cells. PLoS One. 2010;5(1):e8697.
226. Dumont N, Wilson MB, Crawford YG, Reynolds PA, Sigaroudinia M, Tlsty TD. Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. Proc Natl Acad Sci. 2008;105(39):14867–72.
227. Bierie B, Moses HL. Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer. 2006;6 (7):506–20.
228. Bissell MJ, Labarge MA. Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? Cancer Cell. 2005;7(1):17–23.
229. Radisky DC, Bissell MJ. Cancer. Respect thy neighbor! Science. 2004;303(5659):775–7.
230. Massague J. TGFbeta in Cancer. Cell. 2008;134(2):215–30.
231. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer. 2004;4 (2):118–32.
232. Agiostratidou G, Hulit J, Phillips GR, Hazan RB. Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. J Mammary Gland Biol Neoplasia. 2007;12(2–3):127–33.
233. Christofori G. Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J. 2003;22(10):2318–23.
234. Graff JR, Greenberg VE, Herman JG, Westra WH, Boghaert ER, Ain KB, et al. Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. Cancer Res. 1998;58 (10):2063–6.
235. Makrilia N, Kollias A, Manolopoulos L, Syrigos K. Cell adhesion molecules: role and clinical significance in cancer. Cancer Invest. 2009;27(10):1023–37.
236. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin Cancer Res. 2007;13(23):7003–11.
237. Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, et al. Cadherin switching in human prostate cancer progression. Cancer Res. 2000;60(13):3650–4.
238. Pyo SW, Hashimoto M, Kim YS, Kim CH, Lee SH, Johnson KR, et al. Expression of E-cadherin, P-cadherin and N-cadherin in oral squamous cell carcinoma: correlation with the clinicopathologic features and patient outcome. J Craniomaxillofac Surg. 2007;35(1):1–9.
239. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol. 2000;148(4):779–90.
240. Cavallaro U, Niedermeyer J, Fuxa M, Christofori G. N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. Nat Cell Biol. 2001;3(7):650–7.
241. Lehembre F, Yilmaz M, Wicki A, Schomber T, Strittmatter K, Ziegler D, et al. NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. EMBO J. 2008;27 (19):2603–15.
242. Illman SA, Lehti K, Keski-Oja J, Lohi J. Epilysin (MMP-28) induces TGF-β mediated epithelial to mesenchymal transition in lung carcinoma cells. J Cell Sci. 2006;119(Pt 18):3856–65.
243. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2(3):161–74.
244. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr Opin Cell Biol. 2004;16(5):558–64.
245. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev. 2000;14(2):163–76.
246. Dangelo M, Sarment DP, Billings PC, Pacifici M. Activation of TGF-β in chondrocytes undergoing endochondral ossification. J Bone Miner Res. 2001;16(12):2339–47.

247. Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, et al. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci. 2001;114 (Pt 1):111–8.
248. Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature. 2005;436 (7047):123–7.
249. Stuelten CH, DaCosta Byfield S, Arany PR, Karpova TS, Stetler-Stevenson WG, Roberts AB. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-α and TGF-β. J Cell Sci. 2005;118(Pt 10):2143–53.
250. Duivenvoorden WC, Hirte HW, Singh G. TGF-β1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. Clin Exp Metastasis. 1999;17(1):27–34.
251. Kim ES, Sohn YW, Moon A. TGF-β-induced transcriptional activation of MMP-2 is mediated by activating transcription factor (ATF)2 in human breast epithelial cells. Cancer Lett. 2007;252(1):147–56.
252. Kim ES, Kim MS, Moon A. TGF-β in conjunction with H-Ras activation promotes malignant progression of MCF10A breast epithelial cells. Cytokine. 2005;29(2):84–91.
253. Harbeck N, Kates RE, Gauger K, Willems A, Kiechle M, Magdolen V, et al. Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-I: novel tumor-derived factors with a high prognostic and predictive impact in breast cancer. Thromb Haemost. 2004;91(3):450–6.
254. Duffy MJ, Duggan C. The urokinase plasminogen activator system: a rich source of tumour markers for the individualised management of patients with cancer. Clin Biochem. 2004;37(7):541–8.
255. Mitra SK, Lim ST, Chi A, Schlaepfer DD. Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. Oncogene. 2006;25(32):4429–40.
256. Lester RD, Jo M, Montel V, Takimoto S, Gonias SL. uPAR induces epithelial-mesenchymal transition in hypoxic breast cancer cells. J Cell Biol. 2007;178(3):425–36.
257. Ge R, Rajeev V, Ray P, Lattime E, Rittling S, Medicherla S, et al. Inhibition of growth and metastasis of mouse mammary carcinoma by selective inhibitor of TGF-β type I receptor kinase in vivo. Clin Cancer Res. 2006;12(14 Pt 1):4315–30.
258. Nam JS, Terabe M, Mamura M, Kang MJ, Chae H, Stuelten C, et al. An anti-TGF-β antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Res. 2008;68 (10):3835–43.
259. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, et al. Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell. 2008;13(1):23–35.
260. Zhang F, Tom CC, Kugler MC, Ching TT, Kreidberg JA, Wei Y, et al. Distinct ligand binding sites in integrin α3β1 regulate matrix adhesion and cell–cell contact. J Cell Biol. 2003;163 (1):177–88.
261. Whitley BR, Palmieri D, Twerdi CD, Church FC. Expression of active plasminogen activator inhibitor-1 reduces cell migration and invasion in breast and gynecological cancer cells. Exp Cell Res. 2004;296(2):151–62.
262. Descotes F, Riche B, Saez S, De Laroche G, Datchary J, Roy P, et al. Plasminogen activator inhibitor type 1 is the most significant of the usual tissue prognostic factors in node-negative breast ductal adenocarcinoma independent of urokinase-type plasminogen activator. Clin Breast Cancer. 2008;8(2):168–77.
263. Samarakoon R, Higgins CE, Higgins SP, Higgins PJ. Differential requirement for MEK/ERK and SMAD signaling in PAI-1 and CTGF expression in response to microtubule disruption. Cell Signal. 2009;21(6):986–95.
264. Ignotz RA, Massague J. TGF-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem. 1986;261(9):4337–45.
265. Wienke D, Davies GC, Johnson DA, Sturge J, Lambros MB, Savage K, et al. The collagen receptor Endo180 (CD280) Is expressed on basal-like breast tumor cells and promotes tumor growth in vivo. Cancer Res. 2007;67(21):10230–40.
266. Garamszegi N, Garamszegi SP, Samavarchi-Tehrani P, Walford E, Schneiderbauer MM, Wrana JL, et al. Extracellular matrix-induced TGF-β receptor signaling dynamics. Oncogene 2010; PMID: 20101206.
267. Xie L, Law BK, Aakre ME, Edgerton M, Shyr Y, Bhowmick NA, et al. TGF-β-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res. 2003;5 (6):R187–98.
268. Maschler S, Wirl G, Spring H, Bredow DV, Sordat I, Beug H, et al. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene. 2005;24(12):2032–41.
269. Camara J, Jarai G. Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-α. Fibrogenesis Tissue Repair. 2010;3(1):2.
270. Wels J, Kaplan RN, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev. 2008;22(5):559–74.
271. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell. 2009;15(1):35–44.
272. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell. 2009;138(4):645–59.

Oncogene (2010) 29, 6485–6498
© 2010 Macmillan Publishers Limited All rights reserved 0950-9232/10
www.nature.com/onc

ORIGINAL ARTICLE

Transforming growth factor-β-induced epithelial–mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression

MK Wendt, JA Smith and WP Schiemann

Division of General Medical Sciences—Oncology, Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) have critical roles in regulating the metastasis of aggressive breast cancers, yet the impact of epithelial–mesenchymal transition (EMT) induced by TGF-β in altering the response of breast cancer cells to EGF remains unknown. We show in this study that murine metastatic 4T1 breast cancer cells formed compact and dense spheroids when cultured under three-dimensional (3D) conditions, which was in sharp contrast to the branching phenotypes exhibited by their nonmetastatic counterparts. Using the human MCF10A series, we show that epithelial-type and nonmetastatic breast cancer cells were unable to invade to EGF, whereas their mesenchymal-type and metastatic counterparts readily invaded to EGF. Furthermore, EMT induced by TGF-β was sufficient to manifest dense spheroid morphologies, a phenotype that increased primary tumor exit and invasion to EGF. Post-EMT invasion to EGF was dependent on increased activation of EGF receptor (EGFR) and p38 mitogen-activated protein kinase, all of which could be abrogated either by pharmacologic (PF-562271) or by genetic (shRNA) targeting of focal adhesion kinase (FAK). Mechanistically, EMT induced by TGF-β increased cell-surface levels of EGFR and prevented its physical interaction with E-cadherin, leading instead to the formation of oncogenic signaling complexes with TβR-II. Elevated EGFR expression was sufficient to transform normal mammary epithelial cells, and to progress their 3D morphology from that of hollow acini to branched structures characteristic of nonmetastatic breast cancer cells. Importantly, we show that TGF-β stimulation of EMT enabled this EGFR-driven breast cancer model to abandon their inherent branching architecture and form large, undifferentiated masses that were hyperinvasive to EGF and showed increased pulmonary tumor growth upon tail vein injection. Finally, chemotherapeutic targeting of FAK was sufficient to revert the aggressive behaviors of these structures. Collectively, this investigation has identified a novel EMT-based approach to neutralize the oncogenic activities of EGF and TGF-β in aggressive and invasive forms of breast cancer.

Correspondence: Dr WP Schiemann, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, 2103 Cornell Road, Cleveland, OH 44106, USA.
E-mail: william.schiemann@case.edu
Received 14 April 2010; revised 29 June 2010; accepted 19 July 2010; published online 30 August 2010

Oncogene (2010) **29,** 6485–6498; doi:10.1038/onc.2010.377; published online 30 August 2010

Keywords: EGFR; TGF-β; FAK; breast cancer; metastasis; invasion

Introduction

The process of epithelial–mesenchymal transition (EMT) induced by transforming growth factor-β (TGF-β) is well established as a critical mechanism of tumor progression (Zavadil and Bottinger, 2005; Moustakas and Heldin, 2007; Kalluri and Weinberg, 2009; Wendt *et al*., 2009a); however, how these TGF-β-dependent events impact the response to growth factors during metastasis remains incompletely understood (Cowin and Welch, 2007; Moustakas and Heldin, 2007; Wendt *et al*., 2009a). Equally mysterious are the reasons underlying the failure of science and medicine to readily detect the classical mesenchymal and sarcomatoid phenotypes exhibited by fully transitioned carcinoma cells at sites of secondary metastases, which in theory should be enriched in these dedifferentiated and post-EMT cell types (Tarin *et al*., 2005). It therefore stands to reason that deciphering the molecular mechanisms that underlie the interplay between EMT and its counterpart mesenchymal–epithelial transition (Hugo *et al*., 2007) may offer new inroads into targeting tumor metastasis.

Applying genomic analyses to human breast cancers has resulted in the identification and classification of at least five genetically distinct breast cancer subtypes (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003), of which the basal-like 'triple-negative' subtype remains the most intractable to clinical intervention. More recently, elevated expression of ErbB1/epidermal growth factor (EGF) receptor (EGFR) in basal-like tumors has been identified as a highly predictive marker for poor clinical outcomes (Tischkowitz *et al*., 2007). Indeed, synergistic activity between TGF-β and EGF in stimulating EMT has been identified (Saha *et al*., 1999), whereas the actual process of EMT has been associated with the development of chemoresistance to EGFR-targeted therapies in carcinoma cells (Thomson *et al*., 2005, 2008; Buck *et al*., 2007; Barr *et al*., 2008). Because TGF-β is a master

regulator of EMT, we hypothesized that EMT stimulated by TGF-β would induce a fundamental change in how tumor cells sense and respond to their surrounding microenvironment, particularly to EGF (Joo *et al*., 2007; Wang *et al*., 2009). Along these lines, recent studies suggest that aberrant EGFR signaling reflects the inactivation of E-cadherin, a hallmark of EMT (Miettinen *et al*., 1994; Takahashi and Suzuki, 1996; Wilding *et al*., 1996; Bremm *et al*., 2008). However, other studies implicate a novel paracrine signaling loop that transpires between carcinoma cells and tumor-infiltrating macrophages that comprise the actions of TGF-β, EGF and colony-stimulating factor-1 in promoting breast cancer cell migration and invasion to EGF (Wyckoff *et al*., 2000, 2004; DeNardo *et al*., 2009).

Focal adhesion kinase (FAK) is a multifunctional protein tyrosine kinase and scaffolding molecule that links transmembrane signaling inputs arising from integrins and growth factor receptors to intracellular effectors (Mitra *et al*., 2006). Along these lines, FAK has been shown to interact directly with the intracellular domain of EGFR to facilitate its downstream signaling and activation of cell motility (Sieg *et al*., 2000). Recently, analyses performed by our laboratory established a key role for FAK in physically associating integrins with TβR-II (Wendt and Schiemann, 2009). Moreover, we showed that the chemotherapeutic targeting of FAK prevented the infiltration of macrophages into primary mammary tumors (Wendt and Schiemann, 2009). Clearly, these and other studies have established FAK as a key factor in mediating EMT and metastasis stimulated by TGF-β (Cicchini *et al*., 2008; Ding *et al*., 2008); however, whether FAK facilitates the potential pathophysiological activities between TGF-β and EGF remains unknown. Thus, the objectives of this study were to (1) determine how the response of mammary epithelial cells (MECs) to EGF was altered by EMT induced by TGF-β; (2) establish the signaling mechanisms responsible for eliciting the aberrant responses of post-EMT MECs to EGF and (3) characterize the three-dimensional (3D) morphology of resulting hyperinvasive, post-EMT MECs.

Results

Metastatic breast cancer cells grow as dense cell structures manifested by TGF-β-induced EMT

Recent studies strongly suggest that the morphology and cell signaling responses of mammary tumors are more accurately recapitulated *in vitro* using 3D-organotypic systems as compared with growing cells on plastic (Park *et al*. 2000; Kenny *et al*., 2007; Wendt *et al*., 2009b). To this end, we used a well-characterized murine mammary carcinoma progression series comprised of several isogenic cell lines that possess varying metastatic proficiencies and include: (1) noninvasive and nonmetastatic 67NR cells, which form primary tumors that cannot enter the circulation; (2) invasive and nonmetastatic 4T07 cells, which traverse the circulation and fail to establish secondary tumors in the lung and (3) highly metastatic 4T1 cells, which disseminate widely and colonize multiple organ sites (Aslakson and Miller, 1992). Surprisingly, both nonmetastatic cell lines formed highly branched 3D structures, whereas metastatic 4T1 cells formed dense spheroids to lobular-like structures that were devoid of branching (Figure 1a). To verify the metastatic designation of these MEC derivatives, we engineered them to stably express luciferase and then engrafted them onto the mammary fat pad, and pulmonary metastasis was tracked over time using bioluminescent imaging. As expected, only fully metastatic 4T1 cells readily formed secondary metastatic lesions when engrafted onto the mammary fat pads of mice (Figure 1b). Given the recent studies from our lab and others identifying a critical role of EMT in driving breast cancer progression, we sought to examine how EMT induced by TGF-β affected subsequent 3D culture morphologies and tumor metastasis (Padua *et al*., 2008; Wendt and Schiemann, 2009; Wendt *et al*., 2009a). Interestingly, following a prolonged treatment with TGF-β, 4T07 cells showed a 3D morphology that was highly reminiscent of the metastatic 4T1 cells (Figures 1c and a). To verify their EMT status, lysates of TGF-β-treated 4T07 cells were analyzed for decreased expression of E-cadherin and increased expression of vimentin (Figure 1d). Given the somewhat counterintuitive nature of this TGF-β-induced 3D EMT morphology, we used the 67NR cell line that grew as a mixture of independent spheroid and branched structures (Supplementary Figure 1). Indeed, by physically isolating the spheroid structures from 3D cultures, expanding them on a plastic growth surface and then placing them back into 3D cultures we definitively showed that spheroid structures in 3D cultures directly correspond to a classic mesenchymal morphology when cultured plastic (Supplementary Figure 1). Recent studies indicate a prominent role for paracrine EGF production in driving breast cancer metastasis (Wyckoff *et al*., 2004; DeNardo *et al*., 2009). Therefore, we hypothesized that post-EMT, breast cancer cells would be hyperinvasive in response to EGF as compared with pre-EMT cells. Indeed, control 4T07 cells, although highly invasive in response to serum, exhibited little-to-no invasion specifically in response to EGF (Figure 1e). In contrast, post-EMT 4T07 cells readily invaded in response to a solitary EGF stimulus (Figure 1e). Furthermore, and consistent with the establishment of paracrine EGF signaling axes in regulating breast cancer metastasis (Wyckoff *et al*., 2004), we observed significantly elevated quantities of post-EMT 4T07 cells in the lungs of mice at 2 and 4 weeks post-engraftment onto the mammary fat pad (Figure 1f). Although the ultimate fate of disseminated post-EMT cells cannot be ascertained from this experiment, our findings nevertheless show the (1) importance of EMT to enhance metastatic seeding and (2) the inability of EMT to sustain secondary tumor growth (Figure 1f). Consistent with these findings, epithelial-type and nonmetastatic human MCF-7 and MCF10AT1K cells failed to invade in response to EGF, whereas their mesenchymal-type and malignant human

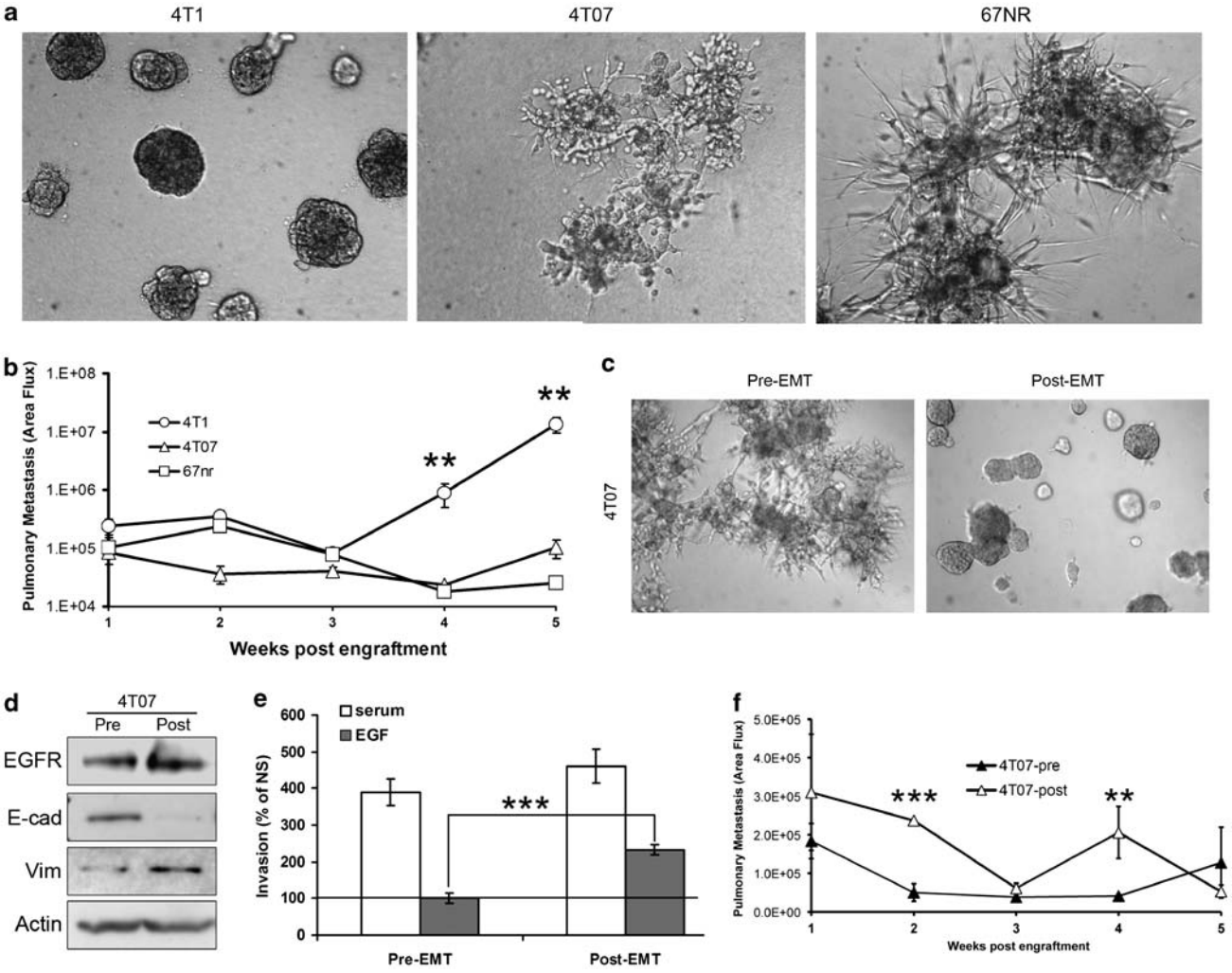


Figure 1 Loss of mammary branching characterizes increasing metastatic potential and is induced by TGF-β1. (**a**) 4T1 (highly metastatic), 4T07 (invasive, but nonmetastatic) and 67NR (noninvasive and nonmetastatic) mammary carcinoma cells were grown in 3D culture for 5 days and representative structures are shown. Data indicate that branched mammary structures are characteristic of nonmetastatic cells, a phenotype that is abandoned by fully metastatic cells. (**b**) The cells described in panel (**a**) expressing firefly luciferase were engrafted onto the mammary fat pad of 4-week-old female Balb/C mice (4T1 = 10 000 cells; 4T07 and 67NR = 100 000 cells). Mean pulmonary luminescence (area flux) is shown at various time points as a measure of metastasis from the primary mammary tumor to the lungs ($n = 5$ mice per group, ± s.e., $^{**}P < 0.01$). (**c**) Pre- and post-EMT 4T07 cells were cultured in 3D-organotypic conditions for 5 days and representative structures are shown. (**d**) The pre- and post-EMT 4T07 cells were analyzed by immunoblot for EGFR, E-cadherin (E-cad), vimentin (vim) and β-actin (actin) as a loading control. Shown are representative immunoblots that were completed three times with similar results. (**e**) Pre- and post-EMT 4t07 cells were allowed to invade through Matrigel-coated synthetic membranes in response to 2% serum or EGF. Data are normalized to a serum-free control (solid line at 100%) and are the mean (± s.e.) of three independent experiments completed in triplicate ($^{***}P < 0.001$). (**f**) The pre- and post-EMT 4T07 cells as shown in panel (c) were engrafted onto the mammary fat pad of Balb/C mice as shown in panel (**b**). Mean pulmonary luminescence (area flux) is shown at various time points following engraftment ($n = 5$ mice per group, ± s.e., $^{***}P < 0.001$, $^{**}P < 0.01$).

MDA-MB-231 and MCF10A-CA1h counterparts readily invaded in response to EGF (Supplementary Figure 2). Collectively, these findings strongly suggest that the process of EMT facilitates the ability of human breast cancer cells to invade aberrantly in response to EGF.

TGF-β stimulation of EMT results in the generation of highly invasive spheroids that possess elevated cell-surface EGFR levels

We next used normal murine mammary gland (NMuMG) cells to more appropriately address the hypothesis that EMT stimulated by TGF-β was sufficient to engender MECs with the ability to invade in response to EGF (Miettinen *et al*., 1994). Figure 2a shows that NMuMG cells endogenously expressed moderate levels of EGFR, which were readily activated along with ERK1/2 in response to EGF (Figure 2a). We demonstrated the specificity of these responses to stimulation by EGF by treating the cells with (1) the Src inhibitor PP2, which uncoupled EGF from phosphorylating EGFR on Y845, but was without effect on EGFR autophosphorylation (Y1116) and ERK1/2 activation; and (2) the EGFR inhibitor AG1478 (AG), which prevented all three EGF-promoted responses (Figure 2a). However, despite their expression of functional EGFR, NMuMG cells did not

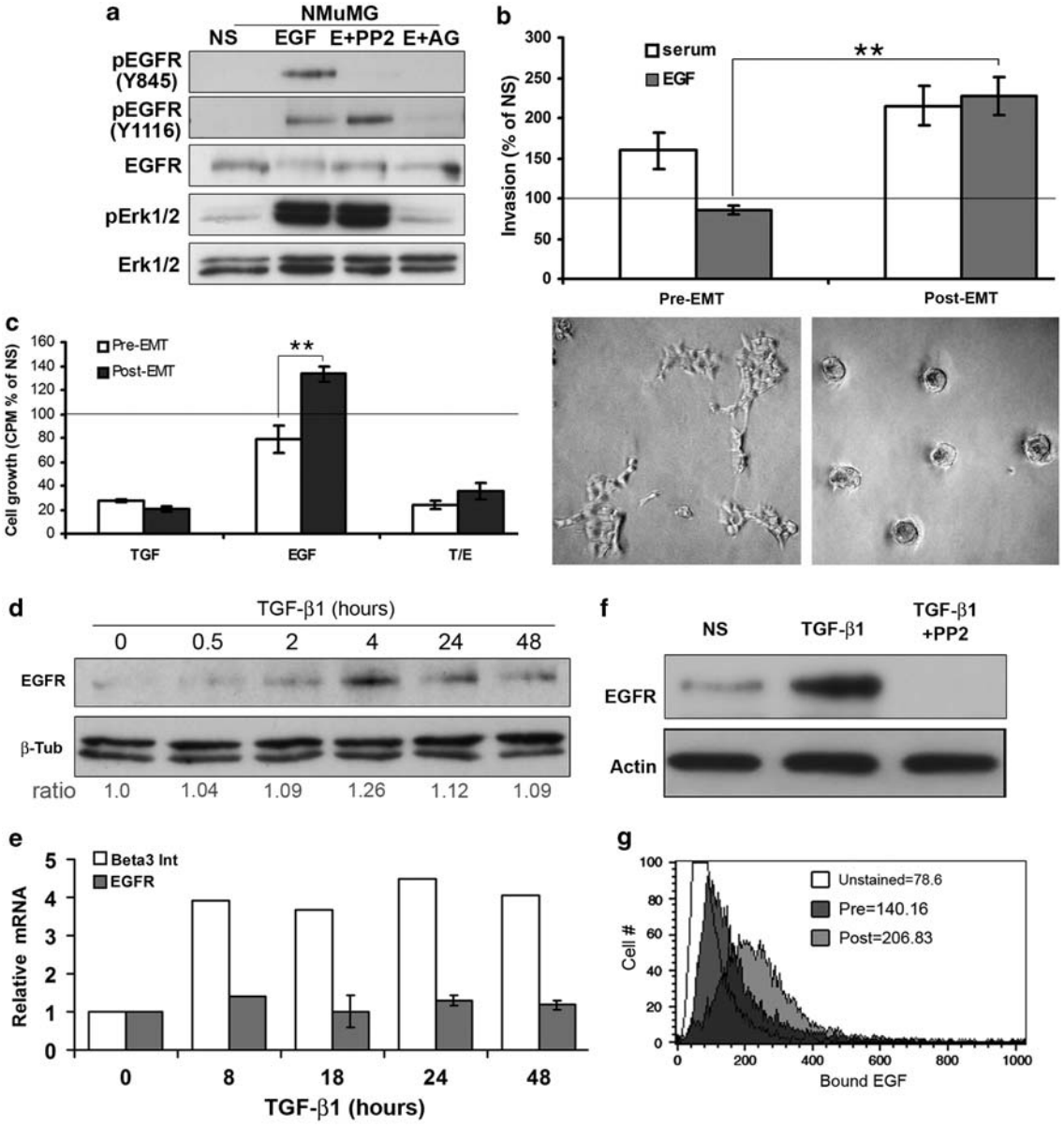


Figure 2 TGF-β stimulation of EMT results in the generation of highly invasive spheroids that possess elevated EGFR cell-surface expression. (**a**) NMuMG cells were serum deprived (0.5% FBS) for 6 h in the absence or presence of the Src inhibitor, PP2 (10 μM) or the EGFR inhibitor, AG1478 (1 μM), at which point they were stimulated for 30 min with EGF and analyzed for the phosphorylation of EGFR (pEGFR(Y845) or pEGFR(Y1116)) or ERK1/2 (pErk1/2). The resulting immunoblots were stripped and reprobed with antibodies against EGFR or ERK1/2, to monitor differences in protein loading. Images are from a representative experiment that was performed at least three times with similar results (NS, no stimulation). (**b**) Pre-EMT and post-EMT NMuMG (48 h pretreatment with TGF-β1 (5 ng/ml)) cells were induced to invade synthetic basement membranes by either serum (2%) or EGF (50 ng/ml) as indicated. Data are the mean (± s.e.) invasion relative to serum-free media for both pre- and post-EMT cells (solid line) observed in three independent experiments completed in triplicate (**$P<0.01$). Accompanying photomicrographs depict the morphology of pre-EMT (lower left) and post-EMT (lower right) NMuMG cells when propagated for 24 h on Matrigel cushions. (**c**) Pre-EMT and post-EMT NMuMG cells were incubated with either TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines for 24 h before labeling cellular DNA by administration of [^{3}H]thymidine. Data are the mean (± s.e.) quantities of incorporated [^{3}H]thymidine normalized to unstimulated controls (solid line) observed in three independent experiments completed in triplicate (**$P<0.01$). (**d**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) for indicated times over a period of 48 h, at which point alterations in EGFR expression were monitored by immunoblotting. Stripped membranes were reprobed with anti-β-tubulin (β-tub) to monitor differences in protein loading. Images are from a representative experiment that was performed at least four times with similar results. (**e**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) as shown in panel (**d**). Afterward, total RNA was isolated and subjected to semiquantitative real-time PCR to monitor the expression of EGFR or β3 integrin, which served as a marker for EMT induced by TGF-β. Data are the mean (± s.d.) fold changes in gene expression relative to untreated control cells observed in at least three independent experiments. (**f**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) for 18 h in the absence or presence of the Src inhibitor, PP2 (10 μM). Alterations in EGFR expression were monitored by immunoblotting as shown in panel (**d**). (**g**) Cell-surface expression of EGFR in pre-EMT and post-EMT NMuMG cells was determined by flow-cytometric analysis of bound Alexa 488-labeled EGF. The presented histogram is representative of three independent experiments.

invade to EGF before their induction of EMT by TGF-β, which conferred robust invasive activities in response to EGF (Figure 2b). Consistent with what we observed in 4T07 cells, post-EMT NMuMG cells showed an 'invasosphere' morphology when cultured on Matrigel (Figure 2b). Moreover, NMuMG cells only showed a proliferative response to EGF subsequent to their induction of EMT (Figure 2c). However, the growth of pre- and post-EMT NMuMG cell populations remained sensitive to the cytostatic activities of TGF-β (Figure 2c).

We next found that TGF-β stimulation of EMT increased the expression of EGFR proteins, a response that was maximal at 4 h and was maintained throughout the 48-h EMT process (Figure 2d). This response was independent of a change in EGFR mRNA (Figure 2e), but was dependent on the activity of Src (Figure 2f). Finally, whole-cell EGF binding assays suggested that TGF-β-induced EMT stabilized EGFR on the cell surface (Figure 2g). Taken together, these findings suggest that the induction of EMT by TGF-β increases stability of EGFR at the cell surface in transitioning cells, which imparts post-EMT MECs with invasive functions in response to EGF.

EMT increases the coupling of EGFR to p38 MAPK activation by FAK

We next sought to determine the function of FAK in regulating EGF signaling in post-EMT MECs. As shown in Figure 3a, EGF-mediated p38 mitogen-activated protein kinase (MAPK) activation and Src-dependent phosphorylation of EGFR at Y845 were greatly augmented in post-EMT NMuMG cells. Importantly, both of these post-EMT EGF signaling events were completely blocked in NMuMG cells depleted in FAK expression (Figure 3a). Recent findings suggest that TGF-β transactivates the EGFR pathway by an extracellular mechanism involving the protease TNF-alpha converting enzyme (TACE)/ADAM17, whose activation by TGF-β mediates the release of EGF ligands (Wang *et al*., 2008, 2009). Supplementary Figure 3 shows that pharmacological antagonism of TACE/ADAM17 or EGFR had no effect on TGF-β-mediated p38 MAPK activation. Furthermore, Figure 3b shows that constitutively elevating EGFR expression in NMuMG cells failed to affect the coupling of TGF-β to p38 MAPK. Interestingly, this same cellular condition specifically enhanced the coupling of EGF to p38 MAPK, but had no affect on the extent of ERK1/2 phosphorylation induced by EGF (Figure 3b). Taken together, these findings suggest that the activation of p38 MAPK by TGF-β takes place by a FAK/Src pathway, whose activation by TGF-β stabilizes EGFR cell-surface expression and enables its coupling to p38 MAPK.

In further addressing the function of FAK in mediating the ability of EGF to induce the invasion of post-EMT MECs, we observed that NMuMGs depleted in FAK expression fail to undergo invasion to EGF in the post-EMT state (Figure 3c). Furthermore, a pharmacological inhibitor of FAK, PF-562271 (PF-271) (Roberts *et al*., 2008) similarly abrogated the invasion of post-EMT control (YFP) NMuMG cells (Figure 3d). Along these lines, inclusion of small molecule inhibitors against TβR-I (SB431542), p38 MAPK (SB203580) or EGFR (AG) also significantly inhibited the invasion of post-EMT control (YFP) NMuMG cells to EGF (Figure 3d). Finally, in light of the elevated expression of EGFR in post-EMT NMuMG cells (Figure 2), we repeated these pharmacological analyses in EGFR-expressing NMuMG cells. Figure 3e shows that constitutive EGFR expression was sufficient to induce invasion to EGF, a cellular reaction that was drastically potentiated in the post-EMT state. As described above, pharmacological inhibition of FAK abrogated the pre- and post-EMT invasion of EGFR-expressing NMuMG cells to EGF (Figure 3e). In stark contrast to their control (YFP) counterparts (Figure 3d), treating post-EMT EGFR-expressing NMuMG cells with inhibitors against either TβR-I or p38 MAPK failed to affect invasion elicited by EGF (Figure 3e). Taken together, these findings suggest that elevated EGFR expression that typically occurs in metastatic breast cancers (Tischkowitz *et al*., 2007) is sufficient in (1) stabilizing the EMT phenotype, (2) allowing persistent invasion to EGF and (3) conferring resistance to small molecules inhibitors against TβR-I and p38 MAPK.

EGFR overexpression transforms NMuMG cells and sensitizes them to EMT by altering EGFR complexes

Given the profound impact constitutive EGFR expression had on EMT-induced invasion to EGF, we next sought to use this NMuMG cell model to further characterize the potential role of EMT in facilitating the ability of EGF to induce breast cancer invasion and metastasis. In doing so, we observed constitutive EGFR expression to be sufficient in transforming NMuMG cells, enabling their production of mammary fat-pad tumors that were comparable with those formed by NMuMG cells engineered to express polyoma middle T (PyMT; Figure 4a). Interestingly, the expression of PyMT in NMuMG cells induced a mesenchymal morphology consistent with increased levels of EGFR and its binding of EGF (Supplementary Figure 4; Salomon *et al*., 1987). Along these lines, constitutive EGFR expression greatly enhanced the mesenchymal character of NMuMG cells stimulated by either TGF-β, EGF or both cytokines together (Figures 4b and c).

Given our findings that suggested that diminished E-cadherin expression determines breast cancer invasion to EGF (Figure 1), we next sought to identify the affects of TGF-β on the physical interaction between EGFR and E-cadherin (Wilding *et al*., 1996; Bremm *et al*., 2008). Figure 4d shows that short-term TGF-β stimulation was unable to affect the expression of E-cadherin or EGFR in confluent cultures (bottom panel). However, we did observe TGF-β to disband the tonic interaction between EGFR and E-cadherin in favor of forming EGFR/TβR-II signaling complexes. Moreover, constitutive EGFR expression alone was sufficient in eliciting a stronger interaction between EGFR and TβR-II that

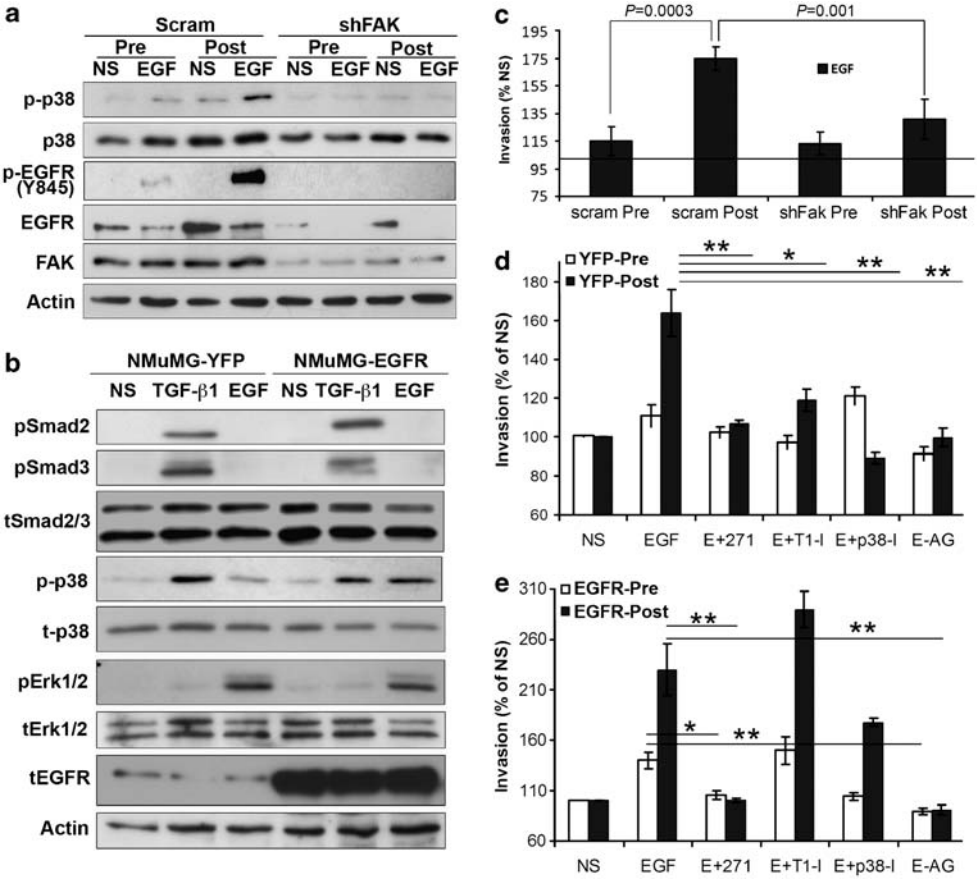


Figure 3 EMT increases the coupling of EGFR to p38 MAPK activation by FAK. (**a**) Control (i.e., scrambled shRNA; scram) and FAK-depleted (shFAK) NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h, at which point they were washed, serum-deprived for 6 h and subsequently stimulated with EGF (50 ng/ml) for 30 min and analyzed for phospho-p38 MAPK (p-p38) or phospho-Y845-EGFR (p-EGFR(Y845)) as indicated. The resulting immunoblots were stripped and reprobed with antibodies against p38 MAPK, EGFR, FAK and β-actin (actin) to monitor differences in protein loading. Images are from a representative experiment that was performed four times with similar results (NS, no stimulation). (**b**) Quiescent control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated for 30 min with either TGF-β1 (5 ng/ml) or EGF (50 ng/ml) and analyzed to monitor the phosphorylation status of Smad2 (pSmad2), Smad3 (pSmad3), p38 MAPK (p-p38) and ERK1/2 (pErk1/2). The resulting immunoblots were stripped and reprobed for total Smad2/3 (tSmad2/3), p38 MAPK (t-p38), ERK1/2 (tErk1/2), EGFR and β-actin (actin) as loading controls. Images are from a representative experiment that was performed at least four times with similar results. (**c**) Control (i.e., scrambled shRNA; scram) and FAK-depleted pre- and post-EMT NMuMG cells were allowed to invade synthetic basement membranes in response to EGF (50 ng/ml). Data are the mean (± s.e.) invasion relative to unstimulated MECs (i.e., serum-free media placed in the bottom chamber, solid line set to 100%) observed in three independent experiments completed in triplicate. (**d**) Pre- and post-EMT control (i.e., YFP) NMuMG cells were induced to invade EGF (50 ng/ml) in the absence or presence of the following pharmacological inhibitors: FAK inhibitor, PF-562271 (271, 1 μM), TβR-I inhibitor, SB431542 (T1-I, 10 μg/ml), p38 MAPK inhibitor, SB203580 (p38-I, 10 μM), or EGFR inhibitor, AG1478 (AG, 1 μM). (**e**) Data consist of the identical invasion experiments as in panel (**d**) completed with the EGFR-expressing (EGFR) NMuMG cells. Presented data in panels (**d**) and (**e**) are the mean (± s.e.) invasion relative to an EGF-free control (i.e., serum-free media placed in the bottom chamber = NS, set to 100%) observed in three independent experiments completed in triplicate (*$P<0.05$; **$P<0.01$).

mirrored the disassociation of E-cadherin from EGFR, an effect that was further exacerbated by TGF-β treatment (Figure 4d). Importantly, the formation of TβR-II/EGFR complexes correlated with increased Smad2/3 transcriptional activity and maintained cytostatic response to TGF-β (Supplementary Figure 5). To further explore the relationship between EGFR and E-cadherin, we performed immunofluorescent analyses to monitor changes in their expression and localization in NMuMG cells before and after TGF-β-stimulated EMT. E-cadherin expression was indeed decreased and delocalized from cell–cell junctions in EGFR-expressing NMuMG cells as compared with their control counterparts, findings that were exacerbated upon TGF-β stimulation (Figure 5a). Along these lines, Figure 5b clearly shows an emergence of $EGFR^{high}$/$E\text{-cadherin}^{low}$ post-EMT NMuMG cells. Moreover, the most morphologically 'mesenchymal' MECs were completely devoid of both E-cadherin and EGFR expression (Figure 5b; Supplementary Figure 6). Taken together, our findings point to the emergence of a post-EMT breast cancer cell population that is E-cadherin-negative, EGFR-positive and poised to exhibit hyperinvasive responses to EGF.

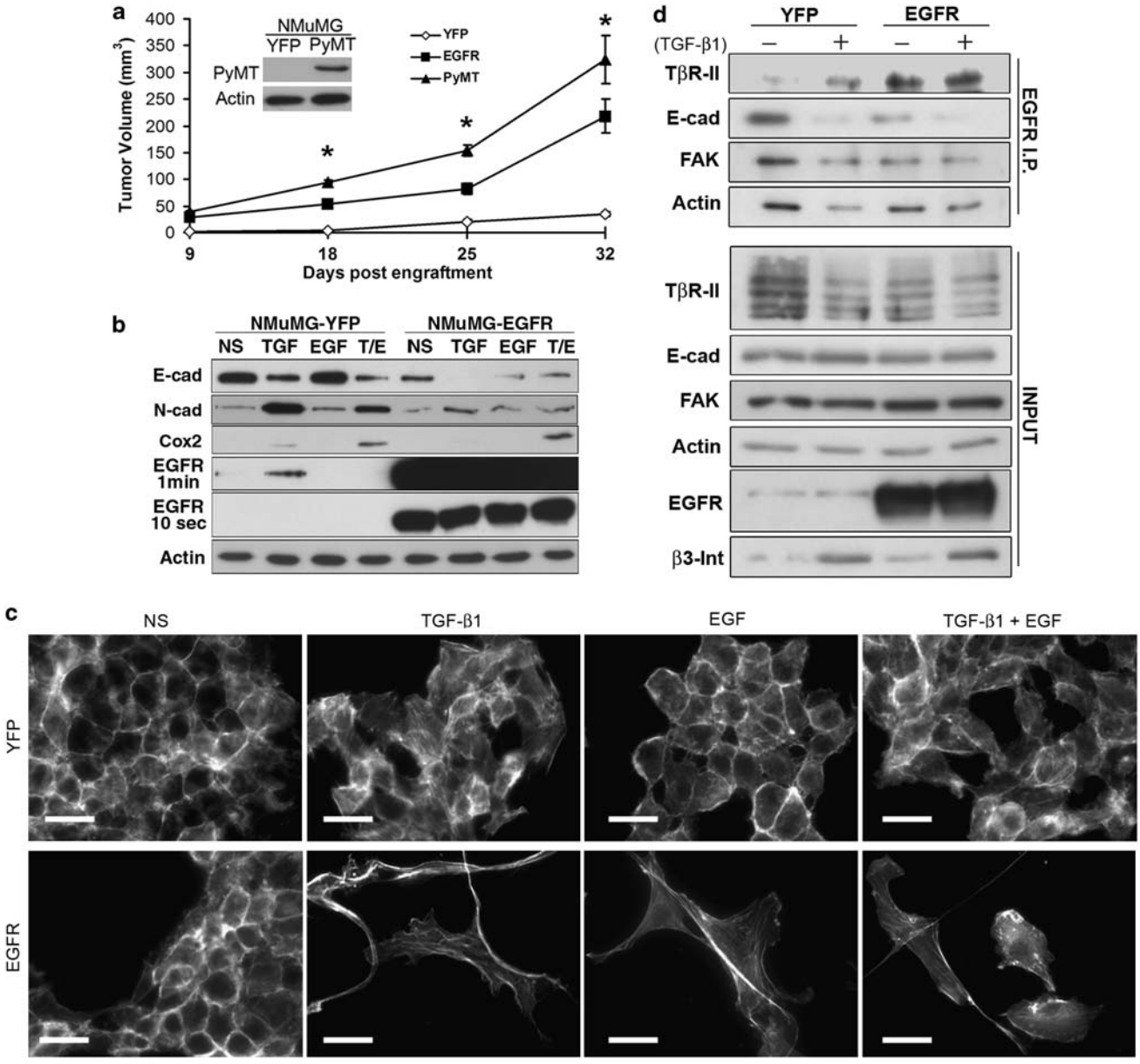


Figure 4 EGFR overexpression transforms NMuMG cells and sensitizes them to EMT by altering EGFR complexes. (**a**) Control (i.e., YFP), PyMT- and EGFR-expressing NMuMG cells were engrafted onto the mammary fat pads of Nu/Nu mice, whose development of mammary tumors was monitored over 32 days. Data are the mean (± s.e.) tumor volumes measured for indicated NMuMG tumor variants. (*$P<0.05$, $n=6$ mice per group). Inset depicts PyMT expression in NMuMG cells, which served as a positive control for tumor formation. (**b**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated with TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines for 48 h as indicated. Afterward, detergent-solubilized whole-cell extracts were prepared and subjected to immunoblot analyses to monitor changes in the expression of E-cadherin (E-cad), N-cadherin (N-cad), cycloxygenase-2 (Cox2), EGFR and β-actin (actin), which served as a loading control. Images are from a representative experiment that was performed at least three times in its entirety with similar results. (**c**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated as described in panel (**b**) before visualizing alterations in the actin cytoskeleton by direct phalloidin fluorescence. Images are representative photomicrographs (× 600) from a single experiment that was performed two times with identical results. (**d**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were allowed to reach confluence to normalize E-cad expression levels and incubated in the absence or presence of TGF-β1 (5 ng/ml) for 24 h before isolating EGFR complexes by immunoprecipitation. The resulting EGFR immunocomplexes (EGFR I.P.) were immunoblotted with antibodies against TβR-II, E-cad, FAK and β-actin (actin; top panel). Aliquots of the original whole-cell extract (input) was also immunoblotted with antibodies against TβR-II, E-cad, FAK, β-actin (actin), EGFR and β3 integrin to control for differences in protein loading (bottom panel). Images are from a representative experiment that was performed three times with similar results.

MEC branching induced by EGFR is dependent on TGF-β/FAK signaling

We next sought to evaluate the effects of EMT and constitutive EGFR expression on the growth and morphology of MECs propagated in 3D-organotypic systems. As we noted previously (Wendt *et al*., 2009b), NMuMG cells readily formed organized and hollow acinar structures with a defined actin cytoskeleton when

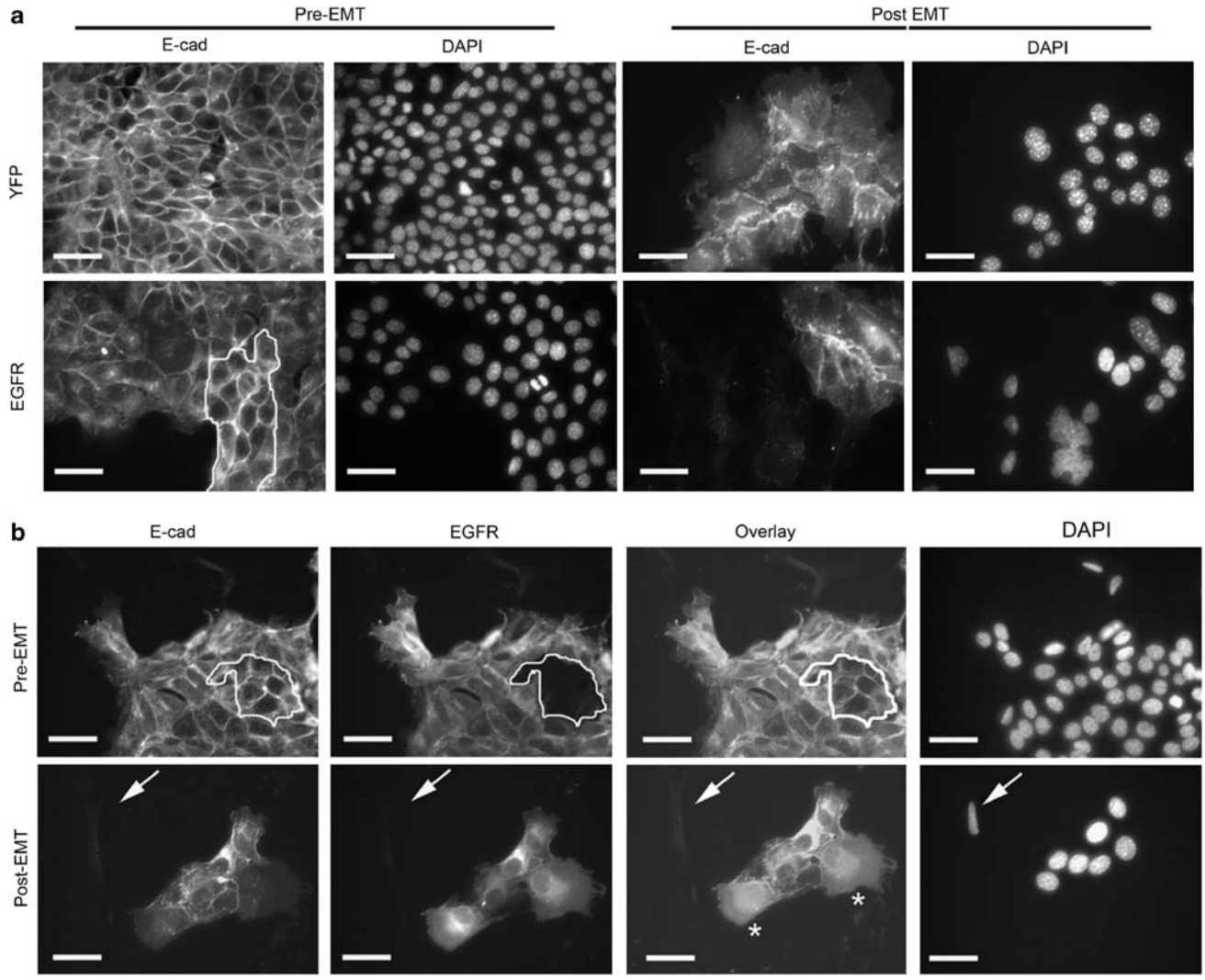


Figure 5 EGFR expression enhanced the delocalization of E-cadherin induced by EMT. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were incubated in the absence (pre-EMT) or presence (post-EMT) of TGF-β1 (5 ng/ml) 24 h, at which point they were processed for E-cadherin (E-cad) and DAPI immunofluorescence (× 400). Junctional localization of E-cadherin was slightly disrupted in EGFR-expressing NMuMG cells as compared with their control counterparts, except for regional pockets of cells designated by white outline. Delocalized and degradation of E-cadherin in response to TGF-β was enhanced in EGFR-expressing cells. (**b**) EGFR-expressing cells were stimulated to undergo EMT as in panel (**a**), at which point they were subjected to dual immunofluorescent staining to visualize E-cadherin (E-cad, red) and EGFR (green). Regions lacking EGFR expression (white outline) exhibit normal junctional localization of E-cad, whereas EMT induction resulted in the appearance two populations of NMuMG cells: one that was EGFR positive and E-cad negative (*), and a second that lacked expression of both EGFR and E-cad (arrows). Photomicrographs (× 400) presented in panels (**a**) and (**b**) are representative of three independent experiments.

grown in 3D-organotypic conditions (Figure 6a). In stark contrast, and reminiscent of what we observed for nonmetastatic breast cancer cells (Figure 1a), EGFR-expressing NMuMG cells were found to form multicellular and highly branched 3D structures (30.18 ± 6.51% of structures were branched; Figure 6a). These branched organoids may represent an exaggerated activation of normal mammary gland branching activities, particularly since EGF stimulation of parental NMuMG cells also generated branched structures that showed a more differentiated phenotype (48.75 ± 4.71% of structures were branched; Figures 6b and 7b). Pharmacological inactivation of either TGF-β or EGFR signaling completely abrogated mammary branching (0% of structures were branched) and was sufficient in restoring normal, hollow acinar development by EGFR-expressing NMuMG cells (Figure 6b). Chemotherapeutic targeting of FAK (PF-271) prevented mammary branching (0% of structures were branched) and acinar hollowing (0% of structures were hollowed; Figure 6b). Finally, although 3D cultures of post-EMT NMuMG cells failed to elicit any branching structures, this system did produce a significant reduction in acinar hollowing (Figure 6c). Collectively, these findings suggest that the selective appearance of these post-EMT cellular aggregates may similarly represent the hyperinvasive spheroids characteristic of metastatic MECs.

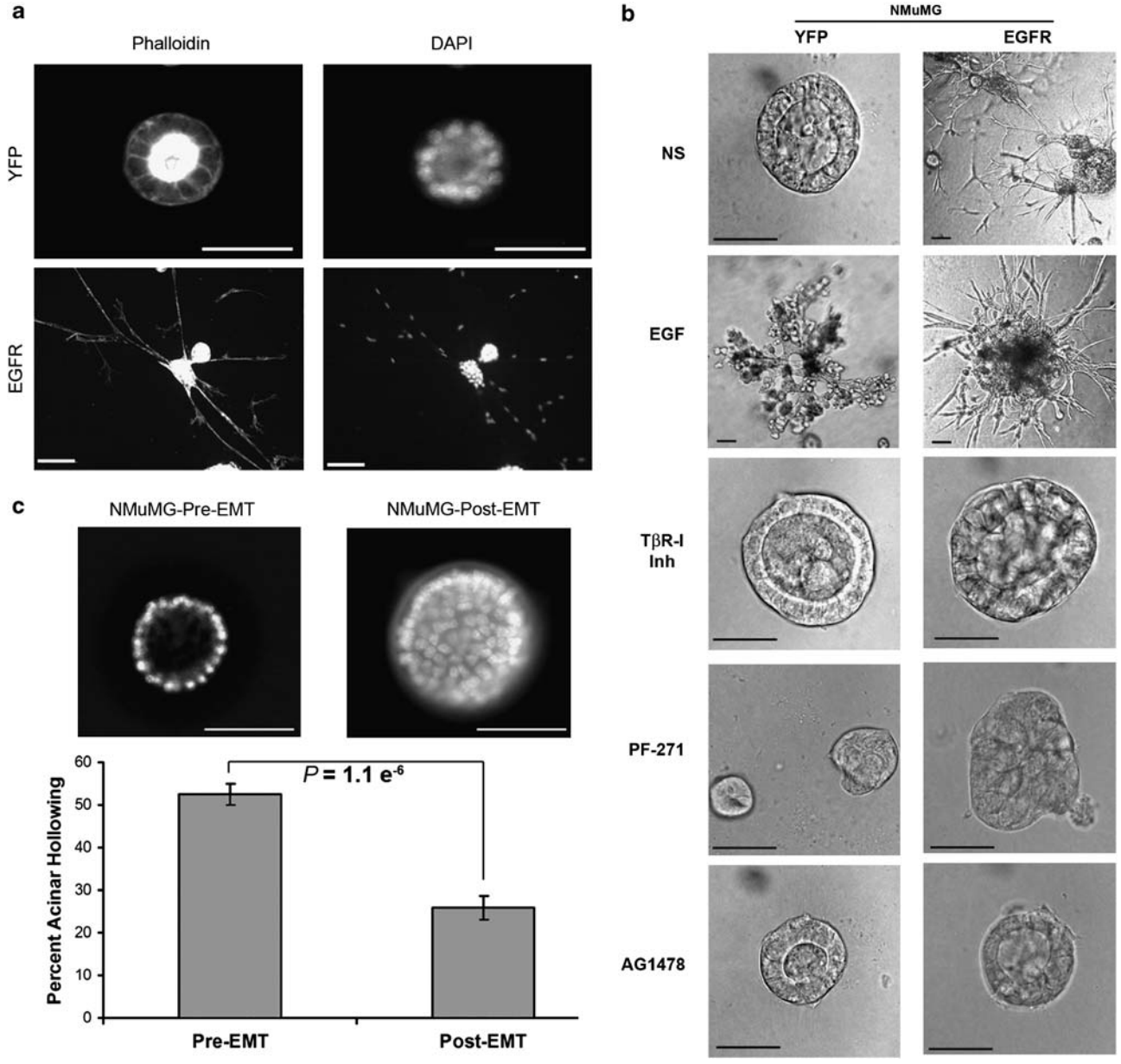


Figure 6 MEC branching induced by EGFR is dependent on TGF-β/FAK signaling. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were propagated for 10 days in 3D-ogranotypic cultures, and subsequently were processed for direct phalloidin and DAPI fluorescence to visualize the actin cytoskeleton and nuclei, respectively. Shown are representative photomicrographs (YFP = × 400; EGFR = × 100) from a single experiment that was performed more than five times with identical results. (**b**) Control (i.e., YFP) and EGFR-expressing cells were propagated as shown in panel (**a**) in the absence (NS) or presence of either (1) EGF (50 ng/ml); (2) the TβR-I inhibitor, SB431542 (TβR-I inh, 10 μM); (3) the FAK inhibitor, PF-562271 (PF-271, 1 μM); or (4) the EGFR inhibitor, AG1478 (1 μM). Shown are representative photomicrographs (small bar = × 100; large bar = × 400) from a single experiment that was performed atleast three times with similar results. (**c**) Parental NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h before their isolation and propagation for 10 days in 3D-organotypic cultures. Afterward, the resulting organoids were stained with DAPI to visualize the nuclei and the percentage of hollowed acini was quantified. Data are the mean (± s.e.; $n = 3$) percent of hollowed acinar structures. Representative acini are shown.

EMT prevents EGF-induced mammary branching and enhances pulmonary tumor growth

We next aimed to determine which 3D morphology (branching or aggregate spheroid) was dominant under EGF-stimulated conditions. Thus, pre- and post-EMT control (YFP) and EGFR-expressing NMuMG cells were propagated in 3D cultures, supplemented with EGF or the EGFR inhibitor, AG. As observed above (Figure 6b), EGF stimulation of control and EGFR-expressing NMuMG cells readily promoted the formation of normal and dysmorphic branching structures, respectively (Figure 7a). More importantly, Figures 7b and c show that TGF-β stimulation of EMT severely blunted the ability of EGF and EGFR to promote

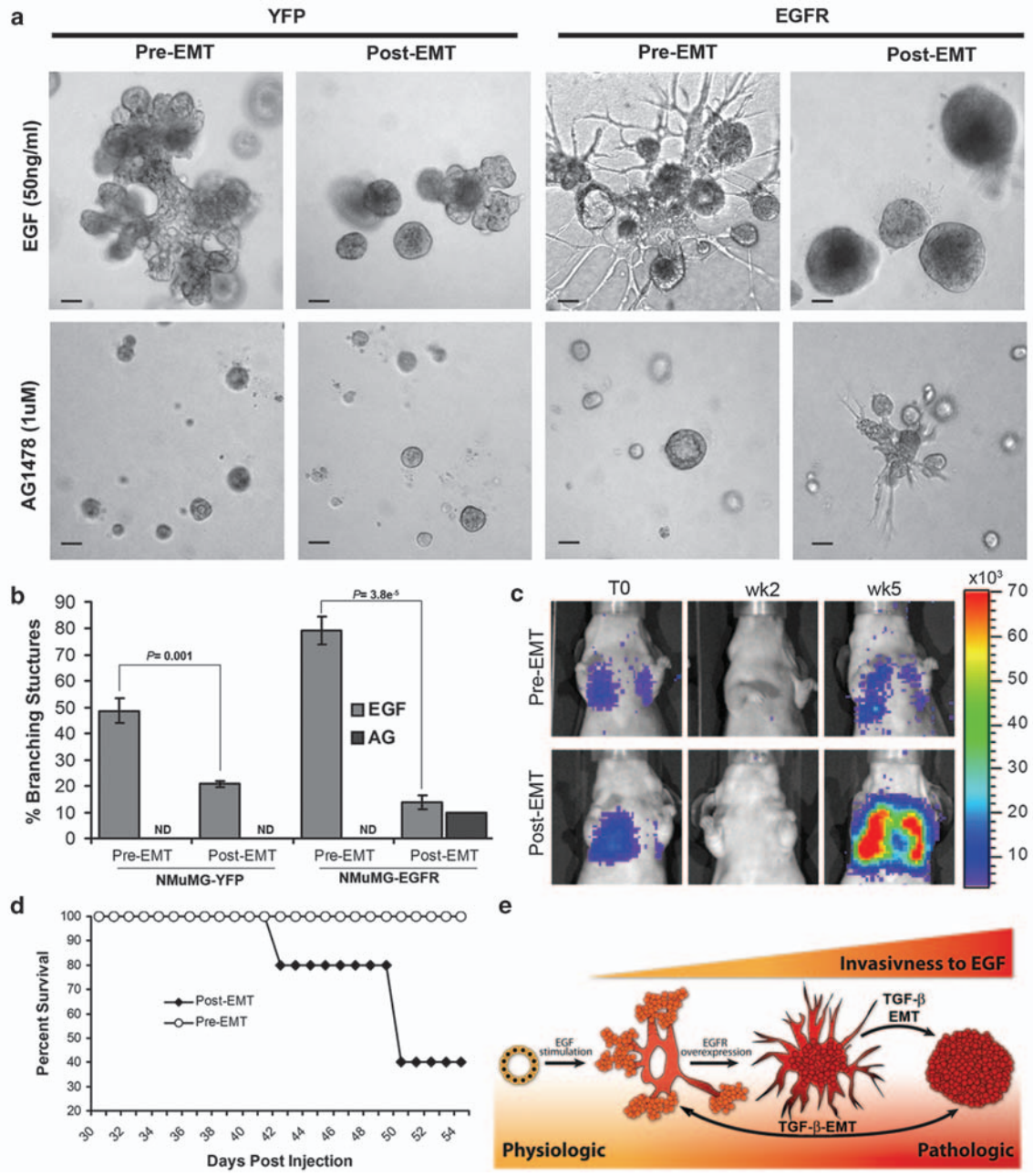


Figure 7 EMT prevents EGF-induced mammary branching and increases pulmonary outgrowth. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h before their isolation and propagation for 10 days in 3D-organotypic cultures supplemented with either EGF (50 ng/ml) or the EGFR antagonist, AG1478 (1 μM) as indicated. Differences in organoid morphology were monitored by phase-contrast microscopy (× 100). (**b**) Alterations in organoid branching were quantified and presented as the mean (± s.e.; $n = 3$) percentage of branched structures. (**c**) NMuMG-EGFR cells were transduced with firefly luciferase, treated with TGF-β1 as described in panel (**a**) and injected into the lateral tail vein of 6-week-old, female, Nu/Nu mice. Shown is a longitudinal study of a representative mouse from each group imaged at the indicated time points ($n = 5$ mice per group). (**d**) A survival curve of mice injected with NMuMG-EGFR cells as in panel (**c**), indicating that induction of EMT in NMuMG-EGFR cells decreases the time in which lethal pulmonary tumor burden is reached. (**e**) Schematic depicts the relationship between MEC invasion, their EMT status induced by TGF-β and their 3D culture morphologies. In particular, EMT stimulated by TGF-β suppressed the branching of developing organoids including that induced by EGF/EGFR and instead resulted in the formation of large, dense spheroids that were hyperinvasive to EGF. This unique invasive morphology and phenotype is metastable (double-sided arrow) in normal mammary epithelial cells and required autocrine TGF-β signaling for its manifestation. In stark contrast, post-EMT invasospheres in mammary carcinoma cells are stabilized in their hyperinvasive phenotype (single-sided arrow), and hence rendered independent of the need for continued TGF-β stimulation. Our findings suggest that these novel invasive spheroids likely represent the post-EMT subpopulation of pathologically invasive and metastatic breast cancer cells.

organoid branching, and instead induced the appearance of large, dense cellular aggregates characteristic of metastatic MECs (Figure 1a). Indeed, induction of EMT enhanced pulmonary tumor growth and decreased the survival rate of mice injected with EGFR-transformed NMuMG cells (Figures 7d and e).

Discussion

The increased ability of TGF-β to induce EMT supports the conversion of TGF-β from restraining tumor formation to encouraging their dissemination to distant secondary sites (Tian and Schiemann, 2009; Wendt *et al*., 2009a). Although TGF-β and EGF ligands have a long-standing pathophysiological association with one another, surprisingly little is known about how these signaling systems cross-talk with one another to impact metastasis. Whereas previous reports suggest that TGF-β transactivates EGFR by liberation of EGF ligands (Thomson *et al*., 2008; Wang *et al*., 2008, 2009), we now show for the first time that TGF-β stimulation of EMT elicits a fundamental change in the coupling of EGFR to its downstream effectors. Furthermore, we show that in 3D-organotypic culture post-EMT MECs manifest as dense cellular aggregates that are characteristic of highly metastatic breast cancer cells. Most importantly, we provide evidence that a two-pronged chemotherapeutic approach that targets FAK in conjunction with EGFR specifically inhibited the oncogenic activities of EGF in these aggressive, post-EMT spheroids.

A key regulatory step during EMT is the loss of E-cadherin expression and activity (Yang and Weinberg, 2008; Thiery *et al*., 2009; Wendt *et al*., 2009a). We observed that EMT induced by TGF-β not only downregulated E-cadherin expression but also prevented its interaction with EGFR, allowing for the formation of EGFR/TβR-II complexes that stabilized EGFR at the cell surface of post-EMT MECs. Along these lines, TGF-β has recently been shown to diminish autocrine EGF-ligand production (Thomson *et al*., 2008). Indeed, under these conditions we show that EGFR exhibits heightened availability and responsiveness to paracrine EGF, a signal initiated *in vivo* by reactive tumor stroma (Wyckoff *et al*., 2004). Accordingly, we observed elevated EGFR expression to transform NMuMG cells effectively, as well as to induce their delocalization and downregulation of E-cadherin expression.

Although the overall levels of EGFR were consistently upregulated in parental NMuMG cells undergoing EMT stimulated by TGF-β, we did identify another highly 'mesenchymal-type' population of post-EMT NMuMG cells that lacked EGFR expression. Indeed, a recent study found that prolonged EMT induced by TGF-β (21 days) could elicit cellular switching of receptor tyrosine kinases from a predominantly EGFR-dependent phenotype to one that is dependent on the receptors for fibroblast growth factor or platelet-derived growth factor (Thomson *et al*., 2008). Whether a similar 'switching' mechanism is transpiring in these highly mesenchymal and EGFR-deficient MECs remains to be determined definitively. However, using isogenic cell lines derived from nonmetastatic 67NR cells, we do show for the first time that highly mesenchymal appearing cells cultured on plastic, manifest as dense, cellular spheroids under 3D culture conditions. Indeed, our findings may offer a novel explanation as to why science and medicine routinely fail to identify EMT in human tumors, particularly in metastatic tumor tissue growing in compliant environments like the lungs. Should this prove to be a universal phenomenon, it stands to reason that determining the molecular mechanisms whereby these novel 'invasospheres' undergo invasion seems particularly meritus. In fact, our preliminary analyses suggest that 'invasospheres' can traverse synthetic basement membranes as a single, cooperating unit (data not shown), which contrasts sharply with the initiation of single-cell-based programs of mesenchymal or amoeboid invasion (Friedl and Brocker, 2000).

In addition to our identification of a unique mode of MEC invasion, our *in vitro* protocol of first eliciting EMT in MECs, followed by their subsequent dissociation and subculture in 3D-organotypic systems, in many respects, recapitulates the steps of breast cancer cell metastasis—that is, primary carcinoma cells undergo EMT, exit the primary tumor, survive anoikis in the circulation and finally invade and grow out in a new compliant microenvironment, such as the lung. Indeed, the propagation of breast cancer cells in 3D cultures has been proposed as a model that strongly recapitulates the outgrowth of breast cancer cells in the lung (Shibue and Weinberg, 2009). As depicted in Figure 7e, we propose that breast cancer cells that have undergone EMT abandon their inherent branching program to instead acquire an 'invasosphere' morphology that enables these structures to form large, undifferentiated metastases at distant locales. This model is further supported by our data showing that induction of EMT not only increases primary tumor exit, but also enhances the outgrowth of pulmonary tumors established by tail vein injection.

In attempting to translate our findings to the clinic, it remains to be determined whether the recent inclusion of elevated EGFR expression to the basal-like/triple-negative gene signatures is indicative of the stabilized EGFR phenotype we observed in post-EMT MECs (Tischkowitz *et al*., 2007). However, EMT stimulated by TGF-β does result in the expression of Snail, which subsequently promotes the downregulation of estrogen receptor-α (Dhasarathy *et al*., 2007). Thus, our findings completely support a model in which distinct subpopulations of breast cancer cells undergo EMT, thereby contributing to the development of a gene signature that is indicative of poor clinical outcomes. More importantly, our findings indicate that chemotherapeutic targeting of the TGF-β signaling system that results in its pan-antagonism may offer little-to-no therapeutic benefit in post-EMT MECs that exhibit elevated EGFR expression. Moreover, our analyses also suggest that

simultaneous targeting of FAK in conjunction with EGFR may provide a highly effective means to inhibit these hyperinvasive, post-EMT 'invasospheres.' Experiments designed to test this clinically relevant hypothesis are currently ongoing.

Materials and methods

Cell lines and retroviral reagents

Normal NMuMG cells were obtained from ATCC (Manassas, VA, USA) and cultured as described previously (Galliher and Schiemann, 2007), as was the construction of NMuMG cells that lacked FAK expression (Wendt and Schiemann, 2009). NMuMG cells were engineered to express elevated levels of EGFR by their transduction with VSVG retroviral particles that encoded for either YFP or EGFR (pBabe-YFP or pBabe-EGFR, and provided by Dr Alexander Sorkin (University of Pittsburg, Pittsburg, PA, USA). Afterward, polyclonal populations of transduced NMuMG cells were isolated by puromycin selection (5 μg/ml) for 14 days. In addition, NMuMG cells were also transduced with murine ecotropic viral particles that encoded for either GFP or PyMT (pMSCV-IRES-GFP or pMIG-PyMT-IRES-GFP) and transduced NMuMG cells expressing GFP were isolated by flow cytometry as described previously (Galliher and Schiemann, 2007). The human MCF10A cell derivates T1k and Ca1h were cultured as described previously (Wendt and Schiemann, 2009), as were the conditions necessary to propagate the human MCF-7 (Micalizzi *et al*., 2009), MDA-MB-231 (Wendt *et al*., 2008), 4T1, 4T07 and 67NR (Wendt *et al*., 2009b) cells. Throughout the study, we refer to post-EMT NMuMG cells as those that were stimulated on plastic for 48 h with TGF-β1 (5 ng/ml), whereas post-EMT 4T07 cells were obtained following 3 weeks of continuous TGF-β stimulation through several passages. In all cases, pre-EMT cells represent their unstimulated counterparts.

Cell signaling, immunoblotting and immunoprecipitation assays

To monitor the activation status of TGF-β and EGF effectors, pre-EMT and post-EMT MECs were serum deprived (0.5% fetal bovine serum) for 6 h before their stimulation with TGF-β1 (5 ng/ml) or EGF (50 ng/ml) for varying times as indicated. Afterward, clarified whole-cell extracts were prepared as described previously (Wendt and Schiemann, 2009; Wendt *et al*., 2009b) and subjected to immunoblot analyses using the primary antibodies listed in Supplementary Materials.

In some experiments, confluent cultures of control (YFP) or EGFR-expressing NMuMG cells were treated for 24 h with TGF-β1 (5 ng/ml) before isolating EGFR complexes using previously described immunoprecipitation conditions (Galliher and Schiemann, 2006). The resulting EGFR immunocomplexes (antiEGFR antibodies: 1:100; Cell Signaling, Danvers, MA, USA) were immunoblotted with antibodies against TβR-II, FAK, E-cadherin and β-actin as described above.

Cell biological assays

The inhibition of DNA synthesis by TGF-β was determined using [^{3}H]thymidine incorporation assays as described previously (Wendt *et al*., 2009b). To monitor the ability of EGF to induce DNA synthesis, pre- and post-EMT NMuMG cells were subcultured onto 24-well plates (20 000 cells per well) and allowed to adhere for 4 h, at which point the growth media were removed and replaced with serum-free media supplemented with TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines. After 24 h, the cells were treated with [^{3}H]thymidine, whose incorporation into cellular DNA was quantified as described previously (Wendt *et al*., 2009b).

The ability of EGF (50 ng/ml) or serum (2%) to alter the invasion of pre- or post-EMT cells was analyzed using a modified Boyden Chamber assay (50 000 cells per well) as described previously (Wendt and Schiemann, 2009), in which all values are normalized to a serum-free control set to 100%. In some experiments, the following pharmacological inhibitors were included with EGF throughout the assay: (1) the TβR-I inhibitor, SB431542 (10 μg/ml; EMD Biosciences, San Diego, CA, USA); (2) the FAK inhibitor, PF-562271 (1 μM; Pfizer, Groton, CT, USA); (3) the p38 MAPK, SB230580 (10 μM; EMD Biosciences) or (4) the EGFR inhibitor, AG1478 (1 μM; Cayman Chemical, Ann Arbor, MI, USA).

The ability of TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines to alter the actin cytoskeleton in NMuMG cells was visualized using TRITC-conjugated phalloidin (0.25 μM; Invitrogen, Carlsbad, CA, USA) as described previously (Galliher and Schiemann, 2006). In some experiments, alterations in the expression and/or localization of E-cadherin and EGFR was monitored by indirect immunofluorescence where the cells were (1) fixed in 4% paraformaldehyde; (2) permeablized in 0.1% Triton X-100 and (3) stained with anti-E-cadherin (1:250) or EGFR (1:100) antibodies. Afterward, E-cadherin immunocomplexes were visualized using biotin-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in combination with Texas-Red-labeled avidin (Vector Laboratories, Burlingame, CA, USA), whereas EGFR immunocomplexes were visualized simultaneously using FITC-labeled donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch).

EGFR expression analyses

We also monitored alterations in EGFR expression using real-time PCR as described in Supplementary Methods. In addition, pre- and post-EMT NMuMG (1×10^6) cells were resuspended in enzyme-free cell disassociation buffer (Invitrogen), and subsequently incubated in phosphate-buffered saline supplemented with 0.1% bovine serum albumin, to which diluent or Alexa 488-labled EGF (50 ng/ml; Invitrogen) was added. The cells were labeled in the dark and kept on ice for 45 min, then were washed extensively with ice-cold phosphate-buffered saline, fixed in 1% paraformaldehyde and analyzed by flow cytometry.

Tumor growth analyses

Control (YFP), PyMT- and EGFR-expressing NMuMG (2×10^6) cells were resuspended in sterile phosphate-buffered saline (50 μl) that contained 5% Matrigel (BD Biosciences, San Jose, CA, USA), and subsequently injected into the mammary fat pad of 6-week-old female Nu/Nu mice (Charles River, Wilmington, MA, USA). Tumor growth was assessed by calculating primary tumor volumes using digital calipers and the following equation: Tumor Volume $= (x^2)(y)(0.5)$, where x is the tumor width and y the tumor length. Alternatively, pre- and post-EMT NmuMG-EGFR (4×10^5) cells were injected into the lateral tail vein of female Nu/Nu mice and pulmonary tumor growth was monitored by bioluminecent analysis using the Xenogen IVIS200 imager (Xenogen, Alameda, CA, USA) as described previously (Wendt and Schiemann, 2009). Luciferase expressing 4T1 (1×10^4), 4T07 (1×10^5) or 67NR (1×10^5) cells were resuspended in sterile phosphate-buffered saline (50 μl) and engrafted onto the mammary fat pad of 6-week-old female Balb/C mice and pulmonary metastasis was monitored as above. All animal studies were performed

according to animal protocol procedures approved by the Institutional Animal Care and Use Committee of the University of Colorado.

3D-organotypic assays

Pre- and post-EMT NMuMG or 4T07 (1×10^4) cells were resuspended in growth media supplemented with 5% Cultrex (R&D Systems, Minneapolis, MN, USA), and subsequently seeded in 48-well plates onto Cultrex cushions (150 μl per well). In some experiments, acinar development was allowed to transpire in the continued presence of EGF (50 ng/ml), SB431542 (10 μg/ml), PF-562271 (1 μM) or AG (1 μM) as indicated. All NMuMG 3D-organotypic cultures were fed on day 7 and were allowed to develop for an additional 3 days, at which point the extent of their hollowing and branching was assessed by phase-contrast microscopy and quantified by three individuals who were masked to the culture conditions. Where indicated, acinar development also transpired on eight-well chamber slides, which were processed on day 10 for TRITC-conjugated phalloidin (0.25 μM; Invitrogen) and 4′,6-diamidino-2-phenylindole staining as described above. All 4T1, 4T07 and 67NR 3D-organotypic cultures were grown for 5 days and similarly assayed for branching as described above.

Statistical analyses

Statistical values were defined using an unpaired Student's *t*-test, in which *P*-value <0.05 was considered significant. *P*-values for all experiments analyzed are indicated.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Pfizer for graciously providing the novel small molecule FAK inhibitor, PF-562271. We also thank Dr Alexander D Sorkin for providing the retroviral construct that encoded human EGFR. WPS was supported in part by grants from the National Institutes of Health (CA129359) and the Susan G Komen for the Cure Foundation (BCTR0706967) and the Department of Defense (BC084561), whereas MKW was supported by a fellowship from the American Cancer Society (PF-09-120-01).

References

Aslakson CJ, Miller FR. (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* **52**: 1399–1405.

Barr S, Thomson S, Buck E, Russo S, Petti F, Sujka-Kwok I *et al*. (2008). Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. *Clin Exp Metastasis* **25**: 685–693.

Bremm A, Walch A, Fuchs M, Mages J, Duyster J, Keller G *et al*. (2008). Enhanced activation of epidermal growth factor receptor caused by tumor-derived E-cadherin mutations. *Cancer Res* **68**: 707–714.

Buck E, Eyzaguirre A, Barr S, Thompson S, Sennello R, Young D *et al*. (2007). Loss of homotypic cell adhesion by epithelial–mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Mol Cancer Ther* **6**: 532–541.

Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A *et al*. (2008). TGF-β-induced EMT requires focal adhesion kinase (FAK) signaling. *Exp Cell Res* **314**: 143–152.

Cowin P, Welch DR. (2007). Breast cancer progression: controversies and consensus in the molecular mechanisms of metastasis and EMT. *J Mammary Gland Biol Neoplasia* **12**: 99–102.

DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N *et al*. (2009). CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* **16**: 91–102.

Dhasarathy A, Kajita M, Wade PA. (2007). The transcription factor Snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor α. *Mol Endocrinol* **21**: 2907–2918.

Ding Q, Gladson CL, Wu H, Hayasaka H, Olman MA. (2008). FAK-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. *J Biol Chem* **283**: 26839–26849.

Friedl P, Brocker EB. (2000). The biology of cell locomotion within three-dimensional extracellular matrix. *Cell Mol Life Sci* **57**: 41–64.

Galliher AJ, Schiemann WP. (2006). β3 integrin and Src facilitate TGF-β mediated induction of epithelial–mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* **8**: R42.

Galliher AJ, Schiemann WP. (2007). Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer Res* **67**: 3752–3758.

Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED *et al*. (2007). Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. *J Cell Physiol* **213**: 374–383.

Joo CK, Kim HS, Park JY, Seomun Y, Son MJ, Kim JT. (2007). Ligand release-independent transactivation of epidermal growth factor receptor by TGF-β involves multiple signaling pathways. *Oncogene* **27**: 614–628.

Kalluri R, Weinberg RA. (2009). The basics of epithelial–mesenchymal transition. *J Clin Invest* **119**: 1420–1428.

Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT *et al*. (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* **1**: 84–96.

Micalizzi DS, Christensen KL, Jedlicka P, Coletta RD, Baron AE, Harrell JC *et al*. (2009). The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial–mesenchymal transition and metastasis in mice through increasing TGF-β signaling. *J Clin Invest* **119**: 2678–2690.

Miettinen PJ, Ebner R, Lopez AR, Derynck R. (1994). TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* **127**: 2021–2036.

Mitra SK, Lim ST, Chi A, Schlaepfer DD. (2006). Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. *Oncogene* **25**: 4429–4440.

Moustakas A, Heldin CH. (2007). Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* **98**: 1512–1520.

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR *et al*. (2008). TGF-β primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133**: 66–77.

Park CC, Bissell MJ, Barcellos-Hoff MH. (2000). The influence of the microenvironment on the malignant phenotype. *Mol Med Today* **6**: 324–329.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA *et al*. (2000). Molecular portraits of human breast tumours. *Nature* **406**: 747–752.

Roberts WG, Ung E, Whalen P, Cooper B, Hulford C, Autry C *et al*. (2008). Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res* **68**: 1935–1944.

Saha D, Datta PK, Sheng H, Morrow JD, Wada M, Moses HL *et al*. (1999). Synergistic induction of cyclooxygenase-2 by TGF-β1 and epidermal growth factor inhibits apoptosis in epithelial cells. *Neoplasia* **1**: 508–517.

Salomon DS, Perroteau I, Kidwell WR, Tam J, Derynck R. (1987). Loss of growth responsiveness to epidermal growth factor and enhanced production of α-transforming growth factors in Ras-transformed mouse mammary epithelial cells. *J Cell Physiol* **130**: 397–409.

Shibue T, Weinberg RA. (2009). Integrin β1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci USA* **106**: 10290–10295.

Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH *et al*. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* **2**: 249–256.

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H *et al*. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* **98**: 10869–10874.

Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A *et al*. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* **100**: 8418–8423.

Takahashi K, Suzuki K. (1996). Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell–cell adhesion. *Exp Cell Res* **226**: 214–222.

Tarin D, Thompson EW, Newgreen DF. (2005). The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* **65**: 5996–6000.

Thiery JP, Acloque H, Huang RY, Nieto MA. (2009). Epithelial–mesenchymal transitions in development and disease. *Cell* **139**: 871–890.

Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N *et al*. (2005). Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res* **65**: 9455–9462.

Thomson S, Petti F, Sujka-Kwok I, Epstein D, Haley JD. (2008). Kinase switching in mesenchymal-like non-small cell lung cancer lines contributes to EGFR inhibitor resistance through pathway redundancy. *Clin Exp Metastasis* **25**: 843–854.

Tian M, Schiemann WP. (2009). The TGF-β paradox in human cancer: an update. *Future Oncol* **5**: 259–271.

Tischkowitz M, Brunet J-S, Begin L, Huntsman D, Cheang M, Akslen L *et al*. (2007). Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC Cancer* **7**: 134.

Wang SE, Xiang B, Guix M, Olivares MG, Parker J, Chung CH *et al*. (2008). TGF-β engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to Trastuzumab. *Mol Cell Biol* **28**: 5605–5620.

Wang SE, Xiang B, Zent R, Quaranta V, Pozzi A, Arteaga CL. (2009). TGF-β induces clustering of HER2 and integrins by activating Src-focal adhesion kinase and receptor association to the cytoskeleton. *Cancer Res* **69**: 475–482.

Wendt M, Schiemann W. (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. *Breast Cancer Res* **11**: R68.

Wendt MK, Allington TM, Schiemann WP. (2009a). Mechanisms of the epithelial–mesenchymal transition by TGF-β. *Future Oncol* **5**: 1145–1168.

Wendt MK, Cooper AN, Dwinell MB. (2008). Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. *Oncogene* **27**: 1461–1471.

Wendt MK, Smith JA, Schiemann WP. (2009b). p130Cas is required for mammary tumor growth and transforming growth factor-beta (TGF-beta)-mediated metastasis through regulation of Smad2/3 activity. *J Biol Chem* **284**: 34145–34156.

Wilding J, Vousden KH, Soutter WP, McCrea PD, Del Buono R, Pignatelli M. (1996). E-cadherin transfection down-regulates the epidermal growth factor receptor and reverses the invasive phenotype of human papilloma virus-transfected deratinocytes. *Cancer Res* **56**: 5285–5292.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER *et al*. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* **64**: 7022–7029.

Wyckoff JB, Segall JE, Condeelis JS. (2000). The collection of the motile population of cells from a living tumor. *Cancer Res* **60**: 5401–5404.

Yang J, Weinberg RA. (2008). Epithelial–mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* **14**: 818–829.

Zavadil J, Bottinger EP. (2005). TGF-β and epithelial-to-mesenchymal transitions. *Oncogene* **24**: 5764–5774.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Cellular Signalling 23 (2011) 951–962

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Review

Transforming growth factor-β and the hallmarks of cancer

Maozhen Tian [a], Jason R. Neil [b], William P. Schiemann [a,*]

[a] Division of General Medical Sciences–Oncology, Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106, United States
[b] Department of Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

ARTICLE INFO

Article history:
Received 31 August 2010
Accepted 1 October 2010
Available online 6 November 2010

Keywords:
Angiogenesis
Apoptosis
Epithelial–mesenchymal transition
Immunosurveillance
Invasion
Metastasis
Signaling transduction
Tumor suppressor

ABSTRACT

Tumorigenesis is in many respects a process of dysregulated cellular evolution that drives malignant cells to acquire six phenotypic hallmarks of cancer, including their ability to proliferate and replicate autonomously, to resist cytostatic and apoptotic signals, and to induce tissue invasion, metastasis, and angiogenesis. Transforming growth factor-β (TGF-β) is a potent pleiotropic cytokine that functions as a formidable barrier to the development of cancer hallmarks in normal cells and tissues. Paradoxically, tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant cells overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the acquisition of cancer hallmarks by developing and progressing human cancers. Here we review the molecular and cellular mechanisms that underlie the ability of TGF-β to mediate tumor suppression in normal cells, and conversely, to facilitate cancer progression and disease dissemination in malignant cells.

© 2010 Elsevier Inc. All rights reserved.

Contents

1. Introduction . . . 951
2. TGF-β signaling system . . . 952
 2.1. Canonical TGF-β signaling . . . 952
 2.2. Noncanonical TGF-β signaling . . . 953
 2.3. Mutated TGF-β signaling components and cancer . . . 953
3. TGF-β and dysregulated cell proliferation . . . 954
 3.1. Resistance to TGF-β-mediated cytostasis . . . 954
 3.2. Cell cycle progression induced by TGF-β . . . 955
4. TGF-β and cancer cell motility . . . 955
 4.1. TGF-β and epithelial-mesenchymal transition . . . 955
 4.2. TGF-β, invasion, and metastasis . . . 955
5. TGF-β and tumor angiogenesis . . . 956
6. TGF-β and cancer cell survival . . . 957
7. TGF-β and dysregulated immunosurveillance . . . 958
8. TGF-β and cancer cell immortality . . . 959
9. Concluding remarks . . . 959
Acknowledgements . . . 959
References . . . 959

1. Introduction

Since the inception of the National Cancer Act of 1971, science and medicine have waged an intense battle aimed at conquering cancer. Although considerable progress has been achieved in terms of our understanding of the molecular mechanisms that underlie cancer development and progression, cancer itself remains a significant

* Corresponding author. Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, Room 2131, 2103 Cornell Road, Cleveland, OH 44106, United States. Tel.: +1 216 368 5763; fax: +1 216 368 1166.
E-mail address: William.Schiemann@case.edu (W.P. Schiemann).

0898-6568/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2010.10.015

health concern and burden in the United States. Indeed, 1 in 4 deaths in the United States results from cancer, which also is the leading cause of death in individuals younger than 65 years of age. Despite these grim statistics, overall cancer incidence and mortality rates have begun to decline over the last decade [1]. Continuing along this positive trend will require the development of new diagnostic and chemotherapeutic regimens, as well as the elucidation of new knowledge of how cancer cells acquire the six essential phenotypes, or hallmarks, necessary to become malignant. Included in this phenotypic list is the ability of cancer cells to (i) grow autonomously; (ii) disregard cytostatic signals; (iii) ignore apoptotic signals; (iv) stimulate angiogenesis; (v) invade and metastasize; and (vi) become immortal [2]. Failure by developing neoplasms to acquire each of these phenotypes prevents their conversion to aggressive states, suggesting that these cancer hallmarks represent various rate-limiting steps during malignant development. As such, pharmacological targeting of cancer hallmarks, both singly and in combination, may offer new inroads to effectively treat the development and dissemination of human malignancies.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates mammalian development, differentiation, and homeostasis. It is also a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. Aberrations in the TGF-β signaling pathway bring about resistance to TGF-β-mediated growth arrest, and thus give rise to human malignances [3–5]. Paradoxically, these genetic and epigenetic aberrations conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, survival, and metastasis. Although the molecular details underlying the oncogenic activities of TGF-β remain to be fully elucidated, recent evidence implicates TGF-β as a principle player involved in regulating the acquisition of cancer hallmarks by malignant cells [3–5]. This review focuses on the complex roles played by TGF-β during cancer progression, particularly its ability to regulate the development and progression of malignant cells through each of the individual hallmarks of cancer (Fig. 1).

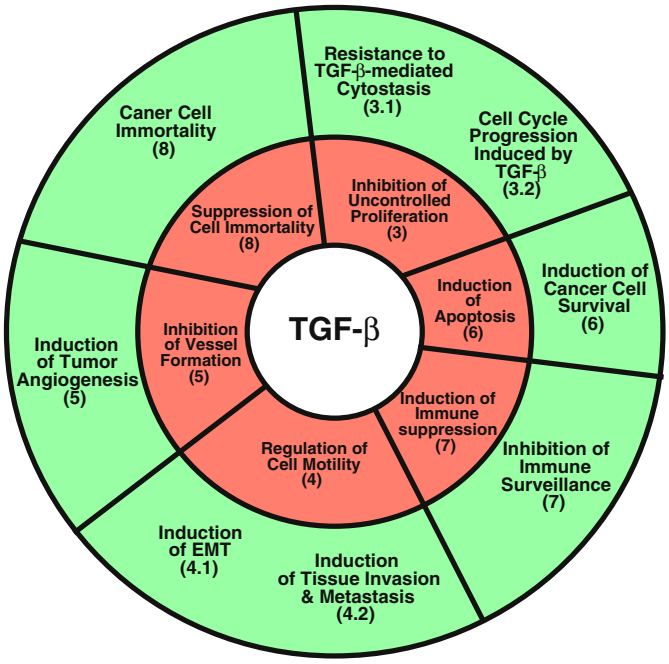


Fig. 1. TGF-β and the hallmarks of cancer. Tumorigenesis converts TGF-β from a powerful tumor suppressor to a lethal tumor promoter that enables evolving cancer cells to acquire the 6 phenotypic traits or hallmarks of cancer. In normal epithelial, endothelial, and hematopoietic cells, TGF-β suppresses the formation of cancer hallmarks (red wheel). However, genetic or epigenetic events conspire to inactivate the tumor suppressing functions of TGF-β, thereby conferring oncogenic behaviors upon this multifunctional cytokine and its ability to stimulate the development of cancer hallmarks (green wheel). Bracketed numbers correspond to individual chapter subheadings that describe the indicated functions of TGF-β.

2. TGF-β signaling system

2.1. Canonical TGF-β signaling

The diverse biological activities of TGF-β are mediated through its stimulation of a deceptively simple signaling system that at its core is comprised of three TGF-β receptors, types I (TβR-I), II (TβR-II), and III (TβR-III), and three latent transcription factors, Smads 2, 3, and 4 (Fig. 2; [4–8]). The initiation of transmembrane signaling by TGF-β takes place upon its binding to TβR-III, which then presents TGF-β to TβR-II [9]. This ligand presentation mechanism is especially important for TGF-β2, which only interacts with TβR-II when bound to TβR-III [10]. It should be noted that the requirement for TβR-III in propagating messages by TGF-β2 is not absolute, particularly in cells that express a TβR-II variant that binds TGF-β2 independently of TβR-II expression [11]. In contrast, TGF-β1 and TGF-β3 both readily bind to TβR-II and induce intracellular signaling in the absence or presence of TβR-III. The differential requirements of individual TGF-β isoforms for TβR-III, coupled with the spatiotemporal differences observed in their expression and activation patterns [12], likely underlies the more than 30 distinct phenotypes observed in TGF-β1-, TGF-β2-, and TGF-β3-deficient mice [13]. Regardless of its mode of activation, ligand-bound TβR-II subsequently associates with and binds to TβR-I. Both TβR-I and TβR-II house intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains, and the conversion of these ligand:receptor ternary complexes from their inactive to active states requires TβR-II to transphosphorylate TβR-I, thereby stimulating its protein kinase activity [14]. Activated TβR-I in turn stimulates Smads 2 and 3 by phosphorylating these latent transcriptional factors at their C-terminal *SXS* motif. Phosphorylated Smads 2 and 3 undergo a rapid conformational change that facilitates their association with common Smad, Smad4. This conformational change also unveils cryptic nuclear localization sequences in Smads 2, 3, and 4 that promotes hetero-complex accumulation in the nucleus [4–8]. Access to the nucleus enables Smad2/3/4 complexes to interact with an expanding list of transcriptional co-activators and -repressors that collectively alter cell fates through the coordinated induction and repression of TGF-β-responsive genes in a cell- and context-specific manner [4–8].

Signaling through this canonical TGF-β pathway is regulated and fine-tuned *via* multiple mechanisms that span all cellular compartments. For instance, several adapter/anchoring proteins such as SARA [15], Hgs [16], and Dab2 [17] bind Smad2/3 and facilitate their phosphorylation and activation by TβR-I. This phosphotransferase reaction can be negated by expression of the inhibitory Smad, Smad7, which (i) interacts physically with TβR-I and occludes its access to Smad2/3 [18–20], and (ii) recruits the E3 ligase Smurf1/2 to facilitate TGF-β receptor ubiquitination and degradation [21,22]. Moreover, the ability of Smad7 to inhibit TGF-β signaling is augmented by its interaction with STRAP [23], and conversely, is attenuated by its association with either AMSH2 [24] or Arkadia [25–27].

The cellular response to TGF-β also is fine-tuned by the continual nucleocytoplasmic shuttling of Smad2/3, which facilitates their ability to sense and respond rapidly to fluctuations in TGF-β receptor activity [28,29]. Moreover, regulated phosphorylation of Smad2/3 linker regions is mediated by a variety of protein kinases, including MAP kinases (*i.e.,* ERK1/2 [30], JNK [31], p38 MAPK [32,33]), Ca^{++}-calmodulin kinase II [34], casein kinase I-ε [35,36], and CDKs 2 and 4 [37], and is readily reversed by SCP1/2/3-mediated dephosphorylation of Smad2/3 linker regions [38]. At present, the precise role of linker phosphorylation in regulating the biology and pathology of TGF-β action remains to be fully clarified. Similar ambiguity exists concerning the role of sumoylation in regulating the function of Smad4, whose transcriptional activity can be strengthened or

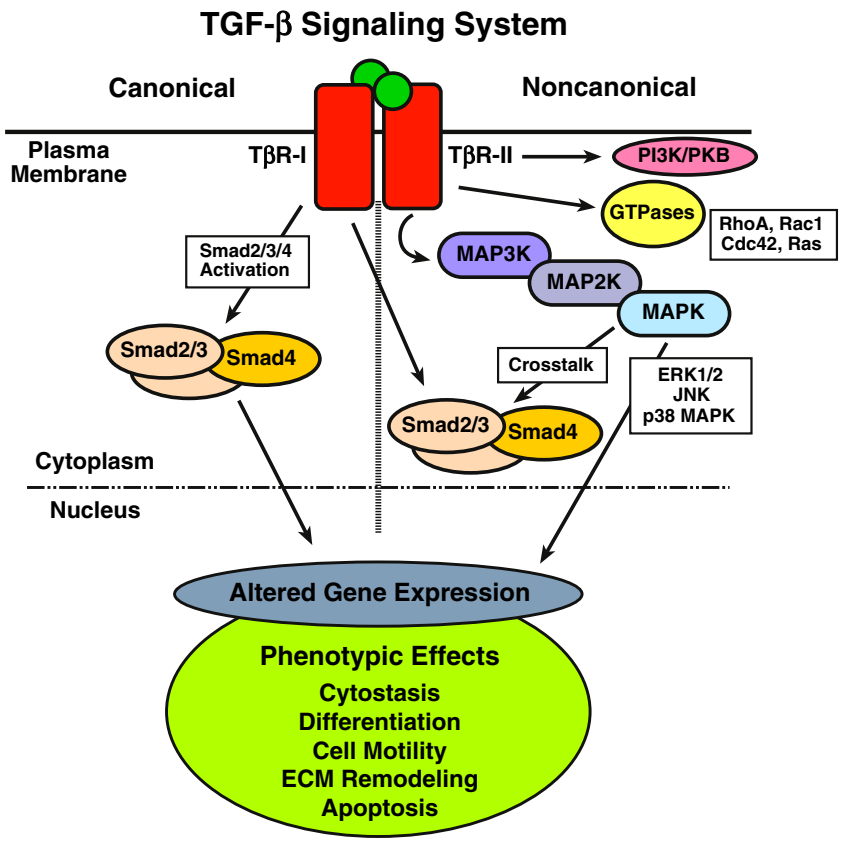


Fig. 2. Canonical and noncanonical TGF-β signaling. TGF-β stimulates responsive cells by binding and activating two transmembrane Ser/Thr protein kinase receptors termed, TGF-β type I (TβR-I) and type II (TβR-II). Activation of these ligand:receptor ternary complexes requires TβR-II to transphosphorylate TβR-I, which phosphorylates and activates Smad2/3. Once activated, Smad2/3 form heterocomplexes with Smad4, which collectively translocate to the nucleus to mediate canonical signaling events by TGF-β (left panel). Noncanonical TGF-β signaling takes place through the ability of TGF-β to stimulate MAP kinases, small GTPases, and PI3K/AKT, and to inhibit NF-κB. Altered coupling of TGF-β to its canonical and noncanonical effector pathways contributes to the development of oncogenic signaling by TGF-β. See text for specific details of TGF-β signaling in normal and malignant cells.

weakened by sumoylation in a promoter-dependent manner [39–43]. Finally, termination of Smad2/3/4 signaling takes place primarily through three distinct mechanisms. First, Smad2/3 are rapidly dephosphorylated and inactivated by the nuclear phosphatase PPM1A [44]. Second, Smad2/3/4 undergo polyubiquitination by the E3 ligases Smurf1, Smurf2, and SCF/Roc1 [45–49], leading to their proteosomal degradation. And third, Smad4 activity is suppressed by Ecto/Tifγ, which monoubiquitinates Smad4 on Lys519 and prevents its association with phosphorylated Smad2 [50]. This inhibitory event is readily reversed by FAM/USP9x, which deubiquitinates Smad4 and restores its responsiveness to TGF-β [50].

2.2. Noncanonical TGF-β signaling

Besides its ability to stimulate the canonical Smad2/3 pathway, TGF-β also alters cell behavior through its activation of Smad2/3-independent signaling systems. Included in this growing list of noncanonical effector molecules stimulated by TGF-β are (i) the MAP kinases ERK1/ERK2, p38 MAPK, and JNK; (ii) the growth and survival kinases PI3K, AKT/PKB, and mTOR; and the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 [51–56]. Additionally, activation of the NF-κB signaling system typically is repressed by TGF-β in normal epithelial cells [57]. Recent evidence also implicates TGF-β in mediating the activation of a number of protein tyrosine kinases, including FAK [58,59], Src [60–63], and Abl [60–62], particularly in mesenchymal or dedifferentiated epithelial cells. Importantly, amplified activation of these noncanonical TGF-β signaling components has been shown to override the normal cellular homeostatic mechanisms governed by Smad2/3 in a manner that figures prominently in the development of human cancers. Precisely how TGF-β couples to the activation of these noncanonical effector systems remains unknown, as is the manner in which their activities becomes dysregulated in response to TGF-β in developing and progressing human cancers. Recent studies linking the inappropriate and/or aberrant activation of alterative TGF-β signaling systems to the acquisition of various cancer hallmarks in developing neoplasms will be presented in the following sections.

2.3. Mutated TGF-β signaling components and cancer

Initial studies aimed at establishing how tumorigenesis negates the cytostatic and tumor suppressing activities of TGF-β were focused primarily on monitoring the expression, mutation, and/or activation status of various TGF-β signaling components. Indeed, considering the fact that TGF-β is a principle player operant in preventing the uncontrolled growth of epithelial cells, and that ~90% of all human malignancies derive from epithelial cell origins [64], it should come as no surprise to learn that components of the TGF-β signaling system are indeed subjected to frequent mutational inactivation. Moreover, many of these genetic defects are heritable and predispose affected individuals to develop cancer [65,66]. This scenario is especially evident in colon cancers that exhibit microsatellite instability, which produces frameshift mutations in *TβR-II* and the synthesis of nonfunctional TβR-II proteins that elicit complete cellular insensitivity to TGF-β [67–69]. Clinically, patients with defective TβR-II proteins present with significantly more polyps and preneoplastic lesions than do their TGF-β-responsive counterparts [70]. However, in an interesting twist of fate, patients housing these TβR-II mutants possess a better overall survival rate due to their failure to progress to aggressive, metastatic disease [71]. As will be discussed later, these

findings point to an essential role for TGF-β signaling in promoting cancer progression and the acquisition of metastatic phenotypes, particularly in late stage cancers that garner a selective advantage by maintaining their ability to respond to TGF-β. In addition, TβR-II defects also are observed in cancers of the stomach, prostate, breast, lung, liver, and pancreas, and in some hematological malignancies (see [3,4,72]).

Likewise, diminished expression and/or activity of TβR-I has been correlated with the development of TGF-β resistance in cancers of the colon, pancreas, ovary, breast, cervix, head and neck, and in T and B cell leukemias [3,72–75]. As a group, TβR-I mutations tend to partition into two general classes, namely those that target its signal sequence [75–78s], and those that target its protein kinase domain [79,80]. Interestingly, an inactivating Ser387Tyr mutation in TβR-I was observed to be more prevalent in metastatic lesions as compared to their corresponding primary tumors, suggesting that this mutation occurs predominantly in late stage cancers where it may play a role in promoting metastasis. Along these lines, cancers of the breast, ovary, prostate, lung, and pancreas frequently lose expression of TβR-III [81–85], an event that enhances the ability of cancer cells to undergo EMT and, consequently, to acquire invasive and metastatic phenotypes. Thus, while TβR-III-deficiency resulting from a loss-of-heterozygousity at or epigenetic silencing of the *TβR-III* locus clearly drives progression of tumors from indolent to aggressive states, the molecular mechanisms underlying the ability of TβR-III to normally suppress these tumorigenic processes remains to be fully elucidated.

Given the importance of Smads 2, 3, and 4 in mediating the tumor suppressing activities of TGF-β, it is not surprisingly to learn that these latent transcription factors also manifest a variety of missense mutations during tumorigenesis. Structurally, Smad2/3/4 are comprised of three distinct domains: (i) a globular N-terminal Mad-homology 1 (MH1) domain, which binds TGF-β-regulated promoters at stimulatory **S**mad-**b**inding **e**lements (SBE; GTCTCAGA, CAGA, or AGAC), or at inhibitory **r**epressive SBE (RSBE; GGCGGG) or **T**GF-β **i**nhibitory **e**lements (TIE; GNNTTGGtGa) [8,86–90]; (ii) a central Pro-rich linker region subject to regulated phosphorylation by a variety of protein kinases (as discussed previously); and (iii) a globular C-terminal MH2 domain, which mediates transactivation through its ability to interact physically with Smad partners, and with various components of the nuclear pore and transcriptional machinery [4,6,8]. Most missense mutations identified to date in Smads 2 and 4 localize to the oligomerization sequences within their MH2 domains [91], and to a lesser extent, to the DNA-binding sequences within their MH1 domains [92]. In either scenario, the ability of TGF-β to mediate cytostasis is compromised severely, which enables malignant cells to progress unabated through the cell cycle even in the continued presence of cytokine. This situation occurs frequently in cancers of the gastrointestinal tract, where Smad4 mutations are observed in 50% of pancreatic cancers [66], in 30% of colorectal cancers [3], and in 25% of patients with juvenile polyposis syndrome [3]. Targeted deletion of Smad4 in mouse keratinocytes [93] or T cells [94], or global Smad4 heterozygousity in combination with APC inactivation [95] further confirmed the essential function of Smad4 in suppressing tumor formation, particularly that in the gastrointestinal track. Although the prevalence of Smad2 mutations is considerably less than that observed for Smad4, mutations in Smad2 are detected in 11% of colorectal cancers, and in 7% of lung cancers [3]. Lastly, despite the fact that Smad3 mutations have thus far remained undetected in human cancers, its expression is lost in 37% of human gastric cancers [96], and in essentially all childhood cases of acute T cell ALL [97]. Furthermore, unlike mice deficient in either Smad2 or Smad4, mice lacking Smad3 are viable and develop colorectal cancers only in response to *Helicobacter* infection and its accompanying inflammatory reaction [98]. More importantly, Smad3-deficiency protects mice from developing chemically-induced skin carcinomas [99]. Collectively, these findings indicate Smad2 and Smad3 mediate distinct aspects of the biology and pathology of TGF-β; they also suggest that retaining selective features of the TGF-β signaling system functions in promoting oncogenic signaling by TGF-β. The molecular mechanisms that underlie oncogenic signaling by TGF-β, as well as their role in achieving the hallmarks of cancers are discussed in the later part.

3. TGF-β and dysregulated cell proliferation

3.1. Resistance to TGF-β-mediated cytostasis

TGF-β is a principle player involved in suppressing autonomous growth by epithelial, endothelial, and hematopoietic cell lineages, doing so primarily through its ability to induce cell cycle arrest, but also through its stimulation of cell differentiation or apoptosis in a cell- and context-specific manner. Importantly, the ability to preneoplastic cells to acquire resistance to growth arrest governed by TGF-β represents a major hallmark in the development of numerous human cancers. The ability of TGF-β to mediate cytostasis takes place late in the G1 phase of the cell cycle and occurs primarily through the initiation of two synchronized events. First, TGF-β downregulates the expression of the growth-promoting transcription factors, c-Myc and Ids 1-3 [4,6,100,101]. In doing so, TGF-β inhibits Myc transcription by inducing the binding of Smad3 to E2F4/5 and p107 at RSBE sites housed in the Myc promoter [87,89]. Smad3 also mediates Id1 repression by TGF-β through a "self-enabled" signaling system that first requires Smad3 to induce the expression of ATF3, which subsequently interacts with Smad3 to inhibit Id1 promoter activation [102]. Loss of Id2 expression requires TGF-β to induce the expression of the Myc antagonists, Mad2 and Mad4, which form heterodimers with Max and prevent Id2 transcription [101].

The second major pathway whereby TGF-β mediates cytostasis occurs through its production of the cyclin-dependent protein kinase (CDK) inhibitors, $p15^{INK4b}$ and $p21^{CIP}$ [4,6,100]. The ability of p15 to prevent cell cycle progression takes place by its binding to CDK4/6, which occludes their access to and activation by cyclin D. TGF-β induces p15 expression *via* a bimodal mechanism involving (i) Smad3-mediated repression of Myc, which together with Miz-1 binds and inactivates p15 transcription; and (ii) the formation of Sp1:Smad3:Miz-1 complexes that stimulate p15 transcription [103,104]. Whereas p15 preferentially inactivates CDK4/6, p21 preferentially targets and antagonizes cyclin E:CDK2 complexes [100]. In doing so, TGF-β stimulates the formation of Sp1:Smad2/3:FoxO complexes that transactivate the p21 promoter [105,106].

Developing and progressing neoplasms have evolved several mechanisms to override the cytostatic activities of TGF-β. For example, dysregulated Myc expression negates cell cycle arrest induced by TGF-β by (i) promoting the formation of Myc:Miz-1 complexes, which inhibit p15 and p21 transcription induced by Smad3:Miz-1 complexes [103,104]; and (ii) recruiting the DNA methyltransferase, Dnmt3a, to Myc:Miz-1 complexes, which methylates and inactivates p21 transcription [107]. In addition, human cancers frequently exhibit hyperactivation of the PI3K/AKT pathway [108], which inactivates the cytostatic activities of TGF-β by (i) phosphorylating FoxO, which subsequently is exported from the nucleus and sequestered in the cytoplasm by 14-3-3, and as such, is unavailable to induce p21 expression with Smad3 [105,109]; and (ii) binding and sequestering inactive Smad3 to prevent its stimulation by active TGF-β receptors [110,111]. Moreover, in conjunction with activated AKT, FoxG1 interacts physically with Smad3:FoxO complexes to inhibit their induction of p21 in glioblastomas [105]. Whether FoxG1 or other Forkhead family members function similarly to inactive p15 and p21 expression in other human cancers remains to be determined definitively.

Cancer cells also evade the cytostatic activities of TGF-β *via* cancer cell-specific alternative splicing of C/EBPβ into transcriptionally active LAP or inactive LIP variants [112]. Under normal circumstances, LAP C/

EBPβ variants participate in TGF-β-mediated cytostasis by cooperating with Smad3:FoxO complexes to induce p15 expression, and with Smad3:E2F4/5 complexes to repress Myc expression [112]. Cancer cells, particularly those of the breast, elevate their expression of inactive LIP C/EBPβ variants, which uncouples TGF-β from regulation Myc and p15 expression and correlates with metastatic disease development [112]. Along these lines, ELAC2 recently was identified as a transcriptional co-factor that mediates p21 expression in conjunction with Smad2:FAST-1 complexes in prostate epithelial cells [113]. Importantly, loss of ELAC2 in developing and progressing prostate cancers inactivates their ability to undergo growth arrest in response to TGF-β [114]. Finally, overexpression or oncogenic activation of the Ski and SnoN induces cellular transformation in part *via* their ability to bind Smad2/3/4 complexes and recruit transcriptional inactivation machinery, including N-Cor, mSin3A, and HDAC, which collectively repress gene induction stimulated by TGF-β [115,116].

3.2. Cell cycle progression induced by TGF-β

In addition to developing resistance to TGF-β-mediated growth arrest, cancer cells routinely acquire the ability to undergo enhanced proliferation when stimulated by TGF-β. The precise mechanisms underlying this phenomenon has yet to be established, but likely reflects a combination of the inactivation of cytostasis mediated by TGF-β coupled to its ability to induce the expression of cytokines and growth factors or their cognate receptors. For instance, TGF-β stimulates the synthesis of IL-1 [117], CTGF [118], bFGF [119], PDGF [120], and TGF-α [121], and of the receptors for PDGF [122] and EGF [123]. Moreover, a common feature of mitogenic signaling systems is their coupling to activation of the Ras/MAP kinase pathways, which results in ERK1/2-mediated phosphorylation of the linker region of Smad2/3 linker and their exclusion from the nucleus, perhaps *via* Smurf-mediated sequestration of Smad2/3 from the nuclear pore machinery [124]. Hyperactivation of the Ras/MAP kinase pathway also promotes ERK1/2-mediated phosphorylation of TGIF, leading to its stabilization and nuclear localization where it functions as a transcriptional co-repressor by recruiting Smad2:HDAC complexes to the p15 promoter [125]. More recently, activation of RTK and Ras/MAP kinase signaling was shown to stimulate CK1ε/δ phosphorylation of p53, which interacted physically with Smad2/3 to promote the initiation of the cytostatic program by TGF-β. Importantly, restoring p53 function to cancer cells devoid of p53 expression or activity reinstated the ability of TGF-β to induce p21 expression and, consequently, cell cycle arrest [36]. Finally, besides their ability to activate the Ras/MAP kinase pathway, mitogens also activate CDKs when promoting cell cycle progression. In doing so, the cytostatic function of TGF-β can be subverted by CDK2- and CDK4-mediated phosphorylation of the linker region of Smad2, which inactivates its ability to induce the expression of p15 and p21, and to repress the expression of Myc [37].

4. TGF-β and cancer cell motility

4.1. TGF-β and epithelial-mesenchymal transition

The process whereby immotile, polarized epithelial cells transdifferentiate into highly motile, apolar mesenchymal cells is known as epithelial-mesenchymal transition (EMT) and was described initially as a phenomenon that took place during chick embryonic development nearly 100 years ago by Frank Lillie [126], and which was first documented as a discrete physiological event in 1982 by Greenburg and Hay [127]. Today, EMT is recognized as a normal and essential physiological process operant in mediating embryonic development, wound healing, and tissue morphogenesis, remodeling, and repair. EMT itself compromises several distinct features, including (i) the loss of cell polarity due to downregulated expression of epithelial cell markers (*e.g.*, E-cadherin, zona occluden-1, and β4 integrin); (ii) cytoskeletal architecture reorganization and intracellular organelle redistribution; (iii) upregulated expression of fibroblast markers (*e.g.,* vimentin, N-cadherin, α-smooth muscle actin); and (iv) elevated cell invasion and migration [126,128–131]. Besides its role in mediating normal tissue morphogenesis and repair, inappropriate initiation of EMT also underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders of the lung, liver, and kidney [126,128–132]. In addition, the acquisition of metastatic phenotypes by dedifferentiated tumors is critically dependent on EMT and its ability to actively remodel tumor microenvironments in a manner that promotes the evolution and selection of metastatic cells [126,128–131].

The essential role of TGF-β in mediating EMT was first described by Miettinen et al. [133] who showed that TGF-β stimulation of normal mammary epithelial cells readily induced their transdifferentiation into fibroblastoid-like cells. Since that time, TGF-β has been identified as a master regulator both of physiological and pathophysiological EMT in a variety of epithelial cell types and tissues. For instance, TGF-β3-deficiency in mice elicits defective palatogenesis and cleft palate formation due to faulty initiation of EMT [134]. Along these lines, neutralization of TGF-β2 function impairs chick heart endocardial cushion development by inhibiting Slug expression and, consequently, its induction of EMT [135]. Finally, targeted-deletion of Smad3 in mice prevents their development of EMT-driven retinal [136,137] and renal [138] fibrosis. Aberrant EMT also is an essential facet of the oncogenic activities of TGF-β, particularly its ability to stimulate the progression and metastasis of late stage cancers. The molecular mechanisms underlying the ability of TGF-β to induce cancer cell EMT and metastasis are described in the following section.

4.2. TGF-β, invasion, and metastasis

The activities of fibroblasts and their associated stromal components play important roles in determining whether TGF-β either suppresses or promotes tumor formation [139,140]. Indeed, the ability of TGF-β to inhibit tumor formation occurs not only through its actions in epithelial cells, but also through its stimulation of adjacent fibroblasts, which synthesize and secrete a variety of cytokines, growth factors, and extracellular matrix (ECM) proteins that mediate tissue homeostasis and suppress cancer development. Importantly, genetic or epigenetic events that inactivate paracrine TGF-β signaling between adjacent epithelial and stromal compartments can lead to the formation of neoplastic cells, as well as to their selection and expansion by promoting their growth, survival, and motility [141,142]. For instance, conditional inactivation of TβR-II in mouse fibroblasts elicits the formation of prostate intraepithelial neoplasias and invasive carcinoma of the forestomach [143]. Similar targeted inactivation of TβR-II in mouse mammary fibroblasts inhibits mammary gland ductal morphogenesis, as well as the formation of terminal end buds [144]. Importantly, grafting mammary carcinoma cells with TβR-II-deficient fibroblasts, not their normal counterparts, significantly enhanced the growth and invasion of breast cancer cells in subrenal capsules [144]. Mechanistically, inactivation of TGF-β signaling in fibroblasts promotes tumorigenesis by upregulating the expression and signaling of TGF-α, MSP, and HGF within cell microenvironments [139,140,143,144]. Furthermore, inactivating TGF-β signaling in fibroblasts also promotes breast cancer metastasis by activating two chemokine receptor axes, namely SDF-1:CXCR4 and CXCL5:CXCR2, which recruit immature GR1 + CD11b + myeloid cells that (i) suppress host tumor immunosurveillance, and (ii) induce MMP expression within tumor microenvironments that promotes the dissemination of breast cancer cells [145].

In addition to its metastasis-promoting activities initiated in fibroblasts and tumor microenvironments, TGF-β also induces EMT and metastasis by directly effecting the activities and behaviors of developing and progressing malignant cells. For instance, TGF-β stimulates human MDA-MB-231 breast cancer cells to metastasize specifically to bone by inducing their expression of PTHrP, IL-11, and CTGF [146–148]. Interestingly, although TGF-β1 had no effect on the growth of mammary tumors induced by transgenic polyomavirus middle T antigen expression, conditional TGF-β1 expression significantly enhanced the ability of these same mammary tumors to metastasize to the lungs [149]. In addition, TGF-β stimulation of EMT in breast cancer cells has been linked to the selection and expansion of cancer initiating/stem cells [150–153], which may underlie breast cancer resistance to neoadjuvant chemotherapies, as well as disease progression and recurrence. Thus, following inactivation of its cytostatic function, TGF-β preferentially promotes the acquisition of metastatic phenotypes in previously nonmetastatic malignant cells.

Accumulating evidence indicates that TGF-β stimulates cancer cell EMT and metastasis through a combination of Smad2/3-dependent and -independent signaling systems. Indeed, engineering metastatic human MCF10ACA1a breast cancer cells to express a dominant-negative Smad3 [154] or a TβR-I mutant incapable of activating Smad2/3 (*i.e.*, L45 mutant) [154] both significantly reduced the ability of MCF10ACA1a cells to colonize the lung. Along these lines, Smad4-deficiency elicited by RNA interference inhibited the ability of MDA-MB-231 xenografts to metastasize to bone in part *via* diminished expression of IL-11 and CTGF in response to TGF-β [146,155]. As previously mentioned, the extent of tumor formation and their rate of growth was unaffected by altering the response of these breast cancer cells to TGF-β [146,155], which again points to the importance of maintaining the fidelity of the TGF-β signaling system during metastasis development. Accordingly, whereas Smad4-deficiency conspires with oncogenic K-Ras to promote pancreatic cancer initiation and development, maintaining intact Smad4 signaling is necessary to induce EMT and TGF-β-dependent growth of advanced pancreatic ductal adenocarcinomas [156]. In addition, overexpression of Smad7, which inhibits TGF-β stimulation of Smad2/3 [18–20], prevents the invasion of breast [157] and head and neck cancers [158,159], as well as impairs the ability of melanoma cells to metastasize to bone [160]. Recently, TGF-β was shown to cooperate with Ras to promote the formation of mutant p53/p63 complexes that are bridged by Smad2/3, and that result the inactivation of p63 and its ability to downregulate the expression of metastasis suppressor genes, Sharp-1 and cyclin G2 [161]. Along these lines, Smad2 has been observed to promote EMT in mammary epithelial cells by stimulating the DNA binding activity of the DNA methyltransferase, DNMT1, leading to the chronic epigenetic silencing of epithelial-associated genes, including *CDH1*, *CGN*, *CLDN4*, and *KLK10* [162]. Collectively, these findings demonstrate the importance of Smad2/3/4 signaling in mediating metastasis stimulated by TGF-β; they also suggest that the development and implementation of novel Smad2/3 antagonists may improve the clinical course of metastatic cancer patients whose tumors house functional Smad2/3 signaling systems.

Equally important to the ability of TGF-β to stimulate cancer cell EMT and metastasis is its coupling to activation of noncanonical signaling systems (Fig. 2; [7]). Indeed, activation of Ras/MAP kinase [51,163–167], PI3K/AKT [52], Rho/ROCK [53]; NF-κB [168], Jagged/Notch [169], Wnt/β-catenin [170], and MDM2/p53 [171] pathways by TGF-β all play essential roles in mediating its induction of cancer cell EMT, invasion, and metastasis. In particular, Ras/MAP kinase signaling cooperates synergistically with TGF-β to elicit EMT and metastasis during the progression of skin cancers from squamous to spindle cell phenotypes [172–174]. In addition, TGF-β stimulation of NF-κB promotes EMT and lung colonization by breast cancer cells that house oncogenic Ras [168]. Furthermore, activation of the Ras effector, c-Raf-1, induces autocrine TGF-β expression and activation that ultimately results in its stimulation of EMT and invasion [175]. Finally, TGF-β-induces elevated MDM2 expression that leads to the destabilization of p53, a key component of EMT [171].

Integrins also have been implicated in regulating oncogenic signaling by TGF-β, particularly its ability to induce EMT, invasion, and metastasis. For instance, administering neutralizing antibodies against β1 integrin prevented TGF-β stimulation of p38 MAPK and EMT in mammary epithelial cells [163]. In addition, TGF-β stimulates the binding of the adapter protein Dab2 [17] to β1 integrin, leading to FAK activation and the induction of EMT in mammary epithelial cells [176]. Similarly, work in our laboratory led to the discovery of a novel αvβ3 integrin:Src:phospho-Y284-TβR-II:Grb2:p38 MAPK signaling axis whose activation mediates oncogenic signaling—*i.e.*, EMT, invasion, and metastasis—by TGF-β in normal and malignant mammary epithelial cells [60–62]. Importantly, the ability of TGF-β to stimulate the growth and pulmonary metastasis of breast cancers in mice absolutely requires activation of this oncogenic signaling complex [62]. In addition to its role in mediating pulmonary metastasis of breast cancers, αvβ3 integrin also mediates breast cancer metastasis to bone [177,178] in a TGF-β-dependent manner. Lastly, new insights into the role of TGF-β in regulating tight junction dissolution during EMT recently was elucidated by Wrana et al. [179] who observed that the tight-junction assembly protein, PAR-6, interacts physically with TβR-I. Activation of TGF-β receptors by ligand results in TβR-II-mediated phosphorylation of PAR-6, which binds Smurf1 and coordinates its ubiquitination and degradation of RhoA. The net effect of these TGF-β-dependent events results in the dissolution of epithelial cell tight junctions and the disassembly of their actin cytoskeleton, leading to the induction of EMT [179]. Future studies need to more thoroughly examine the role of additional phosphorylation events in potentially regulating PAR-6 function, as well as the role of integrins and other adhesion-regulated signaling systems to collaborate with TGF-β in mediating EMT and tight junction dissolution. Similar analyses aimed at determining the importance of these events in regulating metastasis development stimulated by TGF-β also is warranted.

5. TGF-β and tumor angiogenesis

Angiogenesis is a normal physiological process whereby new blood vessels develop from preexisting vessels, and which is an essential component of embryonic development, wound healing, and the female reproductive cycle [180,181]. Pathological activation of angiogenesis also figures prominently in mediating the development of a number of human diseases, including rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration [181,182]. Indeed, inappropriate initiation of angiogenesis perhaps is best known and characterized for its role in mediating the development and progression of tumors, whose growth is limited by the extent of nutrient diffusion into tumor microenvironments. Tumor angiogenesis circumvents this limitation by providing developing neoplasias with an efficient supply of nutrients, and ultimately, with a route for their metastatic spread. Angiogenesis is comprised of two distinct and sequential phases referred to as angiogenesis activation and angiogenesis resolution [180–182]. Angiogenesis activation encompasses the initiation and development of new blood vessels, and is characterized by (i) increased endothelial cell (EC) permeability, proliferation, migration, and invasion, and (ii) reduced EC adhesion and basement membrane integrity. In contrast, angiogenesis resolution encompasses the maturation of newly formed vessels and entails (i) increased EC adhesion and basement membrane deposition, (ii) decreased EC proliferation and motility, and (iii) the recruitment of perivascular cells, namely pericytes, vascular smooth muscle cells, or mural cells, that function in regulating vessel stability and hemodynamics. Importantly, normal endothelium in adult tissues exists in a quiescent, inactive state, a fact that underlies the continued

enthusiasm to selectively target tumor vascular when treating human malignancies.

Consistent with its multifunctional nature, TGF-β has been reported to regulate the activation and resolution phases of angiogenesis [183–186]. In addition, TGF-β also affects ECM production and remodeling in EC microenvironments, and as such, impacts the interactions and communications between ECs and their supporting mesenchymal cells [187]. The ability of TGF-β to regulate angiogenesis was discovered by analyzing the vasculature of TGF-β1-deficient mice, which exhibit severe defects in hematopoiesis and EC differentiation that results in the production of weak, aberrantly formed capillaries [188]. Additional studies demonstrating a crucial role of TGF-β signaling in regulating angiogenesis were gleaned from analyses of mice lacking TβR-I [189], TβRII [190,191], TβR-III [192,193], ALK1 [194,195], endoglin [196], Smad1 [197], or Smad5 [198], all of which exhibited vascular and endothelial defects. Clinically, the development of hereditary hemorrhagic telangiectasia type 1 (HTT1) results from the loss or inactivation of the gene for endoglin [199,200], while the loss or inactivation of the gene for ALK1 elicits HHT2 [201,202]. Moreover, HHT1 and HHT2 both are phenocopied in knockout mice lacking either endoglin [196,203] or ALK1 [204], respectively. Thus, aberrant TGF-β signaling clearly underlies the development of multiple vascular disorders [205].

Genetic inactivation of ALK1 [195] and ALK5 [189] elicits embryonic lethality at E11.5 and E10.5, respectively, demonstrating the indispensable function of these genes in mediating normal embryonic angiogenesis and vasculogenesis. Quite surprisingly, recent studies have established that TGF-β differentially activates TβR-I/ALK5 *versus* ALK1, which elicits dramatically different angiogenic responses by ECs. For instance, TGF-β stimulation of TβR-I/ALK5 activates Smad2/3 and their transcription of the ECM proteins, plasminogen activator inhibitor type 1 (PAI-1) and fibronectin, which mediate angiostasis and vessel maturation [194,206,207]. In addition, Miyazono and colleagues [208] showed that TβR-I/ALK5 mediates TGF-β stimulation of EC gene expression profiles characteristic of angiostasis and vessel maturation, and of periendothelial cell differentiation. Thus, activation of TβR-I/ALK5 by TGF-β functions preferentially in regulating angiogenesis resolution [185,208]. In contrast, activation of ALK1 by TGF-β stimulates Smad1/5/8 transcriptional activity and the expression of angiogenic genes, such as Id1 and interleukin 1 receptor-like 1 [194,206–208]. Thus, activation of ALK1 by TGF-β functions preferentially in regulating angiogenesis activation. Interestingly, the decision by TGF-β to couple to either ALK1 or ALK5 likely depends on the balance between TGF-β and angiogenic cytokines within tumor microenvironments. Indeed, low TGF-β concentrations enhance the ability of bFGF and VEGF to stimulate EC proliferation and angiogenic sprouting, while high TGF-β concentrations inhibit these angiogenic activities [185,186].

In addition to regulating angiogenesis *via* its activation of ALK1 and ALK5, TGF-β also controls vessel development *via* its actions on the co-receptors, TβR-III/betaglycan and endoglin. Several recent studies have shown the importance of epithelial cell expression of TβR-III in suppressing the growth and metastasis of breast [81], lung [82], pancreatic [83], ovarian [84], and prostate [85] cancers. However, the ability of soluble TβR-III to bind and sequester TGF-β has been used successfully to antagonize tumor angiogenesis and, consequently, to inhibit the growth and progression of human tumors produced in mice [209–211]. In contrast, endoglin is expressed predominantly on proliferating ECs, and its expression also can be upregulated by ALK1 [208]. When expressed in human umbilical vein endothelial cells, endoglin inhibits ALK5 signaling and promotes EC proliferation, migration, and tubulogenesis in conjunction with stimulation of ALK1 [212,213]. Moreover, the redundant clinical symptoms of HHT1 (*i.e.,* endoglin defects) and HHT2 (*i.e.,* ALK1 defects) in humans, together with the overlapping phenotypes observed between ALK1- and endoglin-deficient mice, suggest that ALK1 and endoglin both function as negative regulators of TGF-β/ALK5 signaling.

Collectively, these studies highlight the complexities associated with the ability of TGF-β to regulate EC activities coupled to angiogenesis. Future studies clearly need to (i) better define the precise mechanisms that enable TGF-β and its downstream effectors to govern the induction of angiogenic or angiostatic gene expression profiles; (ii) establish the impact of EC and perivascular cell differentiation states to influence the angiogenic response to TGF-β; and (iii) identify the microenvironmental cues and signals that cooperate with TGF-β in mediating angiogenesis activation and resolution.

6. TGF-β and cancer cell survival

Although cancer typically is viewed as a disease that arises from uncontrolled cell proliferation, it also is a disease of dysregulated apoptosis that enhances cancer cell survival by conferring developing neoplasms resistance to stimuli that would normally induce their programmed cell death [2]. Under normal physiological conditions, TGF-β inhibits autonomous cell growth by inducing cell cycle arrest or by stimulating cellular differentiation [3,4,214]; however, TGF-β also guards against the development of dysregulated cell growth through its ability to promote programmed cell death, particularly in lymphocytes and hepatocytes [3,4,214,215]. Importantly, TGF-β stimulation of apoptosis occurs independently of its regulation of EMT and cell motility [216,217], and is mediated through its regulation of a variety of proapoptotic proteins (Table 1).

One of the hallmarks of cancer is the ability of malignant cells to acquire resistance to apoptotic stimuli [2]. As mentioned previously, tumorigenesis frequently subverts the tumor suppressing activity of TGF-β, thereby enabling TGF-β to promote oncogenesis by stimulating the growth, invasion, metastasis, and angiogenesis of developing tumors (as previously discussed). Along these lines, alterations within the TGF-β signaling system also conspire to convert TGF-β from activator of apoptosis to a promoter of cancer cell survival. This altered cellular response to TGF-β is especially evident through its ability to protect and promote the recovery of cancer cells following their targeting by radiation and chemotherapy treatments [218,219]. Thus, the continued reliance on radiation and chemotherapy regimens to treat cancer patients necessitates that science and medicine fully elucidate the molecular mechanisms that affords TGF-β to protect developing neoplasms from apoptotic stimuli.

Numerous studies have established that dysregulated activation of the NF-κB and PI3K/AKT pathways by TGF-β functions in promoting cancer cell survival during tumorigenesis [220–223]. Interestingly, TGF-β routinely suppresses the activation of NF-κB in normal epithelial cells by inducing their expression of IκBα, a phenomenon that is inactivated during oncogenic progression [157,215]. Quite intriguingly, TGF-β stimulation of late stage cancers of the breast, prostate, and liver results in NF-κB activation, leading to the induction of pro-survival and -tumorigenic gene expression profiles [220,224–226]. The aberrant activation of NF-κB by TGF-β has been attributed its stimulation of TAK1, which induces IKK activation [220,224,225]. Indeed, recent work in our laboratory defined a novel TAB1:TAK1:

Table 1
Expression of proapoptotic genes induced by TGF-β.

Gene	Mechanism of action	Reference
SHIP	SH2-containing lipid phosphatase that inhibits cell survival mediated by PI3K	[268]
DAPK	Ca^{++}/calmodulin-dependent protein kinase that regulates mitochondrial-, caspase-, and autophagic cell death	[269,270]
TIEG1	Zinc-finger transcription factor that represses Bcl-2 expression	[271,272]
Daxx	TβR-II-adaptor protein that stimulates JNK	[54]
ARTS	Mitochondrial septin-like protein that inactivates XIAP to elicit caspase activation	[273,274]
TAK1	MAP kinase kinase kinase that stimulates JNK and p38 MAPK	[275–278]

IKKβ:NF-κB signaling axis that forms aberrantly in breast cancer cells, and in normal mammary epithelial cells following their induction of EMT by TGF-β [225]. Once formed, this signaling axis enables oncogenic signaling by TGF-β in part *via* activation of NF-κB and its consequential production of proinflammatory cytokines, and of Cox-2 [227], which engages a PGE2:EP2 signaling axis coupled to breast cancer development and progression [228]. In addition, TAK1-deficiency abrogated the ability of TGF-β to induce the invasion of breast cancer cells, as well as restored their ability to undergo cytostasis in response to TGF-β. More importantly, expression of a dominant-negative TAB1 mutant dramatically reduced the growth of mammary tumors in normal mice, as well as in their immunocompromised counterparts, suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β [225]. Recently, TGF-β was shown to induce xIAP expression in hepatic and endometrial carcinomas, which enhanced their survival and invasiveness *via* xIAP-mediated regulation of PK3K/AKT, TAK1, PKC, JNK, and MMP-9 [229,230]. Along these lines, work in our laboratory identified xIAP as an essential mediator underlying the formation of TAB1:TAK1:IKKβ complexes and, consequently, their activation of NF-κB solely in cancer cells [231]. Thus, pharmacological targeting of xIAP may represent a novel approach to antagonize the oncogenic activities of TGF-β in developing and progressing human malignancies.

Altered coupling of TGF-β that results in its stimulation of AKT [232] and p38 MAPK [233] may also serve in potentiating the activation of IKKβ and NF-κB. Furthermore, activated IKK [234] complexes and AKT [223] both directly phosphorylate FoxO, which subsequently is inactivated and sequestered in the cytoplasm by its binding to 14-3-3 proteins. In doing so, phosphorylated FoxO transcription factors are no longer available to participate in Smad2/3-mediated expression of apoptotic and cytostatic genes, thereby enhancing cancer cell survival. Finally, activated AKT has been shown to bind and sequester inactive, cytoplasmic Smad3, which prevents TGF-β stimulation of programmed cell death [110,111].

In addition to its anti-apoptotic function, NF-κB also plays an important role in mediating TGF-β stimulation of EMT and metastasis [168], and of pro-inflammatory gene expression [226]. For instance, inhibiting NF-κB activity in Ras-transformed mammary epithelial cells blocked the ability of TGF-β to (i) induce MMP-13 and MCP1 expression, and (ii) repress E-cadherin expression. Moreover, TGF-β stimulation of EMT is sufficient in eliciting its ability to stimulate, not inhibit, NF-κB activity in mammary epithelial cells [225]. When stimulated with TGF-β, cancer cells synthesize and secrete an array of pro-inflammatory genes following NF-κB activation. Included in this list of proinflammatory mediators regulated by TGF-β and its activation of NF-κB are Cox-2, GM-CSF, TNF-α, and interleukins 1, 6, and 8 [225,226,235,236], which collectively drive oncogenesis by stimulating tumor angiogenesis and metastasis, as well as by inhibiting host immunosurveillance. Recently, aberrant expression of Cox-2 was shown to negate the cytostatic activities of TGF-β [237], and to promote its ability to induce breast cancer metastasis to bone [235]. Thus, pharmacological targeting of Cox-2 may enhance the tumor suppressing activities of TGF-β. Finally, TGF-β also enhances cancer cell survival through its ability to modulate and suppress immunosurveillance mediated by the adaptive immune system [238,239]. The role of TGF-β in regulating immune system function during tumorigenesis is discussed in the following section.

7. TGF-β and dysregulated immunosurveillance

It has been appreciated from many years now that cancer initiation, promotion, and progression all are linked to aberrant and/or persistent inflammation within tumor microenvironments [240]. Indeed, the balance between host immunosurveillance and proinflammatory activity within the tumor milieu plays an essential role in determining whether tumor development and progression is induced or inhibited [240]. An implicit factor in regulating this delicate balancing act is TGF-β, which governs the activities and behaviors of cancer cells and their associated stromal components [239]. Indeed, while many cytokines and chemokines participate in the inflammatory process, the prominent and essential role of TGF-β in maintaining proper immune system function and homeostasis is underscored by the finding that TGF-β1-deficient mice exhibit lethal multifocal inflammatory disease [241,242], while mice lacking Smad3 exhibit defects in the responsiveness and chemotaxis of their neutrophils, and their T and B cells [243]. In addition, a characteristic feature of cancer cells is their capacity to increase the production and secretion of TGF-β into tumor microenvironments, and into the general circulation of cancer patients [244–246]. Elevated concentrations of active TGF-β also are detected within the tumor milieu due to enhanced ECM degradation mediated by resident and recruited leukocytes—*i.e.*, monocytes/macrophages, dendritic cells, granulocytes, mast cells, T cells, and natural killer (NK) cells—that either promote or suppress tumor development in a context-specific manner [240]. It is interesting to note that the recruitment of leukocytes to developing neoplasms in many respects reflects the processes underlying their normal recruitment to regions of wounding, inflammation, or infection. This observation, coupled with the fact that cancer cells house genetic or epigenetic alterations that elicit persistent tumor inflammation and chronic leukocyte infiltration undoubtedly led to Dvorak to liken “tumors to wounds that never heal” [247].

In general, high levels of TGF-β inactivate host anti-tumor immunosurveillance systems, which confers immune privilege to developing neoplasms and ensures for their continued progression. Mechanistically, TGF-β inhibits the proliferation and differentiation of NK and T cells, as well as their production of cytotoxic effector molecules. Moreover, the ability of TGF-β to affect the behaviors of cytotoxic $CD8^+$ T cells occurs in a manner that reflects their differentiation status. For instance, TGF-β is a potent inhibitor of naïve $CD8^+$ T cell populations, but elicits little to no response in their fully differentiated and activated counterparts, which downregulate expression of TβR-II. The insensitivity of differentiated $CD8^+$ T cells to TGF-β can be overcome by the production of either IL-10 or IL-2, or by the expression of the co-stimulatory molecule CD28 [238,239,248]. Interestingly, TGF-β stimulation of T cells expressing CD28 ultimately promotes the survival of memory/effector phenotypes in thymic and peripheral T cell populations.

The lymphocyte defects observed in Smad3-deficient mice suggest an important role for this latent transcription factor in mediating immunosuppression by TGF-β. Accordingly, activation of Smad3 by TGF-β prevents mitogenesis in $CD8^+$ T cells by (i) inhibiting their production of IL-2; (ii) repressing their expression of c-Myc, cyclin D2, and cyclin E; and (iii) stimulating the expression of the CDKIs p15, p21, and p27 [239,248,249]. In addition, TGF-β also represses the expression and production of cytotoxic effector molecules, including IFN-γ, lymphotoxin-α (LT-α), perforin/granzyme, and Fas ligand [239,248,249]. Although TGF-β has no effect on proliferation of $CD4^+$ T cells, it does inhibit $CD4^+$ T cell differentiation into T helper 1(Th1) and Th2 cell lineages, which readily secrete IFN-γ and LT-α (Th1) or IL-4, IL-5, and IL-13 (Th2), respectively. The inhibitory activities of TGF-β in $CD4^+$ T cells result from its ability to downregulate T cell receptor expression, to diminish intracellular Ca^{++} signaling, and to reduce the expression and activation of transcription factors [239,248,249], which collectively serve to alleviate host immunosurveillance. Accordingly, inactivation of TGF-β in $CD8^+$ or $CD4^+$ T cells results in T cell-mediated eradication of skin [250] and prostate [251] cancer in mice. The ability of TGF-β to suppress the activities of tumor-infiltrating T cells also has been associated with its activation of Tregs, which represent a subset of CD4+ and CD8+ peripheral T cells that also express CD25 and Fox3P, and which routinely localize to tumor microenvironments where they promote immune privilege by inhibiting tumor targeting T and NK cells [248].

In addition to regulating the adaptive immune system, TGF-β also figures prominently in governing the behavior and activation of the innate immune system. Indeed, TGF-β is a potent inhibitor of NK cell cytolytic activity, presumably by attenuating NKp30 and NKG2D receptor activation. Neutralizing TGF-β not only prevents NKG2D downregulation, but also restores NK cell anti-tumor reactivity [252]. In addition, NK cell production of IFN-γ is repressed by TGF-β through cellular mechanisms that remain incompletely understood. Dendritic cells are professional antigen presenting cells whose expression of MHC class II, of the co-stimulatory molecules CD40, CD80, and CD86, and of the cytokines TNF-α, IL-12, and CCL5/Rantes is inactivated following their stimulation with TGF-β [239,248,249,253]. In addition, the increased production of TGF-β within tumor microenvironments serves as a chemoattractant for mast cells that enhance tumor progression through their synthesis and release of a variety of factors, including histamine, proteases, and cytokines (*e.g.*, VEGF and TGF-β) [249,254]. Lastly, TGF-β stimulation of resting monocytes (*i.e.*, non-phagocytic) promotes their chemotaxis to and infiltration into tumor microenvironments where they *(i)* enhance oncogenesis by stimulating ECM degradation, and by inducing tumor angiogenesis, invasion, and metastasis, and *(ii)* contribute to immunosuppression through upregulated production and release of TGF-β into tumor microenvironments [255,256]. Furthermore, activation of differentiated macrophages by TGF-β inhibits their expression of the scavenger receptors, CD36 and SR-A, of the opsonizing IgG receptors, FcγRI and FcγRIII, and of the antigen presenting receptor, MHC class II, which collectively function to enhance the progression and survival of developing neoplasms. In addition to macrophages, TGF-β also induces the infiltration of tumor-associated neutrophils (TAN) that bear a tumor promoting phenotype, and as such, antagonizing TGF-β function facilitates the recruitment of TANs that possess an anti-tumor phenotype [257].

8. TGF-β and cancer cell immortality

An important and widely recognized hallmark of cancer lies in the ability of malignant cells to become immortal, a process achieved through the reactivation of human telomerase reverse transcriptase (hTERT) and its consequential lengthening of chromosomal telomeres. Structurally, telomeres are complex nucleoproteins comprised of single- and double-stranded DNA and associated proteins that function in safeguarding against chromosomal end-to-end fusions. With the exception of germ cells, hTERT expression is silenced and undetectable in normal somatic cells. Thus, in the absence of hTERT and its ability to maintain telomere integrity, each successive cell division results in telomere erosion and, ultimately, in cell senescence, crisis, and apoptosis [258]. Collectively, these events conspire to suppress dysregulated cell growth and survival, and as such, to prevent tumor formation.

In contrast to their normal counterparts, cancer cells readily overcome the negative constraints that repress hTERT expression, resulting in the reinitiation and activation of telomerase activity and the acquisition of cellular immortalization. Human telomerase consists of a telomerase RNA template (hTER) and hTERT, both of which function in coordinating the synthesis of telomere repeats that mediate chromosomal integrity and prolong cellular replication [258,259]. Given the importance of dysregulated hTERT expression and activity to promoting cancer development, it is fitting that TGF-β may in fact be the principle cytokine operant in silencing hTERT expression in normal cells [260]. Indeed, the extent of TGF-β signaling in responsive cells displays an inverse relationship with the levels of hTERT expression observed in cancers of the colon and breast [260–263]. Moreover, elevating hTERT expression in epithelial cells elicits resistance to the cytostatic activities of TGF-β [264].

Current evidence indicates that TGF-β represses hTERT expression *via* several distinct mechanisms. For instance, cellular depletion of SIP1/ZEB2, a zinc-finger transcription factor more commonly associated with induction of EMT, significantly attenuates the ability of TGF-β to repress hTERT expression in human osteosarcoma cells [265]. Moreover as mentioned previously, TAK1 plays a major role in promoting the activation of NF-κB by TGF-β in breast cancer cells, thereby enhancing their tumorigenicity in mice [225]. Quite surprisingly, activation of TAK1 by TGF-β also has been linked to the repression of hTERT expression in human lung cancer cells. In doing so, TAK1 suppresses Sp1 transcriptional activity *via* recruitment of HDAC to the hTERT promoter [266]. Future studies clearly are warranted to delineate how TAK1 participates in mediating tumor suppression (*i.e.*, repressing hTERT expression) and tumor promotion (*i.e.*, NF-κB activation and proinflammatory gene expression) in response to TGF-β. Along these lines, upregulated expression of the negative regulator of TGF-β signaling, Smurf2, contradicts the immortalization activities of hTERT in a manner independent of the ability of Smurf2 to ubiquitinate target proteins and inhibit TGF-β signaling [267]. Thus, the interplay between Smurf2, hTERT, and TGF-β awaits further clarification. Finally, two recent studies have shown that TGF-β represses hTERT transcription by inducing the assembly of Myc:Smad3 complexes on E-box and SBE sites in the hTERT promoter [262]. Thus, dysregulated Myc expression or aberrant Smad3 signaling can promote upregulated telomerase expression and activity.

9. Concluding remarks

Defining the molecular mechanisms that underlie the initiation of the TGF-β paradox in human malignancies remains the most important and unanswered question concerning the biology and pathology of this multifunctional cytokine. In their seminal paper, Hanahan and Weinberg [2] proposed that all neoplasms must evolve 6 physiological traits during the course of their malignant progression. Importantly, the acquisition of each trait—*i.e.*, autonomous cell growth, resistance to cytostatic, apoptotic, and morality signals, and the induction of angiogenesis and invasion/metastasis—represents an essential rate-limiting step operant in mediating cancer development and progression. The studies reviewed herein show that TGF-β plays a major role, both directly and indirectly, in regulating the acquisition by cancer cells of each of these cancer hallmarks, and as such, the development of novel TGF-β chemotherapeutics capable of targeting these cancer hallmarks offers new inroads into alleviating the devastating effects of TGF-β in promoting metastatic disease development in humans.

Acknowledgements

We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Department of Defense (BC084651), the Komen Foundation (BCTR0706967), and the Case Comprehensive Cancer Center.

References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, CA Cancer J. Clin. 57 (1) (2007) 43.
[2] D. Hanahan, R.A. Weinberg, Cell 100 (1) (2000) 57.
[3] G.C. Blobe, W.P. Schiemann, H.F. Lodish, N Engl J. Med. 342 (18) (2000) 1350.
[4] A.J. Galliher, J.R. Neil, W.P. Schiemann, Future Oncol. 2 (6) (2006) 743.
[5] Y. Shi, J. Massague, Cell 113 (6) (2003) 685.
[6] J. Massague, R.R. Gomis, FEBS Lett. 580 (12) (2006) 2811.
[7] A. Moustakas, C.H. Heldin, J. Cell Sci. 118 (Pt 16) (2005) 3573.
[8] X.H. Feng, R. Derynck, Annu. Rev. Cell Dev. Biol. 21 (2005) 659.
[9] F. Lopez-Casillas, J.L. Wrana, J. Massague, Cell 73 (7) (1993) 1435.
[10] S. Sankar, N. Mahooti-Brooks, M. Centrella, T.L. McCarthy, J.A. Madri, J. Biol. Chem. 270 (22) (1995) 13567.
[11] D. Rotzer, M. Roth, M. Lutz, D. Lindemann, W. Sebald, P. Knaus, EMBO J. 20 (3) (2001) 480.
[12] J. Massague, Annu. Rev. Biochem. 67 (1998) 753.
[13] H. Chang, C.W. Brown, M.M. Matzuk, Endocr. Rev. 23 (6) (2002) 787.

[14] J.L. Wrana, L. Attisano, R. Wieser, F. Ventura, J. Massague, Nature 370 (6488) (1994) 341.
[15] T. Tsukazaki, T.A. Chiang, A.F. Davison, L. Attisano, J.L. Wrana, Cell 95 (6) (1998) 779.
[16] S. Miura, T. Takeshita, H. Asao, Y. Kimura, K. Murata, Y. Sasaki, J.I. Hanai, H. Beppu, T. Tsukazaki, J.L. Wrana, K. Miyazono, K. Sugamura, Mol. Cell. Biol. 20 (24) (2000) 9346.
[17] B.A. Hocevar, A. Smine, X.X. Xu, P.H. Howe, EMBO J. 20 (11) (2001) 2789.
[18] H. Hayashi, S. Abdollah, Y. Qiu, J. Cai, Y.Y. Xu, B.W. Grinnell, M.A. Richardson, J.N. Topper, M.A.J. Gimbrone, J.L. Wrana, D. Falb, Cell 89 (1997) 1165.
[19] A. Nakao, M. Afrakht, A. Moren, T. Nakayama, J.L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N.E. Heldin, C.H. Heldin, P. ten Dijke, Nature 389 (1997) 631.
[20] S. Souchelnytskyi, T. Nakayama, A. Nakao, A. Moren, C.H. Heldin, J.L. Christian, P. ten Dijke, J. Biol. Chem. 273 (1998) 25364.
[21] T. Ebisawa, M. Fukuchi, G. Murakami, T. Chiba, K. Tanaka, T. Imamura, K. Miyazono, J. Biol. Chem. 276 (16) (2001) 12477.
[22] P. Kavsak, R.K. Rasmussen, C.G. Causing, S. Bonni, H. Zhu, G.H. Thomsen, J.L. Wrana, Mol. Cell 6 (6) (2000) 1365.
[23] P.K. Datta, H.L. Moses, Mol. Cell. Biol. 20 (9) (2000) 3157.
[24] N. Ibarrola, I. Kratchmarova, D. Nakajima, W.P. Schiemann, A. Moustakas, A. Pandey, M. Mann, BMC Cell Biol. 5 (2004) 2.
[25] D. Koinuma, M. Shinozaki, A. Komuro, K. Goto, M. Saitoh, A. Hanyu, M. Ebina, T. Nukiwa, K. Miyazawa, T. Imamura, K. Miyazono, EMBO J. 22 (24) (2003) 6458.
[26] F.Y. Liu, X.Z. Li, Y.M. Peng, H. Liu, Y.H. Liu, Am. J. Nephrol. 27 (2) (2007) 176.
[27] W. Liu, H. Rui, J. Wang, S. Lin, Y. He, M. Chen, Q. Li, Z. Ye, S. Zhang, S.C. Chan, Y.G. Chen, J. Han, S.C. Lin, EMBO J. 25 (8) (2006) 1646.
[28] G.J. Inman, F.J. Nicolas, C.S. Hill, Mol. Cell 10 (2) (2002) 283.
[29] L. Xu, J. Massague, Nat. Rev. Mol. Cell Biol. 5 (3) (2004) 209.
[30] M. Kretzschmar, J. Doody, I. Timokhina, J. Massaguâe, Genes Dev. 13 (7) (1999) 804.
[31] M.E. Engel, M.A. McDonnell, B.K. Law, H.L. Moses, J. Biol. Chem. 274 (52) (1999) 37413.
[32] F. Furukawa, K. Matsuzaki, S. Mori, Y. Tahashi, K. Yoshida, Y. Sugano, H. Yamagata, M. Matsushita, T. Seki, Y. Inagaki, M. Nishizawa, J. Fujisawa, K. Inoue, Hepatology 38 (4) (2003) 879.
[33] A.K. Kamaraju, A.B. Roberts, J. Biol. Chem. 280 (2) (2005) 1024.
[34] S.J. Wicks, S. Lui, N. Abdel-Wahab, R.M. Mason, A. Chantry, Mol. Cell. Biol. 20 (21) (2000) 8103.
[35] D.S. Waddell, N.T. Liberati, X. Guo, J.P. Frederick, X.F. Wang, J. Biol. Chem. 279 (28) (2004) 29236.
[36] M. Cordenonsi, M. Montagner, M. Adorno, L. Zacchigna, G. Martello, A. Mamidi, S. Soligo, S. Dupont, S. Piccolo, Science 315 (5813) (2007) 840.
[37] I. Matsuura, N.G. Denissova, G. Wang, D. He, J. Long, F. Liu, Nature 430 (6996) (2004) 226.
[38] G. Sapkota, M. Knockaert, C. Alarcon, E. Montalvo, A.H. Brivanlou, J. Massague, J. Biol. Chem. 281 (52) (2006) 40412.
[39] P.S. Lee, C. Chang, D. Liu, R. Derynck, J. Biol. Chem. 278 (30) (2003) 27853.
[40] X. Lin, M. Liang, Y.Y. Liang, F.C. Brunicardi, X.H. Feng, J. Biol. Chem. 278 (33) (2003) 31043.
[41] X. Lin, M. Liang, Y.Y. Liang, F.C. Brunicardi, F. Melchior, X.H. Feng, J. Biol. Chem. 278 (21) (2003) 18714.
[42] J. Long, G. Wang, D. He, F. Liu, Biochem. J. 379 (Pt 1) (2004) 23.
[43] T. Ohshima, K. Shimotohno, J. Biol. Chem. 278 (51) (2003) 50833.
[44] X. Lin, X. Duan, Y.Y. Liang, Y. Su, K.H. Wrighton, J. Long, M. Hu, C.M. Davis, J. Wang, F.C. Brunicardi, Y. Shi, Y.G. Chen, A. Meng, X.H. Feng, Cell 125 (5) (2006) 915.
[45] M. Fukuchi, T. Imamura, T. Chiba, T. Ebisawa, M. Kawabata, K. Tanaka, K. Miyazono, Mol. Biol. Cell 12 (5) (2001) 1431.
[46] L. Izzi, L. Attisano, Oncogene 23 (11) (2004) 2071.
[47] X. Lin, M. Liang, X.H. Feng, J. Biol. Chem. 275 (47) (2000) 36818.
[48] R.S. Lo, J. Massague, Nat. Cell Biol. 1 (8) (1999) 472.
[49] H. Zhu, P. Kavsak, S. Abdollah, J.L. Wrana, G.H. Thomsen, Nature 400 (6745) (1999) 687.
[50] S. Dupont, A. Mamidi, M. Cordenonsi, M. Montagner, L. Zacchigna, M. Adorno, G. Martello, M.J. Stinchfield, S. Soligo, L. Morsut, M. Inui, S. Moro, N. Modena, F. Argenton, S.J. Newfeld, S. Piccolo, Cell 136 (1) (2009) 123.
[51] A.V. Bakin, C. Rinehart, A.K. Tomlinson, C.L. Arteaga, J. Cell Sci. 115(Pt) (15) (2002) 3193.
[52] A.V. Bakin, A.K. Tomlinson, N.A. Bhowmick, H.L. Moses, C.L. Arteaga, J. Biol. Chem. 275 (47) (2000) 36803.
[53] N.A. Bhowmick, M. Ghiassi, A. Bakin, M. Aakre, C.A. Lundquist, M.E. Engel, C.L. Arteaga, H.L. Moses, Mol. Biol. Cell 12 (1) (2001) 27.
[54] R. Perlman, W.P. Schiemann, M.W. Brooks, H.F. Lodish, R.A. Weinberg, Nat. Cell Biol. 3 (8) (2001) 708.
[55] J. Zavadil, M. Bitzer, D. Liang, Y.C. Yang, A. Massimi, S. Kneitz, E. Piek, E.P. Bottinger, Proc. Natl. Acad. Sci. U.S.A. 98 (12) (2001) 6686.
[56] S. Lamouille, R. Derynck, J. Cell Biol. 178 (3) (2007) 437.
[57] M. Azuma, K. Motegi, K. Aota, T. Yamashita, H. Yoshida, M. Sato, Exp. Cell Res. 250 (1) (1999) 213.
[58] J.C. Horowitz, D.S. Rogers, V. Sharma, R. Vittal, E.S. White, Z. Cui, V.J. Thannickal, Cell. Signal. 19 (4) (2007) 761.
[59] V.J. Thannickal, D.Y. Lee, E.S. White, Z. Cui, J.M. Larios, R. Chacon, J.C. Horowitz, R.M. Day, P.E. Thomas, J. Biol. Chem. 278 (14) (2003) 12384.
[60] A.J. Galliher, W.P. Schiemann, Breast Cancer Res. 8 (4) (2006) R42.
[61] A.J. Galliher, W.P. Schiemann, Cancer Res. 67 (8) (2007) 3752.
[62] A.J. Galliher-Beckley, W.P. Schiemann, Carcinogenesis 29 (2008) 244.
[63] S.S. Park, Y.W. Eom, E.H. Kim, J.H. Lee, D.S. Min, S. Kim, S.J. Kim, K.S. Choi, Oncogene 23 (37) (2004) 6272.
[64] B. Elenbaas, R.A. Weinberg, Exp. Cell Res. 264 (1) (2001) 169.
[65] L.L. Myeroff, R. Parsons, S.-J. Kim, L. Hedrick, K.R. Cho, K. Orth, M. Mathis, K.W. Kinzler, J. Lutterbaugh, K. Park, Y.-J. Bang, H.Y. Lee, J.-G. Park, H.T. Lynch, A.B. Roberts, B. Vogelstein, S.D. Markowitz, Cancer Res. 55 (1995) 5545.
[66] K.A. Waite, C. Eng, Nat. Rev. Genet. 4 (10) (2003) 763.
[67] W.M. Grady, L.L. Myeroff, S.E. Swinler, A. Rajput, S. Thiagalingam, J.D. Lutterbaugh, A. Neumann, M.G. Brattain, J. Chang, S.J. Kim, K.W. Kinzler, B. Vogelstein, J.K. Willson, S. Markowitz, Cancer Res. 59 (2) (1999) 320.
[68] S. Markowitz, J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R.S. Fan, E. Zborowska, K.W. Kinzler, B. Vogelstein, M. Brattain, J.K.V. Willson, Science 268 (5215) (1995) 1336.
[69] R. Parsons, L.L. Myeroff, B. Liu, J.K. Willson, S.D. Markowitz, K.W. Kinzler, B. Vogelstein, Cancer Res. 55 (23) (1995) 5548.
[70] W. Grady, J. Willis, P. Trobridge, J. Romero-Gallo, N. Munoz, J. Olechnowicz, K. Ferguson, S. Gautam, S. Markowitz, Int. J. Cancer 118 (3) (2006) 600.
[71] T. Watanabe, T.T. Wu, P.J. Catalano, T. Ueki, R. Satriano, D.G. Haller, A.B. Benson III, S.R. Hamilton, N Engl J. Med. 344 (16) (2001) 1196.
[72] R.L. Elliott, G.C. Blobe, J. Clin. Oncol. 23 (9) (2005) 2078.
[73] J. Seoane, Carcinogenesis 27 (11) (2006) 2148.
[74] W.P. Schiemann, W.M. Pfeifer, E. Levi, M.E. Kadin, H.F. Lodish, Blood 94 (8) (1999) 2854.
[75] W.P. Schiemann, D. Rotzer, W.M. Pfeifer, E. Levi, K.R. Rai, P. Knaus, M.E. Kadin, Cancer Detect. Prev. 28 (1) (2004) 57.
[76] Y. Bian, T. Caldes, J. Wijnen, P. Franken, H. Vasen, V. Kaklamani, K. Nafa, P. Peterlongo, N. Ellis, J.A. Baron, J. Burn, G. Moeslein, P.J. Morrison, Y. Chen, H. Ahsan, P. Watson, H.T. Lynch, A. de la Chapelle, R. Fodde, B. Pasche, J. Clin. Oncol. 23 (13) (2005) 3074.
[77] V.G. Kaklamani, L. Baddi, J. Liu, D. Rosman, S. Phukan, C. Bradley, C. Hegarty, B. McDaniel, A. Rademaker, C. Oddoux, H. Ostrer, L.S. Michel, H. Huang, Y. Chen, H. Ahsan, K. Offit, B. Pasche, Cancer Res. 65 (8) (2005) 3454.
[78] B. Pasche, T.J. Knobloch, Y. Bian, J. Liu, S. Phukan, D. Rosman, V. Kaklamani, L. Baddi, F.S. Siddiqui, W. Frankel, T.W. Prior, D.E. Schuller, A. Agrawal, J. Lang, M.E. Dolan, E.E. Vokes, W.S. Lane, C.C. Huang, T. Caldes, A. Di Cristofano, H. Hampel, I. Nilsson, G. von Heijne, R. Fodde, V.V. Murty, A. de la Chapelle, C.M. Weghorst, Jama 294 (13) (2005) 1634.
[79] T. Chen, D. Carter, L. Garrigue-Antar, M. Reiss, Cancer Res. 58 (21) (1998) 4805.
[80] T. Chen, W. Yan, R.G. Wells, D.L. Rimm, J. McNiff, D. Leffell, M. Reiss, Int. J. Cancer 93 (5) (2001) 653.
[81] M. Dong, T. How, K.C. Kirkbride, K.J. Gordon, J.D. Lee, N. Hempel, P. Kelly, B.J. Moeller, J.R. Marks, G.C. Blobe, J. Clin. Invest. 117 (1) (2007) 206.
[82] E.C. Finger, R.S. Turley, M. Dong, T. How, T.A. Fields, G.C. Blobe, Carcinogenesis 29 (3) (2008) 528.
[83] K.J. Gordon, M. Dong, E.M. Chislock, T.A. Fields, G.C. Blobe, Carcinogenesis 29 (2) (2008) 252.
[84] N. Hempel, T. How, M. Dong, S.K. Murphy, T.A. Fields, G.C. Blobe, Cancer Res. 67 (11) (2007) 5231.
[85] R.S. Turley, E.C. Finger, N. Hempel, T. How, T.A. Fields, G.C. Blobe, Cancer Res. 67 (3) (2007) 1090.
[86] L.J. Jonk, S. Itoh, C.H. Heldin, P. ten Dijke, W. Kruijer, J. Biol. Chem. 273 (33) (1998) 21145.
[87] J.P. Frederick, N.T. Liberati, D.S. Waddell, Y. Shi, X.F. Wang, Mol. Cell. Biol. 24 (6) (2004) 2546.
[88] J. Massague, J. Seoane, D. Wotton, Genes Dev. 19 (23) (2005) 2783.
[89] C.R. Chen, Y. Kang, P.M. Siegel, J. Massague, Cell 110 (1) (2002) 19.
[90] L.A. White, T.I. Mitchell, C.E. Brinckerhoff, Biochim. Biophys. Acta 1490 (3) (2000) 259.
[91] C.-H. Heldin, K. Miyazono, P. ten Dijke, Nature 390 (1997) 465.
[92] W. Friedl, S. Uhlhaas, K. Schulmann, M. Stolte, S. Loff, W. Back, E. Mangold, M. Stern, H.P. Knaebel, C. Sutter, R.G. Weber, S. Pistorius, B. Burger, P. Propping, Hum. Genet. 111 (1) (2002) 108.
[93] L. Yang, C. Mao, Y. Teng, W. Li, J. Zhang, X. Cheng, X. Li, X. Han, Z. Xia, H. Deng, X. Yang, Cancer Res. 65 (19) (2005) 8671.
[94] B.G. Kim, C. Li, W. Qiao, M. Mamura, B. Kasperczak, M. Anver, L. Wolfraim, S. Hong, E. Mushinski, M. Potter, S.J. Kim, X.Y. Fu, C. Deng, J.J. Letterio, Nature 441 (7096) (2006) 1015.
[95] K. Takaku, M. Oshima, H. Miyoshi, M. Matsui, M.F. Seldin, M.M. Taketo, Cell 92 (5) (1998) 645.
[96] S.U. Han, H.T. Kim, H. Seong do, Y.S. Kim, Y.S. Park, Y.J. Bang, H.K. Yang, S.J. Kim, Oncogene 23 (7) (2004) 1333.
[97] L.A. Wolfraim, T.M. Fernandez, M. Mamura, W.L. Fuller, R. Kumar, D.E. Cole, S. Byfield, A. Felici, K.C. Flanders, T.M. Walz, A.B. Roberts, P.D. Aplan, F.M. Balis, J.J. Letterio, N Engl J. Med. 351 (6) (2004) 552.
[98] L. Maggio-Price, P. Treuting, W. Zeng, M. Tsang, H. Bielefeldt-Ohmann, B.M. Iritani, Cancer Res. 66 (2) (2006) 828.
[99] A.G. Li, S.L. Lu, M.X. Zhang, C. Deng, X.J. Wang, Cancer Res. 64 (21) (2004) 7836.
[100] P.M. Siegel, J. Massague, Nat. Rev. Cancer 3 (11) (2003) 807.
[101] P.M. Siegel, W. Shu, J. Massague, J. Biol. Chem. 278 (37) (2003) 35444.
[102] Y. Kang, C.R. Chen, J. Massague, Mol. Cell 11 (4) (2003) 915.
[103] S. Wu, C. Cetinkaya, M.J. Munoz-Alonso, N. von der Lehr, F. Bahram, V. Beuger, M. Eilers, J. Leon, L.G. Larsson, Oncogene 22 (3) (2003) 351.
[104] J. Seoane, C. Pouponnot, P. Staller, M. Schader, M. Eilers, J. Massague, Nat. Cell Biol. 3 (4) (2001) 400.
[105] J. Seoane, H.V. Le, L. Shen, S.A. Anderson, J. Massague, Cell 117 (2) (2004) 211.
[106] A. Moustakas, D. Kardassis, Proc. Natl. Acad. Sci. U.S.A. 95 (12) (1998) 6733.
[107] C. Brenner, R. Deplus, C. Didelot, A. Loriot, E. Vire, C. De Smet, A. Gutierrez, D. Danovi, D. Bernard, T. Boon, P.G. Pelicci, B. Amati, T. Kouzarides, Y. de Launoit, L. Di Croce, F. Fuks, EMBO J. 24 (2) (2005) 336.

[108] J. Luo, B.D. Manning, L.C. Cantley, Cancer Cell 4 (4) (2003) 257.
[109] A. Atfi, L. Abecassis, M.F. Bourgeade, EMBO Rep. 6 (10) (2005) 985.
[110] A.R. Conery, Y. Cao, E.A. Thompson, C.M. Townsend, T.C. Ko, K. Luo, Nat. Cell Biol. 6 (2004) 366.
[111] I. Remy, A. Montmarquette, S.W. Michnick, Nat. Cell Biol. 6 (2004) 358.
[112] R.R. Gomis, C. Alarcon, C. Nadal, C. Van Poznak, J. Massague, Cancer Cell 10 (3) (2006) 203.
[113] D. Noda, S. Itoh, Y. Watanabe, M. Inamitsu, S. Dennler, F. Itoh, S. Koike, D. Danielpour, P. Ten Dijke, M. Kato, Oncogene 25 (41) (2006) 5591.
[114] S.V. Tavtigian, J. Simard, D.H. Teng, V. Abtin, M. Baumgard, A. Beck, N.J. Camp, A.R. Carillo, Y. Chen, P. Dayananth, M. Desrochers, M. Dumont, J.M. Farnham, D. Frank, C. Frye, S. Ghaffari, J.S. Gupte, R. Hu, D. Iliev, T. Janecki, E.N. Kort, K.E. Laity, A. Leavitt, G. Leblanc, J. McArthur-Morrison, A. Pederson, B. Penn, K.T. Peterson, J.E. Reid, S. Richards, M. Schroeder, R. Smith, S.C. Snyder, B. Swedlund, J. Swensen, A. Thomas, M. Tranchant, A.M. Woodland, F. Labrie, M.H. Skolnick, S. Neuhausen, J. Rommens, L.A. Cannon-Albright, Nat. Genet. 27 (2) (2001) 172.
[115] K. Luo, Curr. Opin. Genet. Dev. 14 (1) (2004) 65.
[116] X. Liu, Y. Sun, R.A. Weinberg, H.F. Lodish, Cytokine Growth Factor Rev. 12 (1) (2001) 1.
[117] B. Karakas, A. Weeraratna, A. Abukhdeir, B.G. Blair, H. Konishi, S. Arena, K. Becker, W. Wood III, P. Argani, A.M. De Marzo, K.E. Bachman, B.H. Park, Oncogene 25 (40) (2006) 5561.
[118] A. Igarashi, H. Okochi, D.M. Bradham, G.R. Grotendorst, Mol. Biol. Cell 4 (6) (1993) 637.
[119] F. Strutz, M. Zeisberg, A. Renziehausen, B. Raschke, V. Becker, C. van Kooten, G. Muller, Kidney Int. 59 (2) (2001) 579.
[120] R.A. Seifert, S.A. Coats, E.W. Raines, R. Ross, D.F. Bowen-Pope, J. Biol. Chem. 269 (19) (1994) 13951.
[121] D. Chantry, M. Turner, E. Abney, M. Feldmann, J. Immunol. 142 (12) (1989) 4295.
[122] O. Ishikawa, E.C. LeRoy, M. Trojanowska, J. Cell. Physiol. 145 (1) (1990) 181.
[123] P. Feng, K.J. Catt, M. Knecht, J. Biol. Chem. 261 (30) (1986) 14167.
[124] G. Sapkota, C. Alarcon, F.M. Spagnoli, A.H. Brivanlou, J. Massague, Mol. Cell 25 (3) (2007) 441.
[125] R.S. Lo, D. Wotton, J. Massague, EMBO J. 20 (1–2) (2001) 128.
[126] J.P. Thiery, Nat. Rev. Cancer 2 (6) (2002) 442.
[127] G. Greenburg, E.D. Hay, J. Cell Biol. 95 (1) (1982) 333.
[128] J.P. Thiery, Curr. Opin. Cell Biol. 15 (6) (2003) 740.
[129] J. Zavadil, E.P. Bottinger, Oncogene 24 (37) (2005) 5764.
[130] A. Moustakas, C.H. Heldin, Cancer Sci. 98 (10) (2007) 1512.
[131] P. Savagner, Bioessays 23 (10) (2001) 912.
[132] B.C. Willis, Z. Borok, Am. J. Physiol. Lung Cell. Mol. Physiol. 293 (3) (2007) L525.
[133] P.J. Miettinen, R. Ebner, A.R. Lopez, R. Derynck, J. Cell Biol. 127 (6 Pt 2) (1994) 2021.
[134] V. Kaartinen, J.W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp, J. Groffen, Nat. Genet. 11 (4) (1995) 415.
[135] L.A. Romano, R.B. Runyan, Dev. Biol. 223 (1) (2000) 91.
[136] S. Saika, S. Kono-Saika, Y. Ohnishi, M. Sato, Y. Muragaki, A. Ooshima, K.C. Flanders, J. Yoo, M. Anzano, C.Y. Liu, W.W. Kao, A.B. Roberts, Am. J. Pathol. 164 (2) (2004) 651.
[137] S. Saika, S. Kono-Saika, T. Tanaka, O. Yamanaka, Y. Ohnishi, M. Sato, Y. Muragaki, A. Ooshima, J. Yoo, K.C. Flanders, A.B. Roberts, Lab. Invest. 84 (10) (2004) 1245.
[138] M. Sato, Y. Muragaki, S. Saika, A.B. Roberts, A. Ooshima, J. Clin. Invest. 112 (10) (2003) 1486.
[139] N.A. Bhowmick, H.L. Moses, Curr. Opin. Genet. Dev. 15 (1) (2005) 97.
[140] N.A. Bhowmick, E.G. Neilson, H.L. Moses, Nature 432 (7015) (2004) 332.
[141] I.J. Fidler, Semin. Cancer Biol. 12 (2) (2002) 89.
[142] I.J. Fidler, Differentiation 70 (9-10) (2002) 498.
[143] N.A. Bhowmick, A. Chytil, D. Plieth, A.E. Gorska, N. Dumont, S. Shappell, M.K. Washington, E.G. Neilson, H.L. Moses, Science 303 (5659) (2004) 848.
[144] N. Cheng, N.A. Bhowmick, A. Chytil, A.E. Gorksa, K.A. Brown, R. Muraoka, C.L. Arteaga, E.G. Neilson, S.W. Hayward, H.L. Moses, Oncogene 24 (32) (2005) 5053.
[145] L. Yang, J. Huang, X. Ren, A.E. Gorska, A. Chytil, M. Aakre, D.P. Carbone, L.M. Matrisian, A. Richmond, P.C. Lin, H.L. Moses, Cancer Cell 13 (1) (2008) 23.
[146] Y. Kang, W. He, S. Tulley, G.P. Gupta, I. Serganova, C.R. Chen, K. Manova-Todorova, R. Blasberg, W.L. Gerald, J. Massague, Proc. Natl. Acad. Sci. U. S. A. 102 (39) (2005) 13909.
[147] Y. Kang, P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordon-Cardo, T.A. Guise, J. Massague, Cancer Cell 3 (6) (2003) 537.
[148] J.J. Yin, K. Selander, J.M. Chirgwin, M. Dallas, B.G. Grubbs, R. Wieser, J. Massague, G.R. Mundy, T.A. Guise, J. Clin. Invest. 103 (2) (1999) 197.
[149] R.S. Muraoka-Cook, H. Kurokawa, Y. Koh, J.T. Forbes, L.R. Roebuck, M.H. Barcellos-Hoff, S.E. Moody, L.A. Chodosh, C.L. Arteaga, Cancer Res. 64 (24) (2004) 9002.
[150] S.A. Mani, W. Guo, M.J. Liao, E.N. Eaton, A. Ayyanan, A.Y. Zhou, M. Brooks, F. Reinhard, C.C. Zhang, M. Shipitsin, L.L. Campbell, K. Polyak, C. Brisken, J. Yang, R.A. Weinberg, Cell 133 (4) (2008) 704.
[151] M. Shipitsin, L.L. Campbell, P. Argani, S. Weremowicz, N. Bloushtain-Qimron, J. Yao, T. Nikolskaya, T. Serebryiskaya, R. Beroukhim, M. Hu, M.K. Halushka, S. Sukumar, L.M. Parker, K.S. Anderson, L.N. Harris, J.E. Garber, A.L. Richardson, S.J. Schnitt, Y. Nikolsky, R.S. Gelman, K. Polyak, Cancer Cell 11 (3) (2007) 259.
[152] I. Ben-Porath, M.W. Thomson, V.J. Carey, R. Ge, G.W. Bell, A. Regev, R.A. Weinberg, Nat. Genet. 40 (5) (2008) 499.
[153] A.P. Morel, M. Lievre, C. Thomas, G. Hinkal, S. Ansieau, A. Puisieux, PLoS ONE 3 (8) (2008) e2888.
[154] F. Tian, S. DaCosta Byfield, W.T. Parks, S. Yoo, A. Felici, B. Tang, E. Piek, L.M. Wakefield, A.B. Roberts, Cancer Res. 63 (23) (2003) 8284.
[155] M. Deckers, M. van Dinther, J. Buijs, I. Que, C. Lowik, G. van der Pluijm, P. ten Dijke, Cancer Res. 66 (4) (2006) 2202.
[156] N. Bardeesy, K.H. Cheng, J.H. Berger, G.C. Chu, J. Pahler, P. Olson, A.F. Hezel, J. Horner, G.Y. Lauwers, D. Hanahan, R.A. DePinho, Genes Dev. 20 (22) (2006) 3130.
[157] H. Azuma, S. Ehata, H. Miyazaki, T. Watabe, O. Maruyama, T. Imamura, T. Sakamoto, S. Kiyama, Y. Kiyama, T. Ubai, T. Inamoto, S. Takahara, Y. Itoh, Y. Otsuki, Y. Katsuoka, K. Miyazono, S. Horie, J. Natl Cancer Inst. 97 (23) (2005) 1734.
[158] S.K. Leivonen, R. Ala-Aho, K. Koli, R. Grenman, J. Peltonen, V.M. Kahari, Oncogene 25 (18) (2006) 2588.
[159] S.K. Leivonen, V.M. Kahari, Int. J. Cancer 121 (10) (2007) 2119.
[160] D. Javelaud, K.S. Mohammad, C.R. McKenna, P. Fournier, F. Luciani, M. Niewolna, J. Andre, V. Delmas, L. Larue, T.A. Guise, A. Mauviel, Cancer Res. 67 (5) (2007) 2317.
[161] M. Adorno, M. Cordenonsi, M. Montagner, S. Dupont, C. Wong, B. Hann, A. Solari, S. Bobisse, M.B. Rondina, V. Guzzardo, A.R. Parenti, A. Rosato, S. Bicciato, A. Balmain, S. Piccolo, Cell 137 (1) (2009) 87.
[162] P. Papageorgis, A.W. Lambert, S. Ozturk, F. Gao, H. Pan, U. Manne, Y.O. Alekseyev, A. Thiagalingam, H.M. Abdolmaleky, M. Lenburg, S. Thiagalingam, Cancer Res. 70 (3) (2010) 968.
[163] N.A. Bhowmick, R. Zent, M. Ghiassi, M. McDonnell, H.L. Moses, J. Biol. Chem. 276 (50) (2001) 46707.
[164] M. Davies, M. Robinson, E. Smith, S. Huntley, S. Prime, I. Paterson, J. Cell. Biochem. 95 (5) (2005) 918.
[165] V. Ellenrieder, S.F. Hendler, W. Boeck, T. Seufferlein, A. Menke, C. Ruhland, G. Adler, T.M. Gress, Cancer Res. 61 (10) (2001) 4222.
[166] E. Janda, K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, S. Grunert, J. Cell Biol. 156 (2) (2002) 299.
[167] L. Xie, B.K. Law, A.M. Chytil, K.A. Brown, M.E. Aakre, H.L. Moses, Neoplasia 6 (5) (2004) 603.
[168] M.A. Huber, N. Azoitei, B. Baumann, S. Grunert, A. Sommer, H. Pehamberger, N. Kraut, H. Beug, T. Wirth, J. Clin. Invest. 114 (4) (2004) 569.
[169] J. Zavadil, L. Cermak, N. Soto-Nieves, E.P. Bottinger, EMBO J. 23 (5) (2004) 1155.
[170] K. Kim, Z. Lu, E.D. Hay, Cell Biol. Int. 26 (5) (2002) 463.
[171] S. Araki, J.A. Eitel, C.N. Batuello, K. Bijangi-Vishehsaraei, X.J. Xie, D. Danielpour, K.E. Pollok, D.A. Boothman, L.D. Mayo, J. Clin. Invest. 120 (1) (2010) 290.
[172] M. Oft, R.J. Akhurst, A. Balmain, Nat. Cell Biol. 4 (7) (2002) 487.
[173] W. Cui, D.J. Fowlis, S. Bryson, E. Duffie, H. Ireland, A. Balmain, R.J. Akhurst, Cell 86 (4) (1996) 531.
[174] D.J. Fowlis, W. Cui, S.A. Johnson, A. Balmain, R.J. Akhurst, Cell Growth Differ. 7 (5) (1996) 679.
[175] K. Lehmann, E. Janda, C.E. Pierreux, M. Rytèomaa, A. Schulze, M. McMahon, C.S. Hill, H. Beug, J. Downward, Genes Dev. 14 (20) (2000) 2610.
[176] C. Prunier, P.H. Howe, J. Biol. Chem. 280 (17) (2005) 17540.
[177] E.K. Sloan, N. Pouliot, K.L. Stanley, J. Chia, J.M. Moseley, D.K. Hards, R.L. Anderson, Breast Cancer Res. 8 (2) (2006) R20.
[178] A. Bandyopadhyay, J.K. Agyin, L. Wang, Y. Tang, X. Lei, B.M. Story, J.E. Cornell, B.H. Pollock, G.R. Mundy, L.Z. Sun, Cancer Res. 66 (13) (2006) 6714.
[179] B. Ozdamar, R. Bose, M. Barrios-Rodiles, H.R. Wang, Y. Zhang, J.L. Wrana, Science 307 (5715) (2005) 1603.
[180] P. Carmeliet, Nat. Med. 6 (4) (2000) 389.
[181] P. Carmeliet, Nat. Med. 9 (6) (2003) 653.
[182] P. Carmeliet, R.K. Jain, Nature 407 (6801) (2000) 249.
[183] P. Bertolino, M. Deckers, F. Lebrin, P. ten Dijke, Chest 128 (6 Suppl) (2005) 585S.
[184] F. Lebrin, M. Deckers, P. Bertolino, P. Ten Dijke, Cardiovasc. Res. 65 (3) (2005) 599.
[185] M.S. Pepper, Cytokine Growth Factor Rev. 8 (1) (1997) 21.
[186] M.S. Pepper, J.D. Vassalli, L. Orci, R. Montesano, Exp. Cell Res. 204 (2) (1993) 356.
[187] R.A. Wolff, R.L. Malinowski, N.S. Heaton, D.A. Hullett, J.R. Hoch, J. Vasc. Surg. 43 (5) (2006) 1028.
[188] M.C. Dickson, J.S. Martin, F.M. Cousins, A.B. Kulkarni, S. Karlsson, R.J. Akhurst, Development 121 (6) (1995) 1845.
[189] J. Larsson, M.J. Goumans, L.J. Sjostrand, M.A. van Rooijen, D. Ward, P. Leveen, X. Xu, P. ten Dijke, C.L. Mummery, S. Karlsson, EMBO J. 20 (7) (2001) 1663.
[190] M.J. Goumans, A. Zwijsen, M.A. van Rooijen, D. Huylebroeck, B.A. Roelen, C.L. Mummery, Development 126 (16) (1999) 3473.
[191] M. Oshima, H. Oshima, M.M. Taketo, Dev. Biol. 179 (1) (1996) 297.
[192] C.B. Brown, A.S. Boyer, R.B. Runyan, J.V. Barnett, Science 283 (5410) (1999) 2080.
[193] L.A. Compton, D.A. Potash, C.B. Brown, J.V. Barnett, Circ. Res. 101 (8) (2007) 784.
[194] M.J. Goumans, G. Valdimarsdottir, S. Itoh, F. Lebrin, J. Larsson, C. Mummery, S. Karlsson, P. ten Dijke, Mol. Cell 12 (4) (2003) 817.
[195] S.P. Oh, T. Seki, K.A. Goss, T. Imamura, Y. Yi, P.K. Donahoe, L. Li, K. Miyazono, P. ten Dijke, S. Kim, E. Li, Proc. Natl. Acad. Sci. U. S. A. 97 (6) (2000) 2626.
[196] H.M. Arthur, J. Ure, A.J. Smith, G. Renforth, D.I. Wilson, E. Torsney, R. Charlton, D.V. Parums, T. Jowett, D.A. Marchuk, J. Burn, A.G. Diamond, Dev. Biol. 217 (1) (2000) 42.
[197] R.J. Lechleider, J.L. Ryan, L. Garrett, C. Eng, C. Deng, A. Wynshaw-Boris, A.B. Roberts, Dev. Biol. 240 (1) (2001) 157.
[198] H. Chang, D. Huylebroeck, K. Verschueren, Q. Guo, M.M. Matzuk, A. Zwijsen, Development 126 (8) (1999) 1631.
[199] C.L. Shovlin, J.M. Hughes, J. Scott, C.E. Seidman, J.G. Seidman, Am. J. Hum. Genet. 61 (1) (1997) 68.
[200] K.A. McAllister, K.M. Grogg, D.W. Johnson, C.J. Gallione, M.A. Baldwin, C.E. Jackson, E.A. Helmbold, D.S. Markel, W.C. McKinnon, J. Murrell, et al., Nat. Genet. 8 (4) (1994) 345.

[201] J.N. Berg, C.J. Gallione, T.T. Stenzel, D.W. Johnson, W.P. Allen, C.E. Schwartz, C.E. Jackson, M.E. Porteous, D.A. Marchuk, Am. J. Hum. Genet. 61 (1) (1997) 60.
[202] D.W. Johnson, J.N. Berg, M.A. Baldwin, C.J. Gallione, I. Marondel, S.J. Yoon, T.T. Stenzel, M. Speer, M.A. Pericak-Vance, A. Diamond, A.E. Guttmacher, C.E. Jackson, L. Attisano, R. Kucherlapati, M.E. Porteous, D.A. Marchuk, Nat. Genet. 13 (2) (1996) 189.
[203] A. Bourdeau, D.J. Dumont, M. Letarte, J. Clin. Invest. 104 (10) (1999) 1343.
[204] S. Srinivasan, M.A. Hanes, T. Dickens, M.E. Porteous, S.P. Oh, L.P. Hale, D.A. Marchuk, Hum. Mol. Genet. 12 (5) (2003) 473.
[205] M.J. Goumans, F. Lebrin, G. Valdimarsdottir, Trends Cardiovasc. Med. 13 (7) (2003) 301.
[206] M.J. Goumans, G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras, P. ten Dijke, EMBO J. 21 (7) (2002) 1743.
[207] X. Wu, J. Ma, J.D. Han, N. Wang, Y.G. Chen, Microvasc. Res. 71 (1) (2006) 12.
[208] T. Ota, M. Fujii, T. Sugizaki, M. Ishii, K. Miyazawa, H. Aburatani, K. Miyazono, J. Cell. Physiol. 193 (3) (2002) 299.
[209] A. Bandyopadhyay, F. Lopez-Casillas, S.N. Malik, J.L. Montiel, V. Mendoza, J. Yang, L.Z. Sun, Cancer Res. 62 (16) (2002) 4690.
[210] A. Bandyopadhyay, Y. Zhu, M.L. Cibull, L. Bao, C. Chen, L. Sun, Cancer Res. 59 (19) (1999) 5041.
[211] A. Bandyopadhyay, Y. Zhu, S.N. Malik, J. Kreisberg, M.G. Brattain, E.A. Sprague, J. Luo, F. Lopez-Casillas, L.Z. Sun, Oncogene 21 (22) (2002) 3541.
[212] F. Lebrin, M.J. Goumans, L. Jonker, R.L. Carvalho, G. Valdimarsdottir, M. Thorikay, C. Mummery, H.M. Arthur, P. ten Dijke, EMBO J. 23 (20) (2004) 4018.
[213] C. Li, I.N. Hampson, L. Hampson, P. Kumar, C. Bernabeu, S. Kumar, FASEB J. 14 (1) (2000) 55.
[214] J. Massague, S.W. Blain, R.S. Lo, Cell 103 (2) (2000) 295.
[215] A. Sanchez-Capelo, Cytokine Growth Factor Rev. 16 (2005) 15.
[216] N.G. Docherty, O.E. O'Sullivan, D.A. Healy, M. Murphy, A.J. O'Neill, J.M. Fitzpatrick, R.W. Watson, Am. J. Physiol. Ren. Physiol. 290 (0363-6127 (Print)) (2006) F1202.
[217] Y. Yang, X. Pan, W. Lei, W. J., J. Song, Oncogene 25 (2006) 7235.
[218] S. Biswas, M. Guix, C. Rinehart, T.C. Dugger, A. Chytil, H.L. Moses, M.L. Freeman, C.L. Arteaga, J. Clin. Invest. 117 (5) (2007) 1305.
[219] X. Lei, A. Bandyopadhyay, T. Le, L. Sun, Oncogene 21 (2002) 7514.
[220] M. Arsura, G.R. Panta, J.D. Bilyeu, L.G. Cavin, M.A. Sovak, A.A. Oliver, V. Factor, R. Heuchel, F. Mercurio, S.S. Thorgeirsson, G.E. Sonenshein, Oncogene 22 (2003) 412.
[221] S.J. Lin, C. Chang, A.K. Ng, S.H. Wang, J.J. Li, C.P. Hu, Apoptosis 12 (2007) 1659.
[222] B. Rayet, C. Gelinas, Oncogene 18 (49) (1999) 6938.
[223] I. Shin, A.V. Bakin, U. Rodeck, A. Brunet, C.L. Arteaga, Mol. Biol. Cell 12 (11) (2001) 3328.
[224] Y. Cao, M. Karin, J. Mammary Gland Biol. Neoplasia 8 (2) (2003) 215.
[225] J.R. Neil, W.P. Schiemann, Cancer Res. 68 (2008) 1462.
[226] J.-I. Park, M.-G. Lee, K. Cho, B.-J. Park, K.-S. Chae, D.-S. Byun, B.-K. Ryu, Y.-K. Park, S.-G. Chi, Oncogene 22 (2003) 4314.
[227] J.R. Neil, K.M. Johnson, R.A. Nemenoff, W.P. Schiemann, Carcinogenesis 29 (11) (2008) 2227.
[228] M. Tian, W.P. Schiemann, FASEB J. 24 (2009) 1105.
[229] S. Kaur, F. Wang, M. Venkatraman, M. Arsura, J. Biol. Chem. 280 (0021-9258 (Print)) (2005) 38599.
[230] C. Van Themsche, I. Mathieu, S. Parent, E. Asselin, J. Biol. Chem. 282 (7) (2007) 4794.
[231] J.R. Neil, M. Tian, W.P. Schiemann, J. Biol. Chem. 284 (32) (2009) 21209.
[232] L.V. Madrid, C.Y. Wang, D.C. Guttridge, A.J. Schottelius, A.S. Baldwin Jr., M.W. Mayo, Mol. Cell. Biol. 20 (5) (2000) 1626.
[233] L.V. Madrid, M.W. Mayo, J.Y. Reuther, A.S. Baldwin Jr., J. Biol. Chem. 276 (2001) 18934.
[234] M.C. Hu, D.F. Lee, W. Xia, L.S. Golfman, F. Ou-Yang, J.Y. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, M.C. Hung, Cell 117 (2) (2004) 225.
[235] T. Hiraga, A. Myoui, M.E. Choi, H. Yoshikawa, T. Yoneda, Cancer Res. 66 (2006) 2067.
[236] S. Lu, Z. Dong, Prostate 66 (9) (2006) 996.
[237] G.A. Enders, Br. J. Cancer 97 (10) (2007) 1388.
[238] L. Gorelik, R.A. Flavell, Nat. Rev. Immunol. 2 (2002) 46.
[239] M.O. Li, Y.Y. Wan, S. Sanjabi, A.K. Robertson, R.A. Flavell, Annu. Rev. Immunol. 24 (2006) 99.
[240] W.W. Lin, M. Karin, J. Clin. Invest. 117 (0021-9738 (Print)) (2007) 1175.
[241] L. Gorelik, R.A. Flavell, Immunity 12 (2) (2000) 171.
[242] A.B. Kulkarni, C.G. Huh, D. Becker, A. Geiser, M. Lyght, K.C. Flanders, A.B. Roberts, M.B. Sporn, J.M. Ward, S. Karlsson, Proc. Natl Acad. Sci. 90 (1993) 770.
[243] X. Yang, J.J. Letterio, R.J. Lechleider, L. Chen, R. Hayman, H. Gu, A.B. Roberts, C. Deng, EMBO J. 18 (5) (1999) 1280.
[244] B.I. Dalal, P.A. Keown, A.H. Greenberg, Am. J. Pathol. 143 (2) (1993) 381.
[245] S.M. Gorsch, V.A. Memoli, T.A. Stukel, L.I. Gold, B.A. Arrick, Cancer Res. 52 (1992) 6949.
[246] V. Ivanovic, N. Todorovic-Rakovic, M. Demajo, Z. Neskovic-Konstantinovic, V. Subota, O. Ivanisevic-Milovanovic, D. Nikolic-Vukosavljevic, Eur. J. Cancer 39 (4) (2003) 454.
[247] H.F. Dvorak, N Engl J. Med. 315 (26) (1986) 1650.
[248] B.A. Teicher, Clin. Cancer Res. 13 (21) (2007) 6247.
[249] S.H. Wrzesinski, Y.Y. Wan, R.A. Flavell, Clin. Cancer Res. 13 (18) (2007) 5262.
[250] L. Gorelik, R.A. Flavell, Nat. Med. 7 (10) (2001) 1118.
[251] Q. Zhang, X. Yang, M. Pins, B. Javonovic, T. Kuzel, S.J. Kim, L.V. Parijs, N.M. Greenberg, V. Liu, Y. Guo, C. Lee, Cancer Res. 65 (5) (2005) 1761.
[252] H.G. Kopp, T. Placke, H.R. Salih, Cancer Res. 69 (19) (2009) 7775.
[253] N. Larmonier, D. Cathelin, C. Larmonier, A. Nicolas, D. Merino, N. Janikashvili, S. Audia, A. Bateman, J. Thompson, T. Kottke, T. Hartung, E. Katsanis, R. Vile, B. Bonnotte, Exp. Cell Res. 313 (11) (2007) 2345.
[254] P. Conti, M.I. Castellani, D. Kempuraj, V. Salini, J. Vecchiet, S. Tete, F. Mastrangelo, A. Perrella, M.A. De Lutiis, M. Tagen, T.C. Theoharides, Ann. Clin. Lab. Sci. 37 (4) (2007) 315.
[255] E.Y. Lin, J.F. Li, L. Gnatovskiy, D. Y., L. Zhu, D.A. Grzesik, H. Qian, X.N. Xue, J.W. Pollardc, Cancer Res. 66 (23) (2006) 11238.
[256] J.W. Pollard, Nat. Rev. Cancer 4 (1) (2004) 71.
[257] Z.G. Fridlender, J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G.S. Worthen, S.M. Albelda, Cancer Cell 16 (3) (2009) 183.
[258] S.A. Stewart, R.A. Weinberg, Annu. Rev. Cell Dev. Biol. 22 (2006) 531.
[259] A.L. Ducrest, H. Szutorisz, J. Lingner, M. Nabholz, Oncogene 21 (4) (2002) 541.
[260] H. Li, D. Xu, B.H. Toh, J.P. Liu, Cell Res. 16 (2) (2006) 169.
[261] A.E. Elkak, R.F. Newbold, V. Thomas, K. Mokbel, Cancer Cell Int. 3 (1) (2003) 9.
[262] H. Li, D. Xu, J. Li, M.C. Berndt, J.P. Liu, J. Biol. Chem. 281 (35) (2006) 25588.
[263] H. Yang, S. Kyo, M. Takatura, L. Sun, Cell Growth Differ. 12 (2) (2001) 119.
[264] M.R. Stampfer, J. Garbe, G. Levine, S. Lichtsteiner, A.P. Vasserot, P. Yaswen, Proc. Natl. Acad. Sci. U. S. A. 98 (8) (2001) 4498.
[265] S.Y. Lin, S.J. Elledge, Cell 113 (7) (2003) 881.
[266] T. Fujiki, T. Miura, M. Maura, H. Shiraishi, S. Nishimura, Y. Imada, N. Uehara, K. Tashiro, S. Shirahata, Y. Katakura, Oncogene 26 (36) (2007) 5258.
[267] H. Zhang, S.N. Cohen, Genes Dev. 18 (24) (2004) 3028.
[268] H. Valderrama-Carvajal, E. Cocolakis, A. Lacerte, E.H. Lee, G. Krystal, S. Ali, J.J. Lebrun, Nat. Cell Biol. 4 (2002) 936.
[269] C.W. Jang, C.H. Chen, C.C. Chen, J.Y. Chen, Y.H. Su, R.H. Chen, Nat. Cell Biol. 4 (1465-7392 (Print) (2002) 51.
[270] Y. Kondo, T. Kanzawa, R. Sawaya, S. Kondo, Nat. Rev. Cancer 5 (2005) 726.
[271] E. Chalaux, T. Lopez-Rovira, J.L. Rosa, G. Pons, L.M. Boxer, R. Bartrons, F. Ventura, FEBS Lett. 457 (1999) 478.
[272] I. Tachibana, M. Imoto, P.N. Adjei, G.J. Gores, M. Subramaniam, T.C. Spelsberg, R. Urrutia, J. Clin. Invest. 99 (1997) 2365.
[273] S. Larisch, Y. Yi, R. Lotan, H. Kerner, S. Eimerl, W. Tony Parks, Y. Gottfried, S. Birkey Reffey, M.P. de Caestecker, D. Danielpour, N. Book-Melamed, R. Timberg, C.S. Duckett, R.J. Lechleider, H. Steller, J. Orly, S.J. Kim, A.B. Roberts, Nat. Cell Biol. 2 (2000) 915.
[274] Y. Gottfried, A. Rotem, R. Lotan, H. Steller, S. Larisch, EMBO J. 23 (2004) 1627.
[275] A. Thiefes, S. Wolter, J.F. Mushinski, E. Hoffmann, O. Dittrich-Breiholz, N. Graue, A. Dorrie, H. Schneider, D. Wirth, B. Luckow, K. Resch, M. Kracht, J. Biol. Chem. 18 (2005) 18.
[276] S. Edlund, S. Bu, N. Schuster, P. Aspenstrom, R. Heuchel, N.E. Heldin, P. ten Dijke, C.H. Heldin, M. Landstrom, Mol. Biol. Cell 14 (2003) 529.
[277] T. Moriguchi, N. Kuroyanagi, K. Yamaguchi, Y. Gotoh, K. Irie, T. Kano, K. Shirakabe, Y. Muro, H. Shibuya, K. Matsumoto, E. Nishida, M. Hagiwara, J. Biol. Chem. 271 (23) (1996) 13675.
[278] K. Yamaguchi, K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida, K. Matsumoto, Science 270 (5244) (1995) 2008.

Cells Tissues Organs

Cells Tissues Organs 2011;193:98–113
DOI: 10.1159/000320163

Published online: November 3, 2010

The Cain and Abl of Epithelial-Mesenchymal Transition and Transforming Growth Factor-β in Mammary Epithelial Cells

Tressa M. Allington[a] William P. Schiemann[b]

[a]Department of Pharmacology, Anschutz Medical Campus, University of Colorado Denver, Aurora, Colo., and
[b]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio, USA

Key Words

Breast cancer · c-Abl · Epithelial-mesenchymal transition · Metastasis · Signal transduction · Transforming growth factor-β

Abstract

Transforming growth factor-β (TGF-β) normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, by inducing MEC apoptosis, and by creating cell microenvironments that maintain MEC homeostasis and prevent their uncontrolled growth and motility. Mammary tumorigenesis elicits dramatic alterations in MEC architecture and microenvironment integrity, which collectively counteract the tumor-suppressing activities of TGF-β and enable its stimulation of breast cancer invasion and metastasis. How malignant MECs overcome the cytostatic actions imposed by normal microenvironments and TGF-β, and how abnormal microenvironments conspire with TGF-β to stimulate the development and progression of mammary tumors remains largely undefined. These knowledge gaps have prevented science and medicine from implementing treatments effective in simultaneously targeting abnormal cellular microenvironments, and in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. c-Abl is a ubiquitously expressed nonreceptor protein tyrosine kinase that essentially oversees all aspects of cell physiology, including the regulation of cell proliferation, migration and adhesion, as well as that of cell survival. Thus, the biological functions of c-Abl are highly reminiscent of those attributed to TGF-β, including the ability to function as either a suppressor or promoter of tumorigenesis. Interestingly, while dysregulated Abl activity clearly promotes tumorigenesis in hematopoietic cells, an analogous role for c-Abl in regulating solid tumor development, including those of the breast, remains controversial. Here, we review the functions of c-Abl in regulating breast cancer development and progression, and in alleviating the oncogenic activities of TGF-β and its stimulation of epithelial-mesenchymal transition during mammary tumorigenesis.

Copyright © 2010 S. Karger AG, Basel

Abbreviations used in this paper

BCR	break-point cluster region
CML	chronic myelogenous leukemia
CST-Abl	constitutively active c-Abl
EMT	epithelial-mesenchymal transition
MEC	mammary epithelial cell
PTK	protein tyrosine kinase
TGF-β	transforming growth factor-β

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2010 S. Karger AG, Basel

Accessible online at:
www.karger.com/cto

Dr. William P. Schiemann
Case Comprehensive Cancer Center, Case Western Reserve University
Wolstein Research Building, Room 2131
2103 Cornell Road, Cleveland, Ohio 44106 (USA)
Tel. +1 216 844 8797, Fax +1 216 844 7832, E-Mail William.Schiemann@case.edu

Introduction

Transforming growth factor-β (TGF-β) is a ubiquitous cytokine that fulfills fundamental roles during embryonic development, cellular differentiation, wound healing and tissue remodeling, as well as immune homeostasis [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. In addition, TGF-β also plays an essential function in maintaining normal epithelial cell and tissue architecture, a regulatory mechanism that becomes disrupted in developing neoplasms. Indeed, as neoplastic lesions progress and become invasive, they typically circumvent the tumor-suppressing activities of TGF-β and paradoxically convert this cytokine into a potent promoter of metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Pardali and Moustakas, 2007; Barcellos-Hoff and Akhurst, 2009; Wendt et al., 2009a]. Recent evidence has established epithelial-mesenchymal transition (EMT) as being a vital component involved in initiating oncogenic TGF-β signaling in normal and malignant cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Indeed, TGF-β is a master regulator of EMT and its ability to engender polarized epithelial cells to (1) downregulate their expression of genes associated with epithelial phenotypes, including those operant in forming adherens and tight junctions; (2) remodel their actin cytoskeletons and microtubule networks; and (3) upregulate their expression of genes associated with mesenchymal phenotypes and cell motility [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. The process of EMT has recently been categorized into 3 distinct biological subtypes [Kalluri and Weinberg, 2009], namely type 1 (embryonic and developmental EMT), type 2 (tissue regeneration and fibrotic EMT) and type 3 (cancer progression and metastatic EMT). The linkage of type 3 EMT to the development of metastasis and poor clinical outcomes [Thiery, 2003] has led to intense research efforts aimed at developing novel chemotherapeutics capable of inhibiting oncogenic EMT, and as such, of improving the clinical course of patients with metastatic disease. Alternatively, identifying the molecular mechanisms that promote mesenchymal-epithelial transition (MET), which phenotypically and morphologically reverses the activities of EMT, may also offer new inroads to impede or thwart primary tumor metastasis, an idea echoed by those who attended the 3rd International TEMTIA meeting that was held in Krakow, Poland, in 2007.

c-Abl is a multifunctional nonreceptor protein tyrosine kinase (PTK) that localizes to the plasma membrane, cytoplasm and nucleus where it governs a variety of cellular functions and activities, including the (1) transduction of integrins and growth factor receptor signals; (2) induction of cell cycle arrest initiated by DNA damage; (3) regulation of actin cytoskeletal dynamics; and (4) interaction with numerous adaptor proteins and scaffold complexes [Pendergast, 1996; Plattner et al., 1999; Hamer et al., 2001; Woodring et al., 2001; Pendergast, 2002; Woodring et al., 2002; Zandy and Pendergast, 2008]. In addition, c-Abl and its relative Arg are unique among nonreceptor PTKs in that both molecules house direct actin-binding domains that enable c-Abl to sense and respond to extracellular signals coupled to altered actin cytoskeletal architectures [Woodring et al., 2001, 2002; Zandy and Pendergast, 2008]. It is interesting to note that the diverse and complex biological functions of c-Abl are surprisingly reminiscent of the pathophysiological actions of TGF-β, including its dichotomous behavior exhibited during tumorigenesis. For instance, the tumor-promoting activities of c-Abl are best exemplified by its causal initiation of chronic myelogenous leukemia (CML), wherein c-Abl is translocated and fused to the break-point cluster region (BCR) on chromosome 22, resulting in the generation of a constitutively active Abl kinase fusion protein [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008]. Moreover, the pharmacological development and clinical implementation of imatinib (also known as Gleevec or STI-571), which targets the ATP-binding site in the c-Abl kinase domain and inhibits its phosphotransferase activity [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008], has significantly improved the treatment of CML and served as a model for the rationale design of protein kinase inhibitors [Druker, 2006; Soverini et al., 2008]. Although dysregulated c-Abl activity clearly promotes tumorigenesis in hematopoietic cells, the role of c-Abl in regulating tumorigenesis in solid tumors remains controversial. In fact, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression failed to observe any clinical benefit in imatinib-treated breast cancer patients. Moreover, these same studies found imatinib to cause significant toxicity and elicit disease progression in breast cancer patients [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Along these lines, our recently published study showed that imatinib administration failed to provide any therapeutic benefit to mice bearing aggressive mammary tumors, and instead, this same pharmacological treatment tended to produce larger breast tumors as compared with those observed in their vehicle-treated counterparts (fig. 1) [Allington et al., 2009]. Remarkably,

this same study demonstrated that engineering late-stage metastatic breast cancer cells to stably express a constitutively active c-Abl mutant resulted in their morphologic and phenotypic reversion both in vitro and in vivo, as well as circumvented the oncogenic activities of TGF-β and its ability to induce EMT [Allington et al., 2009]. Clearly, a deeper and more thorough understanding of pathophysiological functions of c-Abl in regulating solid tumor development and progression is needed for science and medicine to successfully advance the use of targeted chemotherapies against c-Abl and against various effectors activated by oncogenic TGF-β signaling.

Here, we review recent findings that directly impact our understanding of the dichotomous roles played by c-Abl during mammary tumorigenesis and discuss emerging evidence suggesting that chemotherapeutic targeting of c-Abl activation, not inhibition, may offer new inroads to suppress breast cancer progression and the oncogenic activities of TGF-β, particularly its induction of EMT and metastasis in developing neoplasms of the breast.

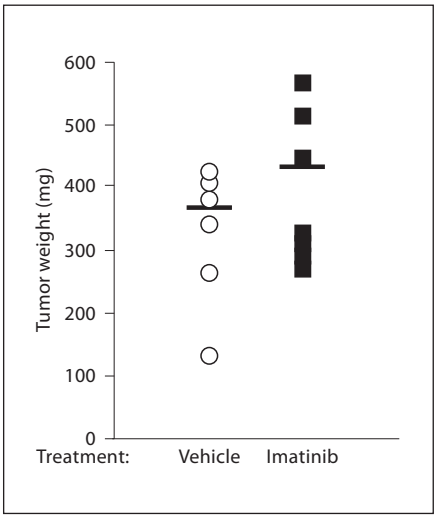
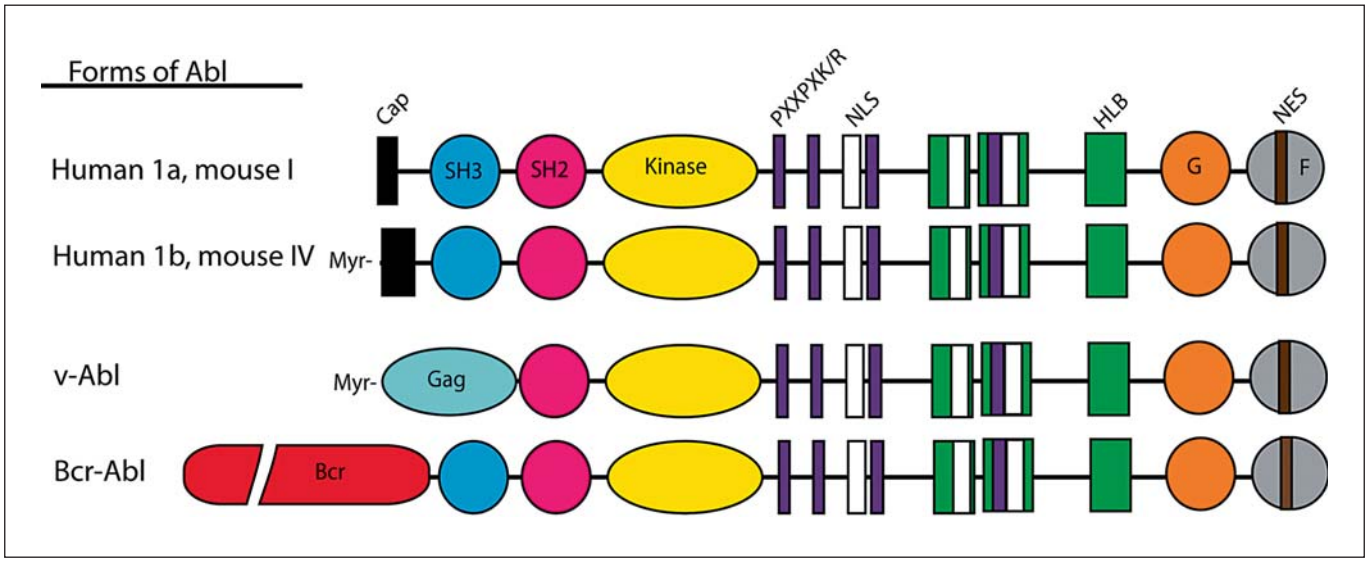


Fig. 1. c-Abl antagonism fails to inhibit mammary tumor growth in mice. Female Balb/c mice were injected orthotopically with syngeneic 4T1 cells and treated daily beginning on day 8 after engraftment with either vehicle or imatinib (50 mg/kg/day) as indicated. Primary tumors were removed surgically and weighed at days 24 and 27 after engraftment. Bars indicate the mean tumor weight per group (6 mice/group). Reprinted with kind permission from Allington et al. [2009].

Abl Signaling and Cancer Progression

c-Abl was originally identified as the cellular counterpart to the Abelson murine leukemia virus, v-Abl [Wang et al., 1984]. Subsequent studies established that c-Abl exists in 2 isoforms in mammals (1a or 1b in humans and I or IV in mice), and that this large (approximately 140 kDa) nonreceptor PTK contains a variety of modular domains that enable c-Abl to bind numerous signaling and scaffolding proteins. Figure 2 depicts the structural features of human and murine c-Abl isoforms, both of which house (1) Src homology 2 (SH2) and SH3 domains; (2) a proline-rich adaptor-binding motif; (3) a PTK domain; (4) 3 nuclear localization signals and 1 nuclear export signal; (5) 3 high-mobility group-like boxes that function cooperatively in binding DNA; and (6) globular and filamentous actin-binding domains [Woodring et al., 2002; Hunter, 2007]. The presence of functional nuclear localization signals and nuclear export signal motifs localizes c-Abl to both the cytoplasmic and nuclear compartments in quiescent cells, as well as enables c-Abl to translocate to the nucleus in response to a variety of extracellular stimuli [Lewis et al., 1996; Wen et al., 1996; Taagepera et al., 1998; Plattner et al., 1999; Woodring et al., 2002]. In nontransformed cells, the activation status of c-Abl is tightly controlled and its PTK activity is retained in an inactive conformation through intramolecular c-Abl interactions [Woodring et al., 2002; Hunter, 2007]. Indeed, displacing either the N-terminal autoinhibitory Cap region or the SH2/SH3 domains of c-Abl by the binding of its substrates or effector molecules rapidly and transiently stimulates this PTK, whose activity is downregulated following its ubiquitination and proteosomal degradation [Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004]. Cytoplasmic c-Abl is activated by a variety of growth factors and cytokines, by reactive oxygen species, and by cell attachment and mechanotransduction that directs c-Abl to adherens complexes and regions of actin cytoskeletal remodeling [Woodring et al., 2002; Zhu and Wang, 2004]. Nuclear c-Abl is also activated by reactive oxygen species and by DNA damage that couples c-Abl to the regulation of cell survival and apoptosis [Agami et al., 1999; Vigneri and Wang, 2001; Truong et al., 2003; Chau et al., 2004]. Interestingly, both of the oncogenic forms of c-Abl, namely BCR-Abl and v-Abl, are unable to enter the nucleus, and their enforced nuclear expression induces apoptosis, not cellular transformation [Woodring et al., 2002; Zhu and Wang, 2004; Suzuki and Shishido, 2007]. Thus, the pathophysiological output of c-Abl activation ultimately reflects a conglomeration of the initiating signal, the cellular context and the cellular locale wherein c-Abl is stimulated.



Color version available online

Fig. 2. Schematic depiction of the functional domains of human (1a and 1b) and murine (I and IV) Abl isoforms. The N terminus of c-Abl contains either an autoinhibitory Cap region or a consensus motif for myristoylation (black), which is followed by SH3 (blue; colors refer to the online version only) and SH2 (pink) domains, which is followed by the catalytic PTK domain (kinase, yellow). The central region of c-Abl possesses 4 PXXPXK/R sequences (purple), 3 nuclear localization sequences (NLS, white), and 3 high-mobility group-like boxes (HLB, green). Finally, the C terminus of c-Abl houses domains for binding globular (G, orange) and filamentous (F, gray) actin, as well as a nuclear export sequence (NES, brown). The oncogenic forms of Abl (v-Abl and BCR-Abl) contain modified N-terminal regions that disrupt the autoinhibitory functions normally mediated by the Cap region, which elicits constitutive PTK activity. The aberrant N terminus in v-Abl comprises a viral Gag sequence (light blue), while that in BCR-Abl comprises a portion of the N terminus of the BCR (red).

As mentioned above, the oncogenic potential of c-Abl was elucidated by the discovery that c-Abl can be fused to the BCR region on chromosome 22, an untoward translocation event that gives rise to BCR-Abl and its ability to induce CML development and progression [Druker, 2006; Hunter, 2007]. The synthesis and implementation of imatinib (Gleevec) to antagonize the phosphotransferase activity of Abl revolutionized the treatment of CML by eliciting response rates of about 98% in patients with the chronic phase of CML at the time of diagnosis [Mauro and Druker, 2001; Mauro et al., 2002; Druker, 2006]. In stark contrast to its causal role in initiating hematopoietic cancers, a definitive function for c-Abl in promoting the formation and progression of solid tumors, including those of the breast, remains an active and controversial area of research. For instance, early cell biology studies found that the phosphorylation of Crk by c-Abl inhibits fibroblast and carcinoma cell motility by preventing the formation of Crk:p130Cas complexes [Kain and Klemke, 2001; Kain et al., 2003]. In addition, hepatocyte growth factor signaling and its stimulation of thyroid cancer cell migration was potentiated significantly in imatinib-treated cells as compared with their vehicle-treated counterparts [Frasca et al., 2001], suggesting that c-Abl activity suppresses carcinoma motility. With respect to cancers of the breast, c-Abl activation has been associated with enhanced breast cancer cell proliferation, invasion, survival and anchorage-independent growth [Plattner et al., 1999; Srinivasan and Plattner, 2006; Lin and Arlinghaus, 2008; Srinivasan et al., 2008], and with their transformation by Src [Sirvent et al., 2007]. In stark contrast, c-Abl was observed to be essential in suppressing mammary tumorigenesis mediated by ephrin B2/ephrin B [Noren et al., 2006]. Moreover, we recently discovered that constitutive c-Abl activity abrogates the oncogenic behaviors of TGF-β in late-stage breast cancer cells, resulting in their phenotypic and morphologic reversion both in vitro and in vivo (see below) [Allington et al., 2009]. These latter findings suggest that imatinib administration may be contraindicated in breast cancer patients. Accordingly, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression have met with disappointing results, including the presence of severe drug

Table 1. Role of c-Abl in solid tumor progression

Study type	Disease/cells	Major finding	Reference
Abl as a tumor promoter			
Preclinical	lung cancer	increased c-Crk expression is associated with aggressive phenotypes	Miller et al., 2003
Preclinical	lung cancer	imatinib and Fus1 inhibit c-Abl and anchorage-independent growth of H1299 cells	Lin et al., 2007b
Preclinical	breast cancer	constitutive c-Abl activation is elicited by dysregulated EGFR, HER2 and Src, leading to increased cell invasion	Srinivasan and Plattner 2006
Preclinical	breast cancer	c-Abl mediates Src-transformation and survival of breast cancer cells	Sirvent et al., 2007
Preclinical	breast cancer	c-Abl mediates IGF-1 receptor-stimulated breast cancer progression	Srinivasan et al., 2008
Abl as a tumor suppressor			
Preclinical	colon cancer	c-Abl activates p73α/GADD45α, leading to apoptosis in response to DNA mismatch repair	Li et al., 2008
Preclinical	colon cancer	c-Abl activates p73α/GADD45α, leading to G2 arrest after induction of DNA mismatch repair	Wagner et al., 2008
Preclinical	breast cancer	ephrin B2/ephrin B4 suppress breast cancer tumorigenicity via activation of a c-Abl/Crk/MMP-2-signaling axis	Noren et al., 2006
Preclinical	thyroid cancer	imatinib enhances thyroid cancer cell motility in response to HGF	Frasca et al., 2001
Preclinical	breast cancer	activated c-Abl suppresses oncogenic TGF-β signaling, inhibits EMT and reverts breast cancer tumorigenicity in vitro and in vivo	Allington et al., 2009
Clinical phase I	breast cancer	imatinib offered no clinical benefit in PDGF receptor-positive metatastic breast cancer	Cristofanilli et al., 2008
Clinical phase II	breast cancer	imatinib provided no therapeutic benefit against invasive breast cancer patients	Modi et al., 2005
Clinical phase II	breast cancer	imatinib and capecitabine treatment failed to improve the clinical course of metastatic breast cancer patients	Chew et al., 2008
Clinical phase I/II	prostate cancer	imatinib administration either alone or in combination promoted disease progression and severe toxicity	Lin et al., 2006, 2007a
Clinical phase II	pancreatic cancer	imatinib administration fails to offer any therapeutic protection against pancreatic cancer	Chen et al., 2006; Gharibo et al., 2008

EGFR = Epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2; IGF-1 = insulin growth factor 1; MMP-2 = matrix metalloproteinase 2; HGF = hepatocyte growth factor; PDGF = platelet-derived growth factor.

toxicities and the initiation of disease progression [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Similar detrimental clinical outcomes were observed in pancreatic [Chen et al., 2006; Gharibo et al., 2008] and prostate [Lin et al., 2006, 2007a] cancer patients subjected to imatinib administration.

Table 1 summarizes the dichotomous roles of c-Abl in regulating solid tumor development and progression, and in doing so, highlights the need to identify novel biomarkers capable of staging and stratifying cancer patients on the basis of their c-Abl expression and signaling profiles.

TGF-β Signaling and EMT

TGF-β is a pluripotent cytokine that plays essential roles in regulating mammalian development and differentiation, and in maintaining tissue homeostasis [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and Akhurst, 2009; Tian and Schiemann, 2009b]. The versatile nature of TGF-β is emphasized by the fact that virtually all cells in the metazoan body are capable of both producing and responding to this cytokine. TGF-β is now recognized as a potent tumor suppressor that prevents the dysregulated growth and survival of cells, particularly those of epithelial, endothelial and hematopoietic origins [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. The process of tumorigenesis and its associated genetic, epigenetic and microenvironmental alterations enable early-stage cancer cells to inactivate the cytostatic activities of TGF-β through mechanisms that remain incompletely understood. As these neoplastic cells continue to evolve towards advanced malignancy, they ultimately acquire the ability to convert the cytostatic functions of TGF-β into those capable of driving neoplastic progression, including the induction of tumor growth, invasion and metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and Akhurst, 2009; Tian and Schiemann, 2009b]. The functional conversion

of TGF-β behavior during tumorigenesis is known as the 'TGF-β paradox', whose eventual interpretation and translation holds the key to developing novel chemotherapies capable of preferentially targeting the oncogenic activities of TGF-β [Schiemann, 2007].

An important consequence of TGF-β signaling is its potential to induce EMT, a process whereby immotile, polarized epithelial cells transdifferentiate into highly motile, apolar fibroblastoid-like cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Essential features exhibited by epithelial cells undergoing EMT include (1) diminished cell polarity owing to the downregulated expression of epithelial cell markers (e.g., E-cadherin, zona occludens 1 and β_4-integrin); (2) remodeled actin cytoskeletal architectures; (3) upregulated expression of fibroblast markers and genes operant in cell motility and invasion (e.g., vimentin, N-cadherin, α-smooth muscle actin, Twist); and (4) acquired expansion of cells that possess stem cell-like properties and phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Importantly, recent studies by our group have shown that the initiation of oncogenic TGF-β signaling coincides with its stimulation of EMT in normal and malignant MECs [Galliher and Schiemann, 2006, 2007; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b], suggesting that the eventual development and implementation of pharmacological agents capable of uncoupling TGF-β from EMT may one day improve the clinical course of breast cancer patients.

The ability of TGF-β to induce EMT commences upon its binding to the TGF-β type II receptor, which then recruits, transphosphorylates and activates TGF-β type I receptor, which then phosphorylates and stimulates the latent TGF-β transcription factors, Smad2 and Smad3. Following their activation, Smad2/3 interact physically with the co-Smad, Smad4, which enables the resulting heterocomplexes to translocate to the nucleus where they associate with a variety of transcriptional activators and repressors to govern the expression of TGF-β-responsive genes in a cell- and context-specific manner [Heldin et al., 2009; Wendt et al., 2009a]. The coupling of TGF-β to Smad2/3 stimulation, which is commonly referred to as either 'Smad2/3-dependent' or 'canonical' TGF-β signaling, plays an essential role in governing all aspects of the pathophysiological activities of TGF-β, including its induction of EMT [Oft et al., 1996; Valcourt et al., 2005; see also Masszi and Kapus, 2011, this issue]. In addition to its stimulation of canonical Smad2/3 signaling, we and others have identified a variety of noncanonical effectors whose activation by TGF-β also mediate fundamental functions of this cytokine [for review, see Lamouille and Derynck, 2011, this issue]. With respect to its induction of EMT, TGF-β must also activate (1) MAP kinase family members, particularly ERK1/2 [Xie et al., 2004] and p38 MAPK [Bhowmick et al., 2001; Galliher and Schiemann, 2007; Galliher-Beckley and Schiemann, 2008]; (2) focal adhesion complex proteins, including β_1- and β_3-integrins [Bhowmick et al., 2001; Galliher and Schiemann, 2006], Src [Galliher and Schiemann, 2006, 2007], focal adhesion kinase [Wendt and Schiemann, 2009] and p130Cas [Wendt et al., 2009b]; (3) nuclear factor-κB [Huber et al., 2004; Neil and Schiemann, 2008] and its downstream effector, cyclooxygenase 2 [Neil et al., 2008], which promotes EMT by initiating an autocrine prostaglandin E_2:EP_2 receptor signaling loop [Tian and Schiemann, 2009a]; (4) phosphoinositide 3-kinase and its downstream effectors, AKT and mTOR [Bakin et al., 2000; Lamouille and Derynck, 2007; Lamouille and Derynck, 2011]; (5) small guanosine triphosphate-binding proteins, including cdc42, Rac1 and RhoA [Wendt et al., 2009a]; and (6) PAR6 and its recruitment of the E3 ligase, Smurf1 [Ozdamar et al., 2005]. Although the precise contribution of canonical and noncanonical TGF-β signaling in mediating the various subtypes of EMT has yet to be clarified, it is known that the activation of both pathways is necessary for the faithful initiation and completion of EMT by TGF-β and its ability to confer stem cell-like properties to epithelial cells, whose newfound plasticity enables metastatic cancer cells to thrive in otherwise hostile secondary sites [Polyak and Weinberg, 2009]. Readers desiring more in-depth discussions of the molecular mechanisms that underlie the ability of TGF-β to induce EMT are directed to several recent and comprehensive reviews [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009; Lamouille and Derynck, 2011].

c-Abl Activation Suppresses EMT and Breast Cancer Progression

An essential component of EMT centers on the dissolution of adherens junctions and the remodeling of the actin cytoskeleton, both of which enable transitioning cells to acquire motile and invasive phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Interestingly, c-Abl activity has been associated with both the assembly and dissolution of adherens junctions and with altered actin dynamics and architectures through its

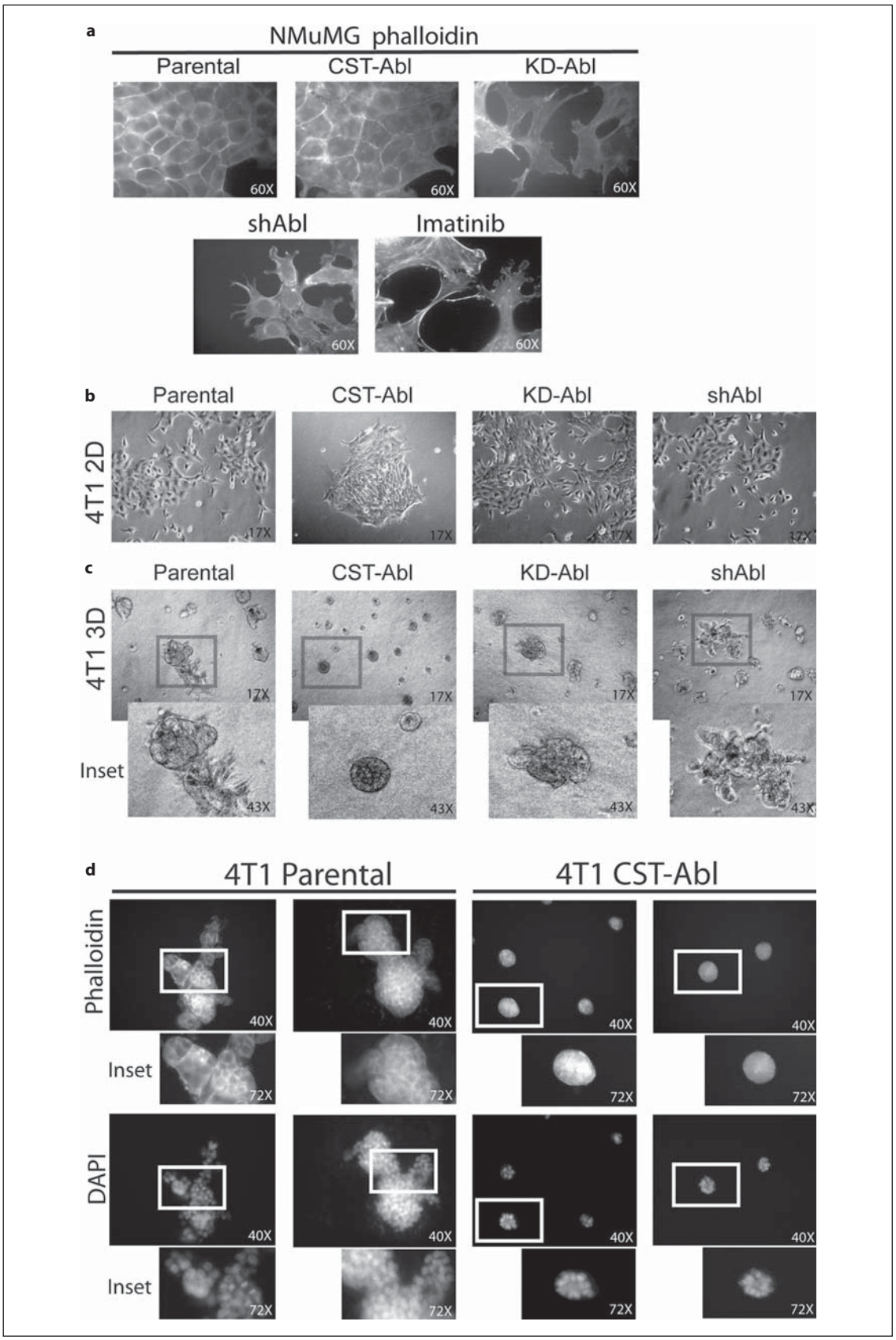
ability to bind globular and filamentous actin [Woodring et al., 2001, 2002, 2004; Zandy et al., 2007; Zandy and Pendergast, 2008]. Thus, the process of EMT presents a unique situation in which the paradoxical functions of c-Abl and TGF-β may intersect during mammary tumorigenesis. For instance, c-Abl essentially governs all biological decisions made by cells, including whether they proliferate, migrate or invade, or even whether they live or die [Zhu and Wang, 2004; Lin and Arlinghaus, 2008]. In fact, the physiological functions performed by c-Abl in many ways parallel those played by TGF-β, including their capacity to serve as tumor suppressors or promoters in a cell- and context-specific manner [Massague, 2008; Tian and Schiemann, 2009b]. Given the obvious pathophysiological similarities that exist between TGF-β and c-Abl in epithelial cells, we hypothesized c-Abl as an essential player in determining the morphologies and phenotypes of MECs, including their ability to undergo EMT in response to TGF-β. We tested this hypothesis by manipulating the expression or activity of c-Abl via several complementary approaches: (1) loss of c-Abl function by pharmacological inhibition (i.e. imatinib), by retroviral transduction of a kinase-dead c-Abl mutant or by lentiviral transduction of a shRNA against c-Abl; or (2) gain of c-Abl function by retroviral transduction of a constitutively active c-Abl mutant (CST-Abl). These c-Abl manipulations were applied to 2 murine MEC cell lines to interrogate the potential linkage between c-Abl and TGF-β: (1) normal murine NMuMG mammary gland cells, which are nontransformed and exhibit normal cuboidal epithelial architectures that readily undergo a robust EMT in response to TGF-β [Sokol et al., 2005; Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Wendt and Schiemann, 2009; Wendt et al., 2009b]; and (2) malignant, metastatic 4T1 cells, which are a late-stage model of TGF-β-responsive breast cancer [Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil and Schiemann, 2008; Yang et al., 2008; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]. Figure 3a shows that c-Abl expression and activity were essential for maintaining normal MEC morphology, such that measures resulting in a loss of c-Abl function elicited an EMT response, while those measures resulting in a gain of c-Abl function produced a 'hyper-epithelial' response that was resistant to EMT and invasion stimulated by TGF-β [Allington et al., 2009]. The morphological alterations induced by inactivating c-Abl also transpired in normal human MECs [Allington et al., 2009], and more importantly, gene expression analyses confirmed the ability of c-Abl deficiency to increase mesenchymal gene expression (table 2). Thus, c-Abl inactivation results in morphological, transcriptional and migrational changes suggestive of those observed during EMT stimulated by TGF-β.

Table 2. c-Abl deficiency elicits an EMT transcriptional signature in NMuMG cells

NMuMG cells		Average relative mRNA, %	
		(–) TGF-β	(+) TGF-β
E-cadherin	scram	100.0	58.1 ± 8.7
	shAbl	104.6 ± 8.7	47.6 ± 7.8
N-cadherin	scram	100.0	856.0 ± 14.6
	shAbl	345.9 ± 21.1	1,050.9 ± 47.4
Vimentin	scram	100.0	475.6 ± 40.9
	shAbl	1,135.1 ± 26.9	858.0 ± 28.9
Twist	scram	100.0	295.3 ± 33.2
	shAbl	569.5 ± 20.3	683.9 ± 49.8

Quiescent parental (scrambled shRNA, scram) or c-Abl-deficient (shAbl) normal murine mammary gland cells (NMuMG) were incubated in the absence or presence of TGF-β_1 (5 ng/ml) for 48 h, at which point total RNA was isolated and subjected to semi-quantitative real-time PCR analysis. Data are the mean (±SE; n = 3) percent change in EMT marker gene expression relative to that observed in untreated parental normal murine mammary gland cells.

Extending these analyses to metastatic 4T1 cells demonstrated that CST-Abl expression was sufficient in reducing cell scattering and promoting stronger cell-cell junctions in traditional 2D culture systems (fig. 3b). The morphological differences induced by c-Abl activation were greatly exaggerated when these same cells were propagated in compliant 3D organotypic cultures. Indeed, in stark contrast to the large and irregularly shaped organoids formed by parental and loss of c-Abl function 4T1 cells, those expressing CST-Abl formed dramatically smaller and perfectly spherical organoids that appeared to undergo a partial hollowing (fig. 3c, d). In addition, CST-Abl expression reinstated the cytostatic activities of TGF-β in 4T1 cells in part by (1) acting as a broad-spectrum suppressor of matrix metalloproteinase expression [Allington et al., 2009], and (2) overriding the tumor-promoting signals engendered by rigid microenvironments [Allington et al., 2009]. Thus, enforced activation of c-Abl in metastatic MECs may provide a novel means to alleviate the oncogenic activities of TGF-β and, consequently,



(For legend see next page.)

to phenotypically and morphologically normalize the malignant behaviors of breast cancer cells.

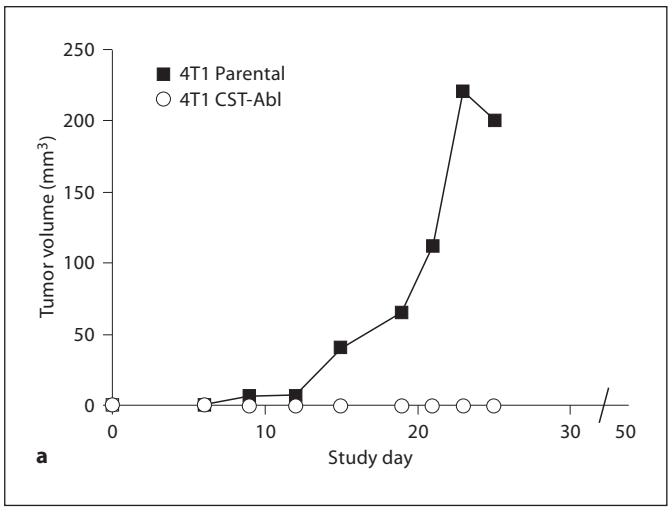
We tested the validity of the above supposition by orthotopically engrafting parental and CST-Abl-expressing 4T1 cells in syngeneic Balb/c mice. As expected [Galliher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Wendt and Schiemann, 2009; Wendt et al., 2009b], parental 4T1 cells rapidly formed palpable tumors that necessitated host euthanization by day 28 due to excessive tumor burden (fig. 4a). Remarkably, every animal injected with CST-Abl-expressing 4T1 cells failed to develop palpable tumors during the course of the study (fig. 4a) and to exhibit overt signs of disease during necropsy [Allington et al., 2009]. Surprisingly, clonogenic assays facilitated the reisolation CST-Abl-expressing 4T1 cells from the mammary fat pads of mice that were euthanized on day 51 (fig. 4b). Collectively, these findings suggest that measures capable of enforcing c-Abl activation may represent a novel means to abrogate the oncogenic activities of TGF-β in cancers of the breast, and as such, to one day to improve the prognosis and treatment of patients with metastatic breast cancer. c-Abl may also influence the latency and dormancy of disseminated breast cancer in the form of micrometastases.

Chemotherapeutic Targeting of c-Abl in Breast Cancer: Friend or Foe?

Since the inception of the National Cancer Act of 1971, science and medicine have waged an all-out battle aimed at conquering cancer. Although considerable progress has been achieved in terms of our understanding of the molecular mechanisms that underlie neoplastic development and progression, cancer itself remains a significant health concern and burden in the United States. In fact, 1 in 4 deaths in the United States results from cancer, which is also the leading cause of death in individuals younger than 85 years of age [Jemal et al., 2009]. Despite these grim statistics, overall cancer incidence and mortality rates have begun to decline over the last decade, including those for breast cancer which annually contributes to more than 40,000 deaths and 192,000 new invasive cases of this deadly disease [Jemal et al., 2009]. Continuing along this positive trend will require the development of new diagnostic and chemotherapeutic regimens, as well as the elucidation of new knowledge of how cancer cells acquire the 6 essential phenotypes, or hallmarks, necessary to become malignant. Included in this phenotypic list is the ability of cancer cells to (1) disregard cytostatic signals; (2) grow autonomously; (3) stimulate angiogenesis; (4) ignore apoptotic signals; (5) become immortal; and (6) invade and metastasize [Hanahan and Weinberg, 2000]. The inability of developing neoplasms to acquire each of these phenotypes prevents their conversion to aggressive states, suggesting that these cancer hallmarks represent various rate-limiting steps during malignant development. Although EMT is not a recognized hallmark of tumorigenesis, type 3 EMT is essential for the acquisition of invasive and metastatic phenotypes by cancer cells and their development of chemoresistance. Thus, pharmacological targeting of individual cancer hallmarks and EMT, both singly and in combination, may offer new inroads to effectively treat the development and dissemination of human malignancies, particularly those of the breast.

Our findings showing that the c-Abl activation circumvents and overrides the oncogenic activities of TGF-β in normal and malignant MECs are intriguing in terms of their scientific and biological significance. For instance, alterations within tumor microenvironments can either restrain or free breast cancer progression in a manner that mirrors the conversion of TGF-β function from a suppressor to a promoter of tumor invasion and metastasis [Bierie and Moses, 2006]. Moreover, mounting evidence indicates that TGF-β promotes breast cancer progression in part via its reprogramming of MEC microenvironments and their cellular architectures. In attempting to circumvent the oncogenic activities of TGF-β in mammary tumors, science and medicine have employed a 'TGF-β centric' approach that is likely to fail

Fig. 3. Constitutive c-Abl activity suppresses EMT and induces MET in metastatic MECs. KD-Abl = Kinase-dead c-Abl mutant; shAbl = c-Abl deficient. **a** Direct FITC-conjugated phalloidin immunofluorescence was performed to monitor the actin cytoskeletal architecture in c-Abl-manipulated normal murine mammary gland (NMuMG) cells, which readily acquired mesenchymal morphologies in loss of c-Abl function MECs. **b** Bright-field images of c-Abl-manipulated 4T1 cells grown in traditional 2D tissue culture systems. Gain of c-Abl function elicited an apparent MET in 4T1 cells. **c**, **d** c-Abl-manipulated 4T1 cells were propagated for 7 days in compliant 3D organotypic cultures prior to analyzing their growth and morphology by capturing bright-field images (**c**), or by staining with FITC-conjugated phalloidin and DAPI (**d**). Gain of c-Abl function suppressed acinar growth and promoted normal acinar development, including partial hollowing of the resulting organoids. All are representative of 2–3 independent experiments and were reprinted with kind permission from Allington et al. [2009].



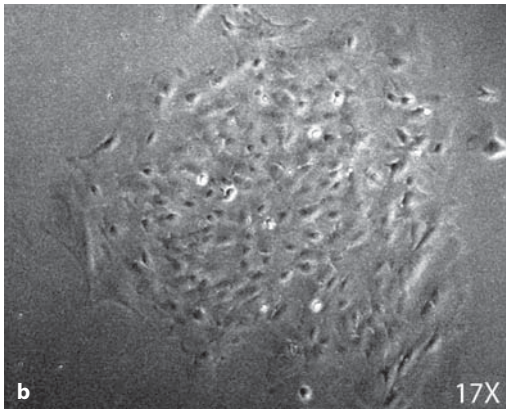


Fig. 4. Enforced c-Abl activation abrogates the growth of metastatic MECs in mice. **a** Female Balb/c mice were injected orthotopically with syngeneic parental (i.e. empty vector) or CST-Abl-expressing 4T1 cells (10,000 cells/animal; 12 animals/group), whose ability to grow as tumors was measured using digital calipers over a period of 30 days. Reprinted with kind permission from Allington et al. [2009]. **b** The mammary glands of mice injected with CST-Abl-expressing 4T1 cells were excised at day 51 after engraftment, and were subsequently dissociated enzymatically to produce a heterogeneous, single-cell suspension that was subjected to a clonogenic assay to reisolate reverted CST-Abl-expressing 4T1 cells.

clinically because targeted TGF-β therapies (i.e. both large and small molecule inhibitors) uniformly function as pan-TGF-β antagonists whose activities are subject to the phenomena underlying the 'TGF-β paradox' – i.e. the ability of mammary tumorigenesis to convert TGF-β from a tumor suppressor to a tumor promoter [Schiemann, 2007]. Pan-TGF-β antagonists are also inadequate in accounting for the pleiotropic functions of TGF-β in (1) governing MEC architectures and microenvironments, and (2) regulating tumor-associated stromal components. Thus, these findings underscore the necessity to design and implement rapid diagnostic tests capable of discriminating cancer patients most likely to benefit from targeted TGF-β therapies from those individuals most likely to be harmed by TGF-β antagonism.

A potential alternative to antagonizing all cellular responses to TGF-β may involve the implementation of a targeted approach that selectively inactivates specific noncanonical TGF-β effectors that preferentially promote its oncogenic activities. We have provided preclinical evidence that supports the therapeutic potential of this alternative approach (e.g., inactivation of αvβ3 integrin, Src, focal adhesion kinase, nuclear factor-κB, cyclooxygenase 2, or EP_2 receptor) [Galliher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]; however, complete disease resolution has yet to be achieved using these applications due to their inability to phenotypically normalize and revert malignant MEC behaviors, architectures and microenvironments. Our findings demonstrate that the enforced activation of c-Abl can fulfill this latter requirement, and in doing so, can promote the phenotypic normalization and reversion of highly malignant, late-stage breast cancers in mice (fig. 4) [Allington et al., 2009]. To our knowledge, c-Abl activation represents the first bona fide tool competent to ablate the oncogenic activities of TGF-β, thereby restoring its cytostatic function in normalized and reverted MECs. Indeed, a bold extension of our findings leads us to propose that the development and implementation of allosteric c-Abl activators may one day provide a paradigm shifting the strategy to treat metastatic breast cancers. Clearly, the notion of chemically stimulating c-Abl is disconcerting to many scientists and clinicians, particularly since c-Abl has been linked to the oncogenic activities of the receptors for epidermal growth factor, platelet-derived growth factor and insulin-like growth factor 1, to the transforming activities of Src and signal transducer and activator of transcription 3, and to the prosurvival activities of ERK5 [Plattner et al., 1999; Srinivasan and Plattner, 2006; Sir-

vent et al., 2007; Lin and Arlinghaus, 2008; Srinivasan et al., 2008]. However, in all cases, it remains difficult to gauge the extent to which off-target effects of imatinib and other c-Abl inhibitors contribute to their apparent effectiveness against breast cancer cells in vitro. Moreover, data obtained in human clinical [Modi et al., 2005; Chen et al., 2006; Lin et al., 2006, 2007a; Chew et al., 2008; Cristofanilli et al., 2008; Gharibo et al., 2008] and murine preclinical trials (fig. 1) [Allington et al., 2009] clearly demonstrate the inability of imatinib to provide any chemotherapeutic benefit towards cancers of the breast. Along these lines, overexpression of c-Abl or the enforced nuclear expression of oncogenic Abl mutants (e.g., BCR-Abl or v-Abl) all fail to elicit cellular transformation [Zhu and Wang, 2004; Suzuki and Shishido, 2007], which points to the possibility that targeted c-Abl activation may in fact be well tolerated by normal MECs. Indeed, we find that MECs are exquisitely sensitive to subtle changes in c-Abl activity [Allington et al., 2009], and as such, we anticipate that even submaximal activation of c-Abl will prove sufficient to induce MET and suppress mammary tumorigenesis stimulated by TGF-β, thereby improving the clinical course of patients with metastatic breast cancer.

Unanswered Questions and Future Directions

Although our ability to accept or reject the aforementioned hypothesis clearly awaits additional experimentation in a variety of genetically distinct human breast cancer subtypes and tissues (e.g., luminal A vs. luminal B vs. ErbB2 vs. basal-like vs. normal-like) [Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003], our findings are nonetheless provocative and potentially paradigm changing with respect to redefining the role of c-Abl in regulating mammary gland development and neoplasia, and to establishing c-Abl as a novel suppressor of oncogenic TGF-β signaling in breast and other epithelial-derived cancers in part by inducing their phenotypic and morphologic normalization [Allington et al., 2009]. Figure 5 depicts our current understanding of the role of c-Abl in regulating normal and malignant MEC behavior and in suppressing the oncogenic activities of TGF-β. Interestingly, we find that inactivating noncanonical TGF-β effectors is sufficient in abrogating the ability of TGF-β to promote breast cancer progression in a manner somewhat reminiscent of that mediated by enforced c-Abl activation. These parallels raise a number of interesting questions, including (1) does c-Abl suppress mammary tumorigenesis by inhibiting EMT, or by stimulating MET; (2) does enforced c-Abl activation alter the selection or expansion of breast cancer stem cells; (3) does cytoplasmic or nuclear c-Abl activation mediate its antitumor activities; (4) does the initiation of oncogenic TGF-β signaling uncouple c-Abl from activation by TGF-β; and (5) do the tumor-suppressing activities of c-Abl require signaling inputs by TGF-β? These questions and their potential for directing the future development and implementation of c-Abl chemotherapies during mammary tumorigenesis are discussed below.

Although c-Abl activation clearly promotes the acquisition of epithelial phenotypes in normal and malignant MECs (fig. 3) [Allington et al., 2009], it remains to be determined whether this event reflects the ability of c-Abl to inhibit EMT, or conversely, to stimulate MET. This question also bears important clinical relevance because inhibiting EMT or stimulating MET are both likely to be most effective in preventing the exit of metastatic cells from the primary tumor; however, the induction of MET may be contraindicated should this process be found to play an essential role in promoting the outgrowth of micrometastatic lesions. Our findings show that c-Abl deficiency or inactivation both elicit EMT, while c-Abl activation induces a 'hyperepithelial' morphology that normalizes and reverts the phenotypes of metastatic breast cancer cells (fig. 3, 4) [Allington et al., 2009]. Along these lines, epithelial cells naturally tend to drift and acquire mesenchymal characteristics in response to extended 2D culture durations, suggesting perhaps that the process of becoming 'mesenchymal' may reflect a more energetically favorable and stable state than that needed to become 'epithelial'. Alternatively, the nearly infinite stiffness of 2D culture systems may serve as an aberrant signal that drives epithelial cells to acquire mesenchymal-like properties as a means to compensate and survive in extremely rigid microenvironments [Butcher et al., 2009; Erler and Weaver, 2009]. In fact, we observed microenvironmental tension to be sufficient in overriding the cytostatic activities of TGF-β, an event that was circumvented by CST-Abl expression [Allington et al., 2009]. Thus, our findings are consistent with the notion that enforced c-Abl activation stimulates MET, as opposed to simply inhibiting EMT. Future studies need to thoroughly address this issue, as well as to explore the potential linkage between c-Abl and known inducers of MET, including Pax-2, Cdx2 and frizzled-7 [Hugo et al., 2007].

Recently, human and mouse MECs were observed to acquire stem cell-like properties when stimulated to un-

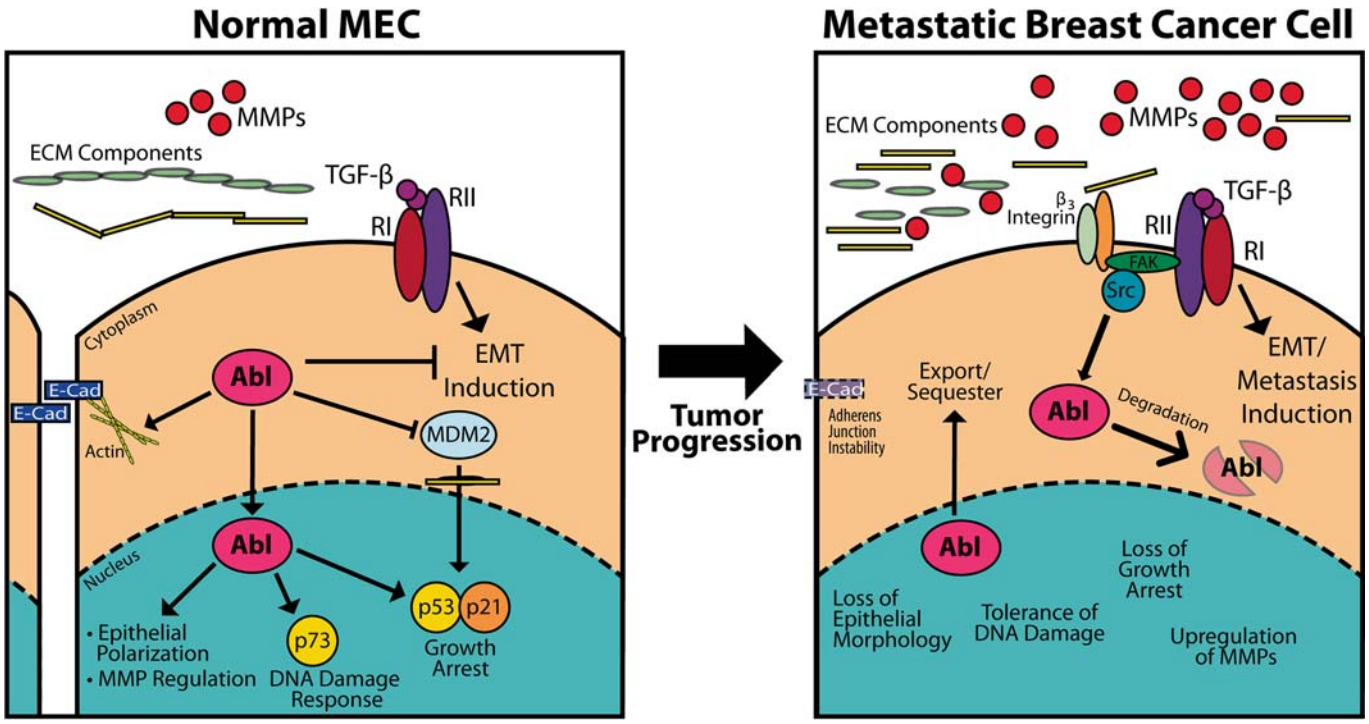


Fig. 5. Schematic depiction of the role of c-Abl in suppressing EMT and oncogenic TGF-β signaling in normal and malignant MECs. In normal MECs, c-Abl activation (1) maintains adherens junction stability and cortical actin architecture; (2) mediates growth arrest in response to TGF-β and DNA damage; and (3) represses MMP expression and secretion. As developing mammary neoplasms become more and more malignant, oncogenic TGF-β signaling in conjunction with focal adhesion complex activation (e.g., β_3-integrin/focal adhesion kinase/Src) may promote the degradation of c-Abl and its uncoupling from the tumor-suppressing activities of TGF-β. The loss of c-Abl function ushers in the initiation of EMT and its dissolution of adherens junctions, its production and activation of matrix metalloproteinases, and its circumvention of cytostasis induced by TGF-β and DNA damage, which collectively enhance breast cancer progression and metastatic dissemination to distant locales. RI/RII = TGF-β type I receptor/TGF-β type II receptor; E-cad = E-cadherin; MDM2 = murine double minute 2.

dergo EMT by TGF-β [Mani et al., 2008]. Mechanistically, upregulated Id1 expression may function in dictating whether TGF-β expands or contracts the pool of cancer stem cells [Tang et al., 2007], and consequently, whether TGF-β suppresses or promotes mammary tumorigenesis. Indeed, inhibiting TGF-β signaling pharmacologically in cancer stem cells elicited MET and their acquisition of less aggressive, more epithelial-like morphologies [Shipitsin et al., 2007]. Our demonstration that activated c-Abl phenotypically and morphologically reverts the malignant behaviors of late-stage breast cancer cells (fig. 3, 4) [Allington et al., 2009] raises 2 interesting questions – does enforced c-Abl activation suppress the selection and expansion of cancer stem cells, and conversely, does imatinib administration promote solid tumor progression by enlarging the population of stem cell-like progenitors in post-EMT carcinoma cells? Thus, future studies clearly need to investigate these important issues as well.

As mentioned above, the strong epithelial morphologies and phenotypes induced by c-Abl activation suggest that this PTK stimulates MET, presumably by functioning in the cytoplasm to affect adherens junctions and cytoskeletal architectures. However, in response to diverse extracellular stimuli (integrins), c-Abl is translocated to the nucleus where it functions in regulating DNA damage and mismatch repair responses [Lewis et al., 1996; Baskaran et al., 1997; Taagepera et al., 1998]. Moreover, c-Abl activation during DNA damage-induced apoptosis requires its coupling to the p53 relative, p73 [Yuan et al., 1999; Shaul, 2000]. Thus, the relative contributions of cytoplasmic and nuclear c-Abl in suppressing mammary tumorigenesis remains an important and unanswered question. With this idea in mind,

we observed the expression of CST-Abl to greatly enhance the induction of p21 expression by TGF-β [Allington et al., 2009] and to induce 4T1 organoids to express robust quantities of p73, suggesting that the morphological reversion of 4T1 acinar structures is partially dependent upon nuclear c-Abl signaling inputs [Allington and Schiemann, unpubl. observation]. Accordingly, we have found that CST-Abl expression is sufficient in resensitizing 4T1 cells to death induced by the DNA-damaging agent, 6-thioguanine (Allington and Schiemann, data not shown). Thus, while 6-thioguanine has previously failed as a single-agent chemotherapeutic for breast cancer, our findings suggest that combining enforced c-Abl activation with 6-thioguanine or other DNA damage-inducing agents may offer new inroads to alleviating breast cancer progression.

Finally, we found that TGF-β administration leads to a very transient activation of c-Abl in MECs [Allington et al., 2009], which is rapidly followed by their degradation of c-Abl in a manner that coincides with initiation of EMT [Allington and Schiemann, unpubl. observation]. Based on these findings, we speculate that the end result of c-Abl activation by TGF-β results in the Src-dependent degradation of c-Abl [Allington and Schiemann, unpubl. observation; Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004], and consequently, in the acquisition of EMT phenotypes stimulated by TGF-β. This model clearly contrasts with the ability of TGF-β to couple to c-Abl activation via a phosphoinositide 3-kinase- and p21-activated kinase-2-dependent pathway in fibroblasts [Daniels et al., 2004; Wang et al., 2005; Wilkes and Leof, 2006]. Despite these disparate activities for c-Abl in MECs and fibroblasts, it remains plausible that the ability of TGF-β to induce EMT requires the inactivation and degradation of c-Abl in epithelial cells (fig. 5). Along these lines, we have recently observed TGF-β stimulation of EMT to drastically reduce the expression and activity of c-Abl in both the cytoplasm and nucleus of normal MECs. Moreover, the kinetics of c-Abl degradation mirrored that for the acquisition of focal adhesion complex signaling by TGF-β, and more importantly, chemotherapeutic targeting of focal adhesion complexes was sufficient in protecting c-Abl from degradation induced by TGF-β [Allington and Schiemann, unpubl. observation]. Interestingly, augmented [Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b] and attenuated [Bhowmick et al., 2004a, 2004b; Cheng et al., 2005; Yang et al., 2008] TGF-β signaling in mammary carcinoma cells has been associated with disease progression, which raises a second interesting question – can c-Abl activation morphologically and phenotypically revert the malignant behaviors of breast cancer cells that can no longer respond to TGF-β? Future studies need to address this important issue, as well as determine how EMT dictates the expression, activation and localization of c-Abl in normal and malignant MECs. Indeed, answering these important questions may provide novel information capable of one day (1) staging and stratifying the treatment of mammary carcinomas based on their c-Abl and TGF-β signatures; and (2) enhancing our understanding of the 'TGF-β paradox' in promoting metastatic disease in breast cancer patients.

Acknowledgements

We thank members of the Schiemann Laboratory for the critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA114039 and CA129359), the Komen Foundation (BCTR0706967) and the Department of Defense (BC084651), while T.M.A was supported by the Department of Defense (BC083323).

References

Agami, R., G. Blandino, M. Oren, Y. Shaul (1999) Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. Nature *399:* 809–813.

Allington, T.M., A.J. Galliher-Beckley, W.P. Schiemann (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling and tumorigenesis in mammary tumors. FASEB J *23:* 4231–4243.

Bakin, A.V., A.K. Tomlinson, N.A. Bhowmick, H.L. Moses, C.L. Arteaga (2000) Phosphatidylinositol 3-kinase function is required for TGF-β-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem *275:* 36803–36810.

Barcellos-Hoff, M.H., R.J. Akhurst (2009) TGF-β in breast cancer: too much, too late. Breast Cancer Res *11:* 202.

Baskaran, R., L.D. Wood, L.L. Whitaker, C.E. Canman, S.E. Morgan, Y. Xu, C. Barlow, D. Baltimore, A. Wynshaw-Boris, M.B. Kastan, J.Y. Wang (1997) Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. Nature *387:* 516–519.

Benson, J.R. (2004) Role of TGF-β in breast carcinogenesis. Lancet Oncol *5:* 229–239.

Bhowmick, N.A., A. Chytil, D. Plieth, A.E. Gorska, N. Dumont, S. Shappell, M.K. Washington, E.G. Neilson, H.L. Moses (2004a) TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science *303:* 848–851.

Bhowmick, N.A., E.G. Neilson, H.L. Moses (2004b) Stromal fibroblasts in cancer initiation and progression. Nature *432:* 332–337.

Bhowmick, N.A., R. Zent, M. Ghiassi, M. McDonnell, H.L. Moses (2001) Integrin β_1 signaling is necessary for TGF-β activation of p38MAPK and epithelial plasticity. J Biol Chem *276:* 46707–46713.

Bierie, B., H.L. Moses (2006) Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer *6:* 506–520.

Buck, M.B., C. Knabbe (2006) TGF-β signaling in breast cancer. Ann NY Acad Sci *1089:* 119–126.

Butcher, D.T., T. Alliston, V.M. Weaver (2009) A tense situation: forcing tumour progression. Nat Rev Cancer *9:* 108–122.

Chau, B.N., T.T. Chen, Y.Y. Wan, J. DeGregori, J.Y. Wang (2004) Tumor necrosis factor-α-induced apoptosis requires p73 and c-ABL activation downstream of RB degradation. Mol Cell Biol *24:* 4438–4447.

Chen, J., C. Rocken, B. Nitsche, C. Hosius, H. Gschaidmeier, S. Kahl, P. Malfertheiner, M.P. Ebert (2006) The tyrosine kinase inhibitor imatinib fails to inhibit pancreatic cancer progression. Cancer Lett *233:* 328–337.

Cheng, N., N.A. Bhowmick, A. Chytil, A.E. Gorksa, K.A. Brown, R. Muraoka, C.L. Arteaga, E.G. Neilson, S.W. Hayward, H.L. Moses (2005) Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks. Oncogene *24:* 5053–5068.

Chew, H.K., W.E. Barlow, K. Albain, D. Lew, A. Gown, D.F. Hayes, J. Gralow, G.N. Hortobagyi, R. Livingston (2008) A phase II study of imatinib mesylate and capecitabine in metastatic breast cancer: Southwest Oncology Group Study 0338. Clin Breast Cancer *8:* 511–515.

Cristofanilli, M., P. Morandi, S. Krishnamurthy, J.M. Reuben, B.N. Lee, D. Francis, D.J. Booser, M.C. Green, B.K. Arun, L. Pusztai, A. Lopez, R. Islam, V. Valero, G.N. Hortobagyi (2008) Imatinib mesylate (Gleevec) in advanced breast cancer-expressing c-Kit or PDGFR-β: clinical activity and biological correlations. Ann Oncol *19:* 1713–1719.

Daniels, C.E., M.C. Wilkes, M. Edens, T.J. Kottom, S.J. Murphy, A.H. Limper, E.B. Leof (2004) Imatinib mesylate inhibits the profibrogenic activity of TGF-β and prevents bleomycin-mediated lung fibrosis. J Clin Invest *114:* 1308–1316.

Druker, B.J. (2006) Circumventing resistance to kinase-inhibitor therapy. N Engl J Med *354:* 2594–2596.

Echarri, A., A.M. Pendergast (2001) Activated c-Abl is degraded by the ubiquitin-dependent proteasome pathway. Curr Biol *11:* 1759–1765.

Erler, J.T., V.M. Weaver (2009) Three-dimensional context regulation of metastasis. Clin Exp Metastasis *26:* 35–49.

Frasca, F., P. Vigneri, V. Vella, R. Vigneri, J.Y. Wang (2001) Tyrosine kinase inhibitor STI571 enhances thyroid cancer cell motile response to hepatocyte growth factor. Oncogene *20:* 3845–3856.

Galliher, A.J., W.P. Schiemann (2006) β_3 integrin and Src facilitate TGF-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res *8:* R42.

Galliher, A.J., W.P. Schiemann (2007) Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res *67:* 3752–3758.

Galliher-Beckley, A.J., W.P. Schiemann (2008) Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis *29:* 244–251.

Gharibo, M., L. Patrick-Miller, L. Zheng, L. Guensch, P. Juvidian, E. Poplin (2008) A phase II trial of imatinib mesylate in patients with metastatic pancreatic cancer. Pancreas *36:* 341–345.

Hamer, G., I.S. Gademan, H.B. Kal, D.G. de Rooij (2001) Role for c-Abl and p73 in the radiation response of male germ cells. Oncogene *20:* 4298–4304.

Hanahan, D., R.A. Weinberg (2000) The hallmarks of cancer. Cell *100:* 57–70.

Heldin, C.H., M. Landstrom, A. Moustakas (2009) Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol *21:* 166–176.

Huber, M.A., N. Azoitei, B. Baumann, S. Grunert, A. Sommer, H. Pehamberger, N. Kraut, H. Beug, T. Wirth (2004) NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest *114:* 569–581.

Hugo, H., M.L. Ackland, T. Blick, M.G. Lawrence, J.A. Clements, E.D. Williams, E.W. Thompson (2007) Epithelial-mesenchymal and mesenchymal-epithelial transitions in carcinoma progression. J Cell Physiol *213:* 374–383.

Hunter, T. (2007) Treatment for chronic myelogenous leukemia: the long road to imatinib. J Clin Invest *117:* 2036–2043.

Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, M.J. Thun (2009) Cancer statistics, 2009. CA Cancer J Clin *59:* 225–249.

Kain, K.H., S. Gooch, R.L. Klemke (2003) Cytoplasmic c-Abl provides a molecular 'Rheostat' controlling carcinoma cell survival and invasion. Oncogene *22:* 6071–6080.

Kain, K.H., R.L. Klemke (2001) Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. J Biol Chem *276:* 16185–16192.

Kalluri, R., R.A. Weinberg (2009) The basics of epithelial-mesenchymal transition. J Clin Invest *119:* 1420–1428.

Lamouille, S., R. Derynck (2007) Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J Cell Biol *178:* 437–451.

Lamouille, S., R. Derynck (2011) Emergence of the phosphoinositide 3-kinase-akt-mammalian target of rapamycin axis in transforming growth factor-β-induced epithelial-mesenchymal transition. Cells Tissues Organs *193:* 8–22.

Lee, Y.H., A.R. Albig, M. Regner, B.J. Schiemann, W.P. Schiemann (2008) Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-β in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis *29:* 2243–2251.

Lewis, J.M., R. Baskaran, S. Taagepera, M.A. Schwartz, J.Y. Wang (1996) Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. Proc Natl Acad Sci USA *93:* 15174–15179.

Li, L.S., J.C. Morales, A. Hwang, M.W. Wagner, D.A. Boothman (2008) DNA mismatch repair-dependent activation of c-Abl/p73α/GADD45alpha-mediated apoptosis. J Biol Chem *283:* 21394–21403.

Lin, A.M., B.I. Rini, M.K. Derynck, V. Weinberg, M. Park, C.J. Ryan, J.E. Rosenberg, G. Bubley, E.J. Small (2007a) A phase I trial of docetaxel/estramustine/imatinib in patients with hormone-refractory prostate cancer. Clin Genitourin Cancer *5:* 323–328.

Lin, A.M., B.I. Rini, V. Weinberg, K. Fong, C.J. Ryan, J.E. Rosenberg, L. Fong, E.J. Small (2006) A phase II trial of imatinib mesylate in patients with biochemical relapse of prostate cancer after definitive local therapy. BJU Int *98:* 763–769.

Lin, J., R. Arlinghaus (2008) Activated c-Abl tyrosine kinase in malignant solid tumors. Oncogene *27:* 4385–4391.

Lin, J., T. Sun, L. Ji, W. Deng, J. Roth, J. Minna, R. Arlinghaus (2007b) Oncogenic activation of c-Abl in non-small cell lung cancer cells lacking FUS1 expression: inhibition of c-Abl by the tumor suppressor gene product Fus1. Oncogene *26:* 6989–6996.

Mani, S.A., W. Guo, M.J. Liao, E.N. Eaton, A. Ayyanan, A.Y. Zhou, M. Brooks, F. Reinhard, C.C. Zhang, M. Shipitsin, L.L. Campbell, K. Polyak, C. Brisken, J. Yang, R.A. Weinberg (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133:* 704–715.

Massague, J. (2008) TGF-β in Cancer. Cell *134:* 215–230.

Masszi, A., A. Kapus (2011) Smaddening complexity: The role of Smad3 in epithelial-myofibroblast transition. Cells Tissues Organs *193:* 41–52.

Mauro, M.J., B.J. Druker (2001) STI571: targeting BCR-ABL as therapy for CML. Oncologist *6:* 233–238.

Mauro, M.J., M. O'Dwyer, M.C. Heinrich, B.J. Druker (2002) STI571: a paradigm of new agents for cancer therapeutics. J Clin Oncol *20:* 325–334.

Miller, C.T., G. Chen, T.G. Gharib, H. Wang, D.G. Thomas, D.E. Misek, T.J. Giordano, J. Yee, M.B. Orringer, S.M. Hanash, D.G. Beer (2003) Increased C-CRK proto-oncogene expression is associated with an aggressive phenotype in lung adenocarcinomas. Oncogene *22:* 7950–7957.

Modi, S., A.D. Seidman, M. Dickler, M. Moasser, G. D'Andrea, M.E. Moynahan, J. Menell, K.S. Panageas, L.K. Tan, L. Norton, C.A. Hudis (2005) A phase II trial of imatinib mesylate monotherapy in patients with metastatic breast cancer. Breast Cancer Res Treat *90:* 157–163.

Neil, J.R., K.M. Johnson, R.A. Nemenoff, W.P. Schiemann (2008) Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis *29:* 2227–2235.

Neil, J.R., W.P. Schiemann (2008) Altered TAB1:IκB kinase interaction promotes TGF-β-mediated NF-κB activation during breast cancer progression. Cancer Res *68:* 1462–1470.

Neil, J.R., M. Tian, W.P. Schiemann (2009) X-linked inhibitor of apoptosis protein and its E3 ligase activity promote TGF-β-mediated NF-κB activation during breast cancer progression. J Biol Chem *284:* 21209–21217.

Noren, N.K., G. Foos, C.A. Hauser, E.B. Pasquale (2006) The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway. Nat Cell Biol *8:* 815–825.

Oft, M., J. Peli, C. Rudaz, H. Schwarz, H. Beug, E. Reichmann (1996) TGF-β_1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev *10:* 2462–2477.

Ozdamar, B., R. Bose, M. Barrios-Rodiles, H.R. Wang, Y. Zhang, J.L. Wrana (2005) Regulation of the polarity protein Par6 by TGF-β receptors controls epithelial cell plasticity. Science *307:* 1603–1609.

Pardali, K., A. Moustakas (2007) Actions of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta *1775:* 21–62.

Pendergast, A.M. (1996) Nuclear tyrosine kinases: from Abl to WEE1. Curr Opin Cell Biol *8:* 174–181.

Pendergast, A.M. (2002) The Abl family kinases: mechanisms of regulation and signaling. Adv Cancer Res *85:* 51–100.

Perou, C.M., T. Sorlie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, C.A. Rees, J.R. Pollack, D.T. Ross, H. Johnsen, L.A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S.X. Zhu, P.E. Lonning, A.L. Borresen-Dale, P.O. Brown, D. Botstein (2000) Molecular portraits of human breast tumours. Nature *406:* 747–752.

Plattner, R., L. Kadlec, K.A. DeMali, A. Kazlauskas, A.M. Pendergast (1999) c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. Genes Dev *13:* 2400–2411.

Polyak, K., R.A. Weinberg (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer *9:* 265–273.

Schiemann, W.P. (2007) Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? Expert Rev Anticancer Ther *7:* 609–611.

Shaul, Y. (2000) c-Abl: activation and nuclear targets. Cell Death Differ *7:* 10–16.

Shipitsin, M., L.L. Campbell, P. Argani, S. Weremowicz, N. Bloushtain-Qimron, J. Yao, T. Nikolskaya, T. Serebryiskaya, R. Beroukhim, M. Hu, M.K. Halushka, S. Sukumar, L.M. Parker, K.S. Anderson, L.N. Harris, J.E. Garber, A.L. Richardson, S.J. Schnitt, Y. Nikolsky, R.S. Gelman, K. Polyak (2007) Molecular definition of breast tumor heterogeneity. Cancer Cell *11:* 259–273.

Sirvent, A., A. Boureux, V. Simon, C. Leroy, S. Roche (2007) The tyrosine kinase Abl is required for Src-transforming activity in mouse fibroblasts and human breast cancer cells. Oncogene *26:* 7313–7323.

Sokol, J.P., J.R. Neil, B.J. Schiemann, W.P. Schiemann (2005) The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by TGF-β. Breast Cancer Res *7:* R844–R853.

Sorlie, T., C.M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, T. Thorsen, H. Quist, J.C. Matese, P.O. Brown, D. Botstein, P. Eystein Lonning, A.L. Borresen-Dale (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA *98:* 10869–10874.

Sorlie, T., R. Tibshirani, J. Parker, T. Hastie, J.S. Marron, A. Nobel, S. Deng, H. Johnsen, R. Pesich, S. Geisler, J. Demeter, C.M. Perou, P.E. Lonning, P.O. Brown, A.L. Borresen-Dale, D. Botstein (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA *100:* 8418–8423.

Soverini, S., G. Martinelli, I. Iacobucci, M. Baccarani (2008) Imatinib mesylate for the treatment of chronic myeloid leukemia. Expert Rev Anticancer Ther *8:* 853–864.

Srinivasan, D., R. Plattner (2006) Activation of Abl tyrosine kinases promotes invasion of aggressive breast cancer cells. Cancer Res *66:* 5648–5655.

Srinivasan, D., J.T. Sims, R. Plattner (2008) Aggressive breast cancer cells are dependent on activated Abl kinases for proliferation, anchorage-independent growth and survival. Oncogene *27:* 1095–1105.

Suzuki, J., T. Shishido (2007) Regulation of cellular transformation by oncogenic and normal Abl kinases. J Biochem *141:* 453–458.

Taagepera, S., D. McDonald, J.E. Loeb, L.L. Whitaker, A.K. McElroy, J.Y. Wang, T.J. Hope (1998) Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. Proc Natl Acad Sci USA *95:* 7457–7462.

Tang, B., N. Yoo, M. Vu, M. Mamura, J.S. Nam, A. Ooshima, Z. Du, P.Y. Desprez, M.R. Anver, A.M. Michalowska, J. Shih, W.T. Parks, L.M. Wakefield (2007) TGF-β can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res *67:* 8643–8652.

Thiery, J.P. (2003) Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol *15:* 740–746.

Tian, M., W.P. Schiemann (2009a) PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J *24:* 1105–1116.

Tian, M., W.P. Schiemann (2009b) The TGF-β paradox in human cancer: an update. Future Oncol *5:* 259–271.

Truong, T., G. Sun, M. Doorly, J.Y. Wang, M.A. Schwartz (2003) Modulation of DNA damage-induced apoptosis by cell adhesion is independently mediated by p53 and c-Abl. Proc Natl Acad Sci USA *100:* 10281–10286.

Valcourt, U., M. Kowanetz, H. Niimi, C.H. Heldin, A. Moustakas (2005) TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell *16:* 1987–2002.

Vigneri, P., J.Y. Wang (2001) Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase. Nat Med *7:* 228–234.

Wagner, M.W., L.S. Li, J.C. Morales, C.L. Galindo, H.R. Garner, W.G. Bornmann, D.A. Boothman (2008) Role of c-Abl kinase in DNA mismatch repair-dependent G2 cell cycle checkpoint arrest responses. J Biol Chem *283:* 21382–21393.

Wang, J.Y. (2006) Eph tumour suppression: the dark side of Gleevec. Nat Cell Biol *8:* 785–786.

Wang, J.Y., F. Ledley, S. Goff, R. Lee, Y. Groner, D. Baltimore (1984) The mouse c-Abl locus: molecular cloning and characterization. Cell *36:* 349–356.

Wang, S., M.C. Wilkes, E.B. Leof, R. Hirschberg (2005) Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis in vivo. FASEB J *19:* 1–11.

Wen, S.T., P.K. Jackson, R.A. Van Etten (1996) The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the p53 and Rb tumor suppressor gene products. EMBO J *15:* 1583–1595.

Wendt, M.K., T.M. Allington, W.P. Schiemann (2009a) Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol *5:* 1145–1168.

Wendt, M.K., W.P. Schiemann (2009) Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. Breast Cancer Res *11:* R68.

Wendt, M.K., J.A. Smith, W.P. Schiemann (2009b) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem *284:* 34145–34156.

Wilkes, M.C., E.B. Leof (2006) TGF-β activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures. J Biol Chem *281:* 27846–27854.

Woodring, P.J., T. Hunter, J.Y. Wang (2001) Inhibition of c-Abl tyrosine kinase activity by filamentous actin. J Biol Chem *276:* 27104–27110.

Woodring, P.J., E.D. Litwack, D.D. O'Leary, G.R. Lucero, J.Y. Wang, T. Hunter (2002) Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. J Cell Biol *156:* 879–892.

Woodring, P.J., J. Meisenhelder, S.A. Johnson, G.L. Zhou, J. Field, K. Shah, F. Bladt, T. Pawson, M. Niki, P.P. Pandolfi, J.Y. Wang, T. Hunter (2004) c-Abl phosphorylates Dok1 to promote filopodia during cell spreading. J Cell Biol *165:* 493–503.

Xie, L., B.K. Law, A.M. Chytil, K.A. Brown, M.E. Aakre, H.L. Moses (2004) Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. Neoplasia *6:* 603–610.

Xu, J., S. Lamouille, R. Derynck (2009) TGF-β-induced epithelial to mesenchymal transition. Cell Res *19:* 156–172.

Yang, L., J. Huang, X. Ren, A.E. Gorska, A. Chytil, M. Aakre, D.P. Carbone, L.M. Matrisian, A. Richmond, P.C. Lin, H.L. Moses (2008) Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell *13:* 23–35.

Yuan, Z.M., H. Shioya, T. Ishiko, X. Sun, J. Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum, D. Kufe (1999) p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature *399:* 814–817.

Zandy, N.L., A.M. Pendergast (2008) Abl tyrosine kinases modulate cadherin-dependent adhesion upstream and downstream of Rho family GTPases. Cell Cycle *7:* 444–448.

Zandy, N.L., M. Playford, A.M. Pendergast (2007) Abl tyrosine kinases regulate cell-cell adhesion through Rho GTPases. Proc Natl Acad Sci USA *104:* 17686–17691.

Zhu, J., J.Y. Wang (2004) Death by Abl: a matter of location. Curr Top Dev Biol *59:* 165–192.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2010/12/14/M110.168682.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 8, pp. 6414–6422, February 25, 2011
© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Fibromodulin Suppresses Nuclear Factor-κB Activity by Inducing the Delayed Degradation of IKBA via a JNK-dependent Pathway Coupled to Fibroblast Apoptosis*[S]

Received for publication, July 26, 2010, and in revised form, December 13, 2010 Published, JBC Papers in Press, December 14, 2010, DOI 10.1074/jbc.M110.168682

Yong-Hun Lee and William P. Schiemann[1]

From the Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106

Fibulin-5 (FBLN5) belongs to the Fibulin family of secreted extracellular matrix proteins, and our laboratory first established FBLN5 as a novel target for TGF-β in fibroblasts and endothelial cells. To better understand the pathophysiology of FBLN5, we carried out microarray analysis to identify fibroblast genes whose expressions were regulated by FBLN5 and TGF-β. In doing so, we identified fibromodulin (Fmod) as a novel target gene of FBLN5, and we validated the differential expression of Fmod and 12 other FBLN5-regulated genes by semi-quantitative real time PCR. Fmod belongs to the small leucine-rich family of proteoglycans, which are important constituents of mammalian extracellular matrices. Interestingly, parental 3T3-L1 fibroblasts displayed high levels of nuclear factor-κB (NF-κB) activity, although those engineered to express Fmod constitutively exhibited significantly reduced NF-κB activity, suggesting that Fmod functions to inhibit NF-κB signaling. By monitoring alterations in the activation of NF-κB and the degradation of its inhibitor, IκBα, we demonstrate for the first time that Fmod contributes to the constitutive degradation of IκBα protein in 3T3-L1 fibroblasts. Mechanistically, we observed Fmod to delay the degradation of IκBα by promoting the following: (i) activation of c-Jun N-terminal kinase; (ii) inhibition of calpain and casein kinase 2 activity; and (iii) induction of fibroblast apoptosis. Taken together, our study identified a novel function for Fmod in directing extracellular signaling, particularly the regulation of NF-κB activity and cell survival.

Fibromodulin (Fmod)[2] is a keratan sulfate proteoglycan that is highly concentrated in cartilage, tendon, skin, cornea and sclera (1). Fmod belongs to the small leucine-rich repeat family of extracellular matrix (ECM) proteoglycans and glycoproteins, whose members also include decorin, biglycan, and lumican (2, 3). Fmod plays a central role in organizing the structure of type I and II collagen fibrils, and its expression is ubiquitous in a variety of connective tissues, such as cartilage, tendons, ligaments, and dermal tissues (4, 5). Along these lines, transforming growth factor-β (TGF-β) isoforms interact with Fmod physically to regulate the fibrotic responses of several tissues, including the kidney, lung, and skin (6–8). Moreover, Fmod also affects the differentiation of tendon stem/progenitor cells by modulating the signals transduced by bone morphogenic proteins, ultimately enhancing tendon repair and formation *in vivo* (9). Thus, Fmod expression figures prominently in regulating tissue homeostasis in part by impacting the activities of TGF-β superfamily members.

TGF-β itself is a multifunctional cytokine that regulates tissue morphogenesis and differentiation by effecting cell proliferation and survival and by altering the production of ECM proteins within cell and tissue microenvironments (10, 11). In addition, our laboratory first identified the ECM protein Fibulin-5 (FBLN5) as a novel target gene for TGF-β in fibroblasts (12) and endothelial cells (12, 13). Moreover, we established FBLN5 as a multifunctional signaling molecule that does the following: (i) regulates the proliferation, motility, and invasion of normal and malignant cells both *in vitro* and *in vivo* (12–14); (ii) antagonizes endothelial cell activities coupled to angiogenesis both *in vitro* and *in vivo* (13, 14); (iii) inhibits the growth of fibrosarcomas in mice (14); and (iv) induces epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells via a matrix metalloproteinase-dependent mechanism (15). In an attempt to further our understanding of the pathophysiological functions of FBLN5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression. In doing so, we identified Fmod as a novel target gene of FBLN5 in fibroblast cells. Whereas previous studies of Fmod action have been performed primarily in connective tissues (16) and endothelial cells (17), we show here that Fmod expression in fibroblasts potently suppressed their activation of nuclear factor-κB (NF-κB) by preventing the degradation of IκBα via a c-Jun N-terminal kinase (JNK)-, casein kinase-2 (CK2)-, and calpain-dependent pathway that serves in diminishing fibroblast survival.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Normal murine 3T3-L1 fibroblasts were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 3T3-L1 fibroblasts were engineered to stably express either GFP or Fmod using a bi-

* This work was supported, in whole or in part, by National Institutes of Health Grant CA129359. This work was also supported by Department of Defense Grant BC084651 and Komen Foundation Grant BCTR0706967 (to W. P. S.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S6.

[1] To whom correspondence should be addressed: Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Bldg., Rm. 2131, 2103 Cornell Rd., Cleveland, OH 44121. Tel.: 216-368-5763; Fax: 216-368-1166; E-mail: William.Schiemann@case.edu.

[2] The abbreviations used are: Fmod, fibromodulin; CHX, cycloheximide; ECM, extracellular matrix; NF-κB, nuclear factor-κB; TUNEL, terminal dUTP nick end-labeling.

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

cistronic retroviral vector (pMSCV-IRES-GFP) as described previously (12). The NF-κB promoter-driven luciferase reporter was provided by Dr. John M. Routes (Medical College of Wisconsin, Milwaukee). The generation of 3T3-L1 fibroblasts that lacked Fmod expression was accomplished by their transduction with lentiviral particles that encoded for either a scrambled (*i.e.* nonsilencing) or Fmod-directed shRNA in pLKO.1 (Thermo Scientific, Huntsville, AL). The generation of pLKO.1 lentiviral particles and their transduction into 3T3-L1 fibroblasts were accomplished as described previously (18, 19), although the extent of Fmod depletion was monitored by immunoblotting whole-cell extracts prepared from parental (*i.e.* nonsilencing shRNA) and Fmod-deficient 3T3-L1 fibroblasts with anti-Fmod antibodies.

Microarray Analysis and Semi-quantitative Real Time PCR Assays—Total RNA from parental (*i.e.* GFP) and FBLN5-expressing 3T3-L1 fibroblast cells was purified using the RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The creation of polyclonal populations of 3T3-L1 cells that constitutively expressed FBLN5 and its role in promoting fibroblast proliferation and MAPK activation were described previously (12). In some experiments, GFP- or FBLN5-expressing 3T3-L1 fibroblasts were stimulated with TGF-β1 (5 ng/ml). RNA samples were reverse-transcribed and labeled for microarray analysis using standard techniques (20, 21). For real time PCR, cDNAs were synthesized by iScript reverse transcription (Bio-Rad), which then were diluted 10-fold in H_2O and employed in semi-quantitative real time PCRs (25 μl) using the SYBR Green system (Bio-Rad) supplemented with 5 μl of diluted cDNA and 0.1 μM of oligonucleotide pairs provided in supplemental Table S1. PCRs were performed and analyzed on a Bio-Rad Mini-Opticon detection system, and differences in RNA concentrations were monitored by normalizing individual gene signals to their corresponding GAPDH signals.

Immunoblot Assays—Parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were lysed and solubilized in Buffer H/Triton X-100 (22) for 30 min on ice. Clarified whole-cell extracts were resolved on 10% SDS-polyacrylamide gels, transferred electrophoretically onto nitrocellulose membranes, and blocked in 5% milk before incubation with the following primary antibodies (dilutions): (*a*) anti-Fmod (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (*b*) anti-IκBα (1:3000; Santa Cruz Biotechnology); (*c*) anti-phospho-IκBα (1:1000; Cell Signaling, Danvers, MA); (*d*) anti-phospho-ERK (1:500; Cell Signaling); (*e*) anti-phospho-p38 (1:500; Cell Signaling); (*f*) anti-phospho-JNK (1:500; Cell Signaling); (*g*) anti-p65 (1:1,000; Santa Cruz Biotechnology); and (*h*) anti-histone H1 (1:200; Santa Cruz Biotechnology). The resulting immunocomplexes were visualized by enhanced chemiluminescence. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies (1:1000; Rockland Immunochemicals, Gilbertsville, PA). Where specified, parental (*i.e.* GFP), Fmod-expressing, or Fmod-deficient (Fmod shRNA) 3T3-L1 fibroblasts were incubated for various times in the absence or presence of the following: (i) protein synthesis inhibitor, cycloheximide (CHX); (ii) proteosome inhibitor, MG-132; (iii) JNK inhibitor, SP600125; (iv) calpain inhibitor, EST (Calbiochem); and (v) CK2 inhibitor, TBBz (Calbiochem) at the provided concentrations.

Luciferase Reporter Gene Assays—Analysis of luciferase activity driven by a synthetic NF-κB promoter was accomplished as described previously (23). Briefly, parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts (25–30,000 per well) were cultured overnight onto 24-well plates and subsequently were transiently transfected the following morning by overnight exposure to LT1 liposomes (Mirus, Madison, WI) containing pNF-κB-luciferase cDNA (300 ng/well) and CMV-β-gal cDNA (50 ng/well,) which was used to control for differences in transfection efficiency. Afterward, the resulting luciferase and β-gal activities contained in detergent-solubilized cell extracts were determined. As above and where specified, parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of the inhibitors to JNK (SP600125), calpain (EST), and CK2 (TBBz). Data are the mean (±S.E.) luciferase activities of at least three independent experiments normalized to untreated cells.

NF-κB-biotinylated Oligonucleotide Capture Assay—DNA binding activity of NF-κB was monitored in parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts as described previously (23). Briefly, the cells were incubated for 24 h in the absence or presence of JNK inhibitor (SP600125) and subsequently were collected and fractionated into cytoplasmic and nuclear extracts using a nuclear extraction kit according to the manufacturer's instructions (Chemicon). NF-κB binding activity was determined by incubating 60 μg of nuclear extract with 1 μg of biotinylated double-stranded DNA oligonucleotides that contained an NF-κB consensus sequence site under continuous rotation at 4 °C (forward probe, 5′-GATT-AGGGACTTTCCGCTGGGGACTTTCCAGTCGA; reverse probe, 5′-TCGACTGGAAAGTCCCCAGCGGAAAGTC-CCTAGATC). The resulting NF-κB-oligonucleotide complexes were captured by addition of streptavidin-agarose beads (Pierce) and collected by microcentrifugation. Washed complexes were fractionated through 10% SDS-PAGE before their immobilization to nitrocellulose membranes, which were subsequently probed with anti-p65 antibodies (1:500). Differences in extract loading were monitored by immunoblotting 25 μg of resolved nuclear extract aliquots with antibodies against histone H1 (1:200).

Cell Biological Assays—The effect of Fmod on calpain activity in 3T3-L1 fibroblasts was determined using the Calpain-Glo™ protease assay kit (Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, 3T3-L1 fibroblasts (30,000 cells/well in 96-well plates) were lysed and mixed with proluminescent calpain substrate, and the resulting luminescence was measured as described previously (12). Along these lines, the effect of Fmod on 3T3-L1 fibroblast apoptosis was determined using two complementary methods. First, 3T3-L1 fibroblasts (10,000 cells/well) were incubated for 24 h in the absence or presence of the JNK inhibitor SP600125 prior to addition of the proluminescent caspase-3/7 DEVD-aminoluciferin substrate as instructed by the Caspase-Glo® assay kit (Promega) (24). Second, parental (*i.e.* GFP),

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

TABLE 1

Validation of select gene targets regulated by FBLN5 expression in 3T3-L1 fibroblasts

Among the genes whose expression was controlled by FBLN5 as detected in microarray analyses, the expression of 13 genes was confirmed by semi-quantitative real-time PCR.

Gene name	NCBI no.	Microarray expression	Real time PCR expression
Unknown	BB503935	9.2-Fold decrease	22.2-Fold decrease
Unknown	BB533736	5.4-Fold increase	3.2-Fold increase
Unknown	BB831146	9.2-Fold increase	2.1-Fold increase
Pleckstrin homology domain-containing family A member	BC010215	7.5-Fold decrease	5.8-Fold decrease
Transglutaminase 2	BC016492	5.8-Fold decrease	33.6-Fold decrease
Homeobox D9	BC019150	9.4-Fold increase	2.1-Fold increase
Rho GTPase-activating protein 24	BC025502	5.5-Fold decrease	3.3-Fold decrease
Thrombospondin 1	M87276	10.7-Fold increase	10.5-Fold increase
Procollagen, type XI, a 1	NM_007729	9.9-Fold increase	2.3-Fold increase
Angiopoietin 1	NM_009640	8.3-Fold increase	2.1-Fold increase
Cysteine-rich protein 61	NM_010516	10.4-Fold increase	1.8-Fold increase
Dickkopf homolog 3	NM_015814	6.9-Fold increase	6.7-Fold increase
Fibromodulin	NM_021355	9.6-Fold increase	10.6-Fold increase

Fmod-expressing, or Fmod-deficient (*i.e.* Fmod shRNA) 3T3-L1 cells were again subjected to JNK inhibitor prior to their processing for TUNEL assays according to the manufacturer's recommendations (Invitrogen).

RESULTS

Fmod Is a Novel Target Gene of FBLN5 in 3T3-L1 Fibroblasts—Our laboratory first established FBLN5 as a novel gene target for TGF-β in fibroblasts and endothelial cells (12). Moreover, we demonstrated that constitutive FBLN5 expression was sufficient to enhance 3T3-L1 cell proliferation and activation of MAPKs (12). In an effort to enhance our understanding of the pathophysiological actions of FBLN5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression or by constitutive FBLN5 expression plus added TGF-β1. Afterward, total RNA was collected and prepared for hybridization to Affymetrix microarrays, which enabled us to identify ~1200 genes whose expression was controlled by constitutive FBLN5 expression. The altered expression of 13 genes was confirmed by semi-quantitative real time PCR (Table 1), including that of Fmod (NCBI accession number NM_021355). Here, we limited our analyses to Fmod because this ECM protein and FBLN5 both play prominent roles in regulating cell-cell and cell-matrix signaling in ECM. Interestingly, FBLN5 only coupled to Fmod expression in 3T3-L1 fibroblasts but not in normal (NMuMG) or malignant (MDA-MB-231) mammary epithelial cells or in endothelial (MB114) cells (see supplemental Fig. S1). Collectively, these findings identify Fmod as a novel and fibroblast-specific gene target of FBLN5.

Fmod Suppresses NF-κB Activity by Stabilizing IκBα—To study the function of Fmod, we first established Fmod-expressing fibroblasts using a retroviral expression system according to our published procedures (12). As expected, fibroblasts transduced with Fmod-encoding viral particles constitutively expressed Fmod proteins at significantly higher levels as compared with their parental (*i.e.* GFP) counterparts (Fig. 1*A*). We then measured the production of luciferase whose expression was driven by Smad2/3 or AP1 transcription factors in these cells before and after their stimulation of with TGF-β1 because this cytokine is known to readily interact with Fmod (25). In doing so, we observed Fmod-expressing fibroblasts to exhibit significantly elevated luciferase expression driven by the Smad2/3 and AP1 transcription factors both basally and in response to TGF-β (data not shown). We also examined the effects of Fmod in altering the expression of luciferase driven by NF-κB. In comparison with parental (*i.e.* GFP) fibroblasts, those engineered to express Fmod exhibited a 75% reduction of luciferase activity directed by NF-κB (Fig. 1*B*). Along these lines, the ability of NF-κB to mediate and induce the expression of MMP-2 and −9 in fibroblasts has previously been established (26), and as such, Fmod-mediated inhibition of NF-κB activity would be anticipated to reduce fibroblast expression of MMP-2 and −9. Accordingly, Fmod-expressing fibroblasts did indeed express significantly reduced levels of MMP-2 and −9 as compared with their parental (*i.e.* GFP) counterparts (see supplemental Fig. S2).

NF-κB activity is regulated primarily by its interaction with inhibitory κB (IκB) proteins (27). Extracellular signals that stimulate NF-κB must first induce the release of IκB proteins from NF-κB, thereby allowing for its nuclear translocation and regulation of gene expression. This initial dissociation event requires the activation of IκB kinases, which phosphorylate IκB proteins and lead to their subsequent release from NF-κB, ubiquitination, and proteosomal degradation (27). In light of the dramatic reduction in NF-κB measured in Fmod-expressing fibroblasts, we immunoblotted whole-cell extracts prepared from parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts with anti-IκBα antibodies. Fig. 1*C* shows that Fmod-expressing fibroblasts contain significantly higher quantities of IκBα as compared with their parental counterparts, a finding consistent with the diminished NF-κB activity measured in Fmod-expressing fibroblasts. Interestingly, although IκBα levels were considerably higher in Fmod-expressing *versus* parental fibroblasts, we observed the phosphorylation status of IκBα to be equivalent between these cell lines (Fig. 1*C*). This finding suggests that the ability of Fmod to inhibit NF-κB activity may reflect dysregulated IκBα dynamics. As such, we monitored the stability of IκBα proteins in parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with CHX, which inhibits protein synthesis and enables assessment of the rate of IκBα turnover. As shown in Fig. 2*A*, IκBα was rapidly degraded in parental (*i.e.* GFP) 3T3-L1 fibroblasts treated with CHX; how-

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

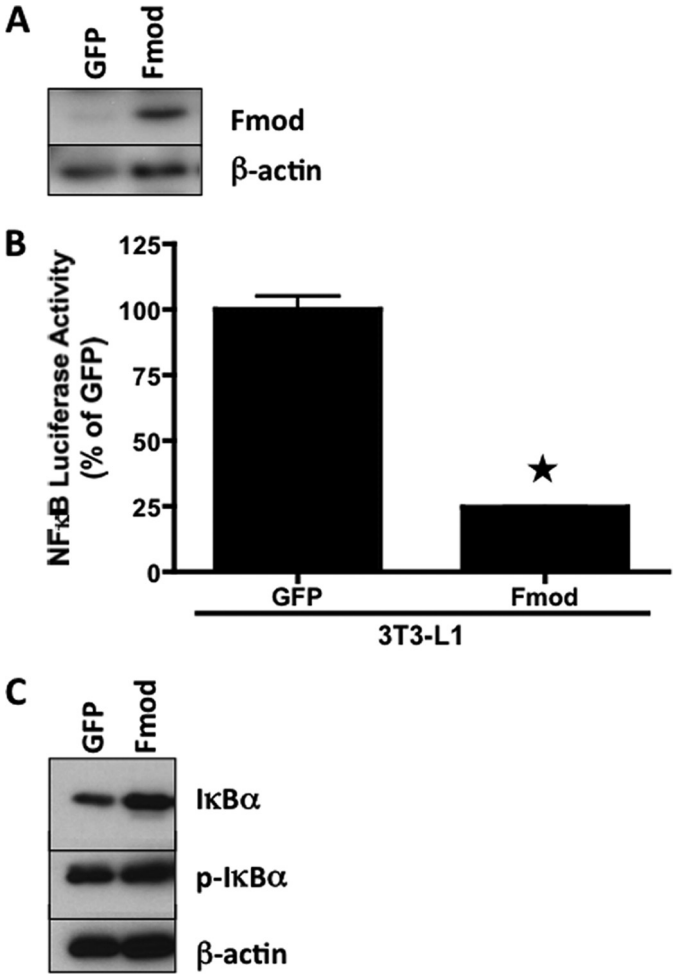


FIGURE 1. **Fmod expression suppresses NF-κB activity in fibroblasts.** *A,* immunoblot analysis of parental (*i.e.* GFP) or Fmod-expressing 3T3-L1 cell extracts with anti-Fmod antibodies showed that Fmod protein expression was abundant in fibroblasts transduced by bicistronic retroviral vector (pMSCV-IRES-GFP) encoding for murine Fmod. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are representative images from a single experiment that was performed four times. *B,* parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal. Forty eight hours later, luciferase activities were measured and normalized to that of β-gal. Data are the mean (±S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (⋆, $p < 0.05$). *C,* whole-cell extracts prepared from parental (*i.e.* GFP) and Fmod-expressing fibroblasts were fractionated through 10% SDS-PAGE and immobilized onto nitrocellulose. The expression level of IκBα, phospho-IκBα, and β-actin was monitored by immunoblot analysis. Data are representative images from a single experiment that was performed four times.

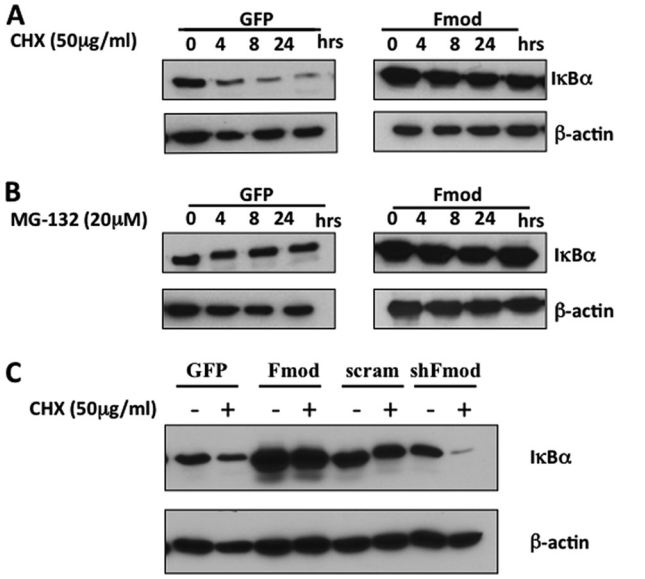


FIGURE 2. **Fmod prolongs the half-life of IκBα in fibroblasts.** Parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for varying times (0–24 h) in the absence or presence of CHX (50 μg/ml; *A*) or MG-132 (20 μM; *B*) as indicated. Afterward, whole-cell extracts were prepared, fractionated, and immobilized to nitrocellulose prior to their immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are representative images from a single experiment that was performed four times. *C, i.e.* GFP, Fmod-expressing (Fmod, *scram*) or Fmod-depleted (*shFmod*) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of CHX (50 μg/ml). The expression levels of IκBα were visualized by immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed four times with similar results.

ever, this same experimental treatment elicited little to no change in the levels of IκBα detected in Fmod-expressing fibroblasts. Likewise, administration of MG-132, which inhibits proteosomally mediated protein degradation, also failed to alter the levels IκBα in Fmod-expressing fibroblasts (Fig. 2*B*). Collectively, these findings suggest that Fmod inhibits NF-κB activity by preventing the degradation of IκBα. To more thoroughly investigate the merits of this supposition, we transduced Fmod-expressing 3T3-L1 fibroblasts with scrambled or Fmod-directed shRNAs to neutralize the activities of constitutive Fmod expression. The extent of Fmod deficiency mediated by individual Fmod-directed shRNAs was monitored by immunoblotting with antibodies against Fmod (see supplemental Fig. S3 and Table S2). Based on these analyses, polyclonal populations of 3T3-L1 cells that stably expressed Fmod shRNA2 were selected and used for all subsequent Fmod deficiency studies. As shown in Fig. 2*C*, elevating Fmod expression (*i.e.* Fmod) prevented IκBα degradation in CHX-treated fibroblasts, a reaction that was readily reversed in fibroblasts depleted of Fmod expression (*i.e.* shFmod). Moreover, the enhanced stability of IκBα was specific to Fmod expression because the introduction of nonsilencing shRNA (*i.e.* scram) failed to restore IκBα degradation (Fig. 2*C*). Taken together, these findings indicate that Fmod repressed the activation of NF-κB by increasing the stability of IκBα.

Fmod Stabilizes IκBα by Activating JNK—Mammalian MAPKs play important roles in many different signaling pathways, including the control of gene expression, and of cell proliferation, differentiation, and apoptosis (28, 29). NF-κB and JNK are two key regulators of the pathophysiology of cells, and cross-talk between these two signaling molecules has been reported in the scientific literature (30–32). These connections prompted us to test whether the ability of Fmod to inhibit NF-κB transpired through MAPKs, particularly JNK. In doing so, we monitored the activation status of individual MAPKs by immunoblotting extracts prepared from parental (*i.e.* GFP) or Fmod-expressing fibroblasts with phospho-specific anti-MAPK antibodies. Fig. 3*A* shows that although Fmod expression failed to activate ERK1/2 and elic-

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

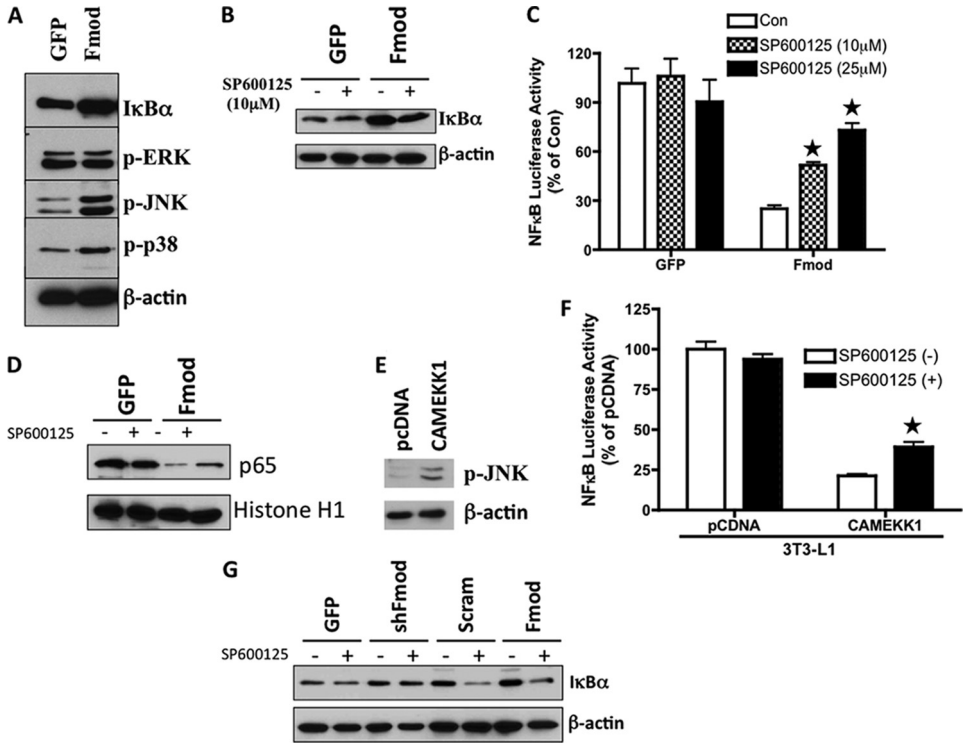


FIGURE 3. **Fmod stabilizes IκBα by activating JNK.** *A,* whole-cell extracts prepared from parental (*i.e.* GFP) and Fmod-expressing cells were fractionated through 10% SDS-PAGE and immobilized onto nitrocellulose. Immunoblot analysis with antibodies against IκBα, phospho-ERK (*p-ERK*), phospho-JNK (*p-JNK*), and phospho-p38 MAPK (*p-p38*) showed that Fmod specifically couples to JNK activation. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed three times with identical results. *B,* parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated in the absence or presence of the JNK inhibitor SP600125 (10 μM) for 24 h. Afterward, the expression levels of IκBα were detected by immunoblot analysis using anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed three times with similar results. *C,* parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the JNK inhibitor (SP600125) as indicated. Data are the mean (±S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (★, $p < 0.05$). *D,* parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h with the JNK inhibitor (SP600125) as indicated, and nuclear extracts were prepared and incubated with biotinylated NF-κB oligonucleotide probes. Afterward, p65/RelA-oligonucleotide complexes were captured with streptavidin-agarose beads and visualized by immunoblotting with antibodies against total p65/RelA. Data are from a representative experiment that was performed three times with similar results. *E,* control (*i.e.* pcDNA) or constitutively active MEKK1 (*CAMEKK1*) were transiently transfected into 3T3-L1 cells, whose subsequent level of JNK phosphorylation was monitored by immunoblot analysis using anti-phospho-JNK antibodies (*p-JNK*). Differences in protein loading were controlled by immunoblotting for β-actin. Images are from a single experiment that was performed four times with identical results. *F,* control (*i.e.* pcDNA) and constitutively active MEKK1 (*CAMEKK1*) were co-transfected with pNF-κB-luciferase and pCMV-β-gal and subsequently were incubated for 24 h with the JNK inhibitor SP600125 (25 μM) as indicated. Data are the mean (±S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (★, $p < 0.05$). *G,* Fmod-expressing (Fmod, *scram*) and Fmod-depleted (*shFmod* and *GFP*) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor SP600125 (25 μM). The expression levels of IκBα were analyzed by immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are from a representative experiment that was performed three times with similar results. *Con*, control.

ited only modest activation of p38 MAPK, this ECM protein did significantly stimulate the phosphorylation of JNK in a manner that correlated with elevated IκBα expression. Thus, JNK activation may underlie diminished NF-κB activity in Fmod-expressing fibroblasts. To address this hypothesis, we treated parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts with the JNK inhibitor, SP600125, prior to monitoring changes in IκBα expression. Interestingly, inhibiting JNK activity elicited only modest alterations in IκBα levels in parental (*i.e.* GFP) fibroblasts; however, this same experimental treatment induced a dramatic reduction of IκBα in Fmod-expressing cells (Fig. 3*B*), suggesting that Fmod-mediated activation of JNK prevents IκBα degradation. Moreover, this finding contrasts sharply with the failure of inhibitors against p38 MAPK (SB203580) or MEK1/2 (U0126) to alter IκBα expression in Fmod-expressing 3T3-L1 fibroblasts (data not shown). Along these lines, we observed SP600125 to dose-dependently increase NF-κB activity specifically in Fmod-expressing fibroblasts but not in their parental (*i.e.* GFP) counterparts (Fig. 3*C*). Similarly, transient expression of Fmod in 3T3-L1 fibroblasts significantly reduced their resting levels of NF-κB activity by ~50%, an effect that was significantly reversed following administration of SP600125 (see supplemental Fig. S4). We also observed significantly less NF-κB to be captured from nuclear fractions of Fmod-expressing 3T3-L1 cells as compared with their GFP-expressing counterparts (Fig. 3*D*). Moreover, inhibiting JNK activity augmented the activation and nuclear accumulation of NF-κB specifically in Fmod-expressing 3T3-L1 cells (Fig. 3*D*). To further address the role of JNK in prolonging the half-life of IκBα, we also expressed a constitutively active mutant of MEKK1 (CAMEKK1), which couples to JNK activation (33). As expected, elevated JNK phosphorylation was readily detected in fibroblasts transiently transfected with CAMEKK1 as com-

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

pared with mock (*i.e.* empty pcDNA)-transfected cells (Fig. 3*E*). Moreover, expression of CAMEKK1 in 3T3-L1 fibroblasts reduced their NF-κB activity by ~75%, a response that was partially neutralized by administration of SP600125 (Fig. 3*F*). Thus, enforced activation of JNK by expression of CAMEKK1 in parental (*i.e.* GFP) fibroblasts recapitulated the actions of Fmod on NF-κB signaling in fibroblasts. Finally, to determine the role of Fmod in promoting JNK-mediated IκBα stabilization, we once again depleted constitutive Fmod expression in 3T3-L1 fibroblasts using shRNAs directed against this ECM protein. As shown in Fig. 3*G*, administering SP600125 to Fmod-expressing cells (*i.e.* Fmod and Fmod/scram) readily promoted IκBα degradation; however, this same treatment protocol failed to effect IκBα levels in cells depleted of Fmod. Taken together, these findings strongly suggest that Fmod expression stimulates JNK and its ability to prolong the half-life of IκBα, leading to reduced NF-κB activity in fibroblasts.

Constitutive Degradation of IκBα in Fibroblasts Is Mediated by Calpain—Several reports established calpain as a mediator of constitutive IκBα degradation (34–36). In light of our findings that demonstrated the ability of Fmod to suppress IκBα degradation, we next determined whether calpain activity was differentially regulated between parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts using the Calpain-Glo™ protease assay. As shown in Fig. 4*A*, Fmod-expressing 3T3-L1 fibroblasts exhibited 40% less calpain activity as compared with their parental (*i.e.* GFP) counterparts, a finding consistent with the ability of Fmod to enhance IκBα stability (Figs. 1–3). As such, we determined whether administering the calpain inhibitor, EST, could alter NF-κB activity in a JNK-dependent manner. Fig. 4, *B* and *C*, shows that inhibiting calpain alone or in combination with JNK had no effect on NF-κB activity in parental (*i.e.* GFP) fibroblasts. In stark contrast, NF-κB activity was readily induced in Fmod-expressing cells treated with the JNK inhibitor SP600125. More importantly, the effects of JNK inactivation on NF-κB activity were readily reversed by administration of the calpain inhibitor, EST (Fig. 4, *B* and *C*). Identical findings were obtained by treating Fmod-expressing fibroblasts with a second independent calpain inhibitor, calpain inhibitor III (see supplemental Fig. S5). Taken together, these findings suggest that the elevated expression of Fmod inhibited calpain protease activity and its degradation of IκBα, which manifests significantly reduced NF-κB activity in 3T3-L1 fibroblasts.

Along these lines, the ability of calpain to degrade IκBα has been associated with the activation of CK2 (36, 37). Our findings thus far suggest that parental 3T3-L1 fibroblasts likely contain high levels of active CK2, which couples to calpain activation and the rapid degradation of IκBα, resulting in constitutive NF-κB activity. Thus, constitutively active NF-κB activity in parental (*i.e.* GFP) fibroblasts is predicted to be inhibited by administration of CK2 antagonists, although their Fmod-expressing counterparts are predicted to be refractory to CK2 inhibition. To determine the validity of these predictions, we measured NF-κB luciferase activity in parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with the CK2 inhibitor, TBBz. Fig. 4*D*

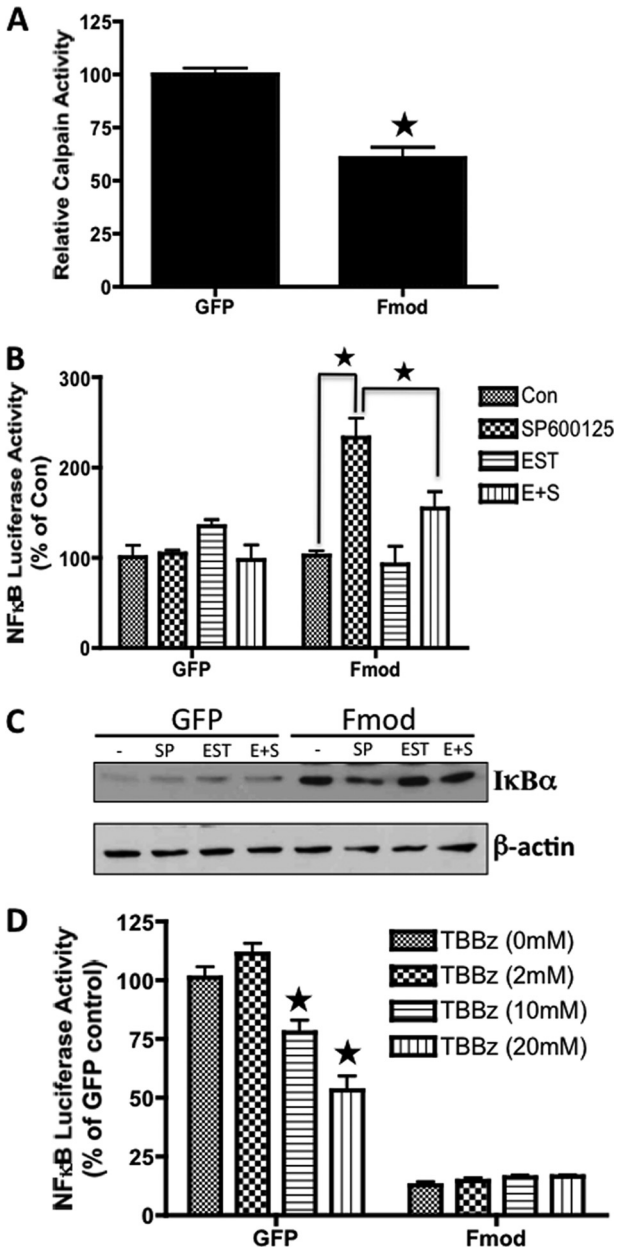


FIGURE 4. **Constitutive degradation of IκBα in fibroblasts is mediated by calpain.** *A*, calpain activity was measured using the Calpain-Glo™ protease assay kit according to the manufacturer's protocol (Promega). Data are the mean (±S.E.; $n = 3$) calpain activity relative to that measured in parental (*i.e.* GFP) cells. *B*, parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently they were incubated for 24 h with inhibitors to JNK (SP600125, 25 μM), calpain (EST, 50 μM), or both compounds as indicated. Data are the mean (±S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (★, $p < 0.05$). *Con*, control. *C*, parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h with inhibitors to JNK (SP600125, 25 μM), calpain (EST, 50 μM), or both compounds as indicated. Afterward, whole-cell extracts were prepared, fractionated, and immobilized to nitrocellulose prior to their immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. *D*, parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal and subsequently were incubated for 24 h with the CK2 inhibitor TBBz as indicated. Data are the mean (±S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (★, $p < 0.05$).

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

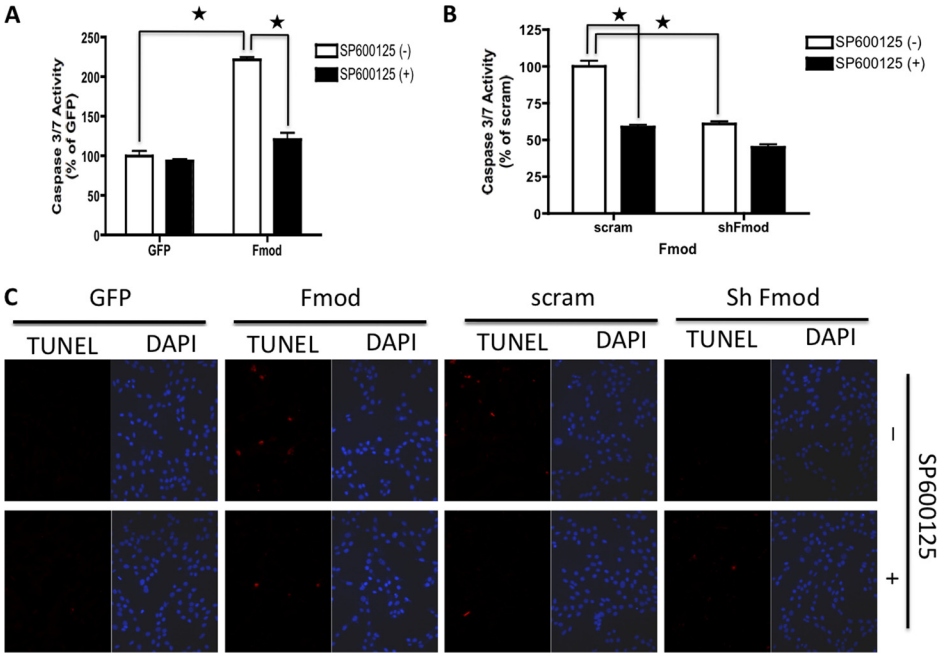


FIGURE 5. **Fmod induces fibroblast apoptosis.** *A,* parental (*i.e.* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated in the absence or presence of JNK inhibitor SP600125 (25 μM) for 24 h, at which point caspase-3/7 activity was measured using Caspase-Glo™ 3/7 assay according to the manufacturer's recommendations (Promega). Data are the mean (±S.E.) of activity observed in three independent experiments completed in triplicate (★, $p < 0.05$). *B,* Fmod-expressing (*scram*) and Fmod-depleted (*shFmod*) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor SP600125 (25 μM). Afterward, caspase-3/7 activity was measured as above. Data are the mean (±S.E.) activity observed in three independent experiments completed in triplicate (★, $p < 0.05$). *C,* Fmod-expressing (Fmod, *scram*) and Fmod-depleted (GFP, *shFmod*) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor SP600125 (25 μM). Afterward, TUNEL staining was performed. Data are from a representative experiment that was performed three times with similar results.

shows that NF-κB activity in Fmod-expressing fibroblasts was insensitive to the administration of TBBz; however, this same experimental regimen significantly inhibited the transcriptional activity of NF-κB in parental (*i.e.* GFP) fibroblasts. Moreover, identical findings were obtained by treating parental (*i.e.* GFP) and Fmod-expressing fibroblasts with a second independent CK2 inhibitor, TBCA (see supplemental Fig. S6). Collectively, these findings suggest that the constitutive turnover of IκBα is mediated by a CK2-calpain signaling axis that is inactivated in Fmod-expressing fibroblasts in a JNK-dependent manner.

Fmod Induces Fibroblast Apoptosis—Recent findings have linked CK2 to the regulation of cell survival in part via its ability to promote IκBα degradation and NF-κB activation (38). Because constitutive Fmod expression promotes JNK activation coupled to reduced calpain activity and enhanced IκBα stability, we investigated the effect of Fmod on fibroblast survival using the Caspase-Glo™ 3/7 assay kit, which showed that Fmod-expressing fibroblasts possessed significantly higher caspase-3/7 activities as compared with parental (*i.e.* GFP) fibroblasts (Fig. 5*A*). In contrast to parental (*i.e.* GFP) fibroblasts, SP600125 administration significantly reduced caspase-3/7 activity in Fmod-expressing fibroblasts, suggesting that Fmod-mediated activation of JNK couples to caspase-3/7 activation. We also determined the relative reliance of caspase-3/7 activation on Fmod by once again depleting its expression in Fmod-expressing cells via introduction of Fmod-directed shRNA. Fig. 5*B* clearly shows that Fmod expression was essential for JNK-dependent caspase-3/7 activation in 3T3-L1 fibroblasts. Along these lines, we observed Fmod-expressing 3T3-L1 cells to possess elevated TUNEL staining as compared with their parental and Fmod-depleted counterparts (Fig. 5*C*). As above, inhibiting JNK activity significantly reduced TUNEL staining only in Fmod-expressing 3T3-L1 cells (Fig. 5*C*), suggesting that Fmod-mediated activation of JNK promotes apoptosis in fibroblasts. Collectively, these findings demonstrate that Fmod expression is necessary for the activation of JNK (Fig. 3) and, consequently, for activation of caspase-3/7 to initiate fibroblast apoptosis (Fig. 5).

DISCUSSION

NF-κB is a ubiquitously expressed transcription factor that governs the transcription of genes involved in immune responses, angiogenesis, cell transformation, invasion, migration, apoptosis, and cell cycle (39). As such, the inappropriate activation of NF-κB has been linked to a variety of inflammatory and autoimmune diseases and of human malignancies (40). The signaling of NF-κB is normally suppressed by a family of inhibitory molecules termed IκB proteins, such as IκBα that binds NF-κB and prevents its activation in the cytoplasm. In response to a variety of stimuli, including proinflammatory cytokines (*e.g.* TNF-α or IL-1), T and B cell mitogens, lipopolysaccharide, viral infections, and cellular stresses (*e.g.* ionizing radiation or chemotherapies) (41, 42), IκBα molecules are rapidly degraded to facilitate the activation and nuclear translocation of NF-κB (39). The extent to which ECM molecules regulate NF-κB activity and the molecular mechanisms whereby these events transpire remain incompletely understood. To this end, we show here for the first time that Fmod is a novel gene target of FBLN5 (Table 1), which itself func-

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

tions as an effector of TGF-β. More importantly, we established a novel Fmod signaling axis that significantly enhances the stability and accumulation of IκBα proteins via the activation of JNK, which suppresses the ability of CK2 to stimulate calpain and its induction of IκBα turnover. The net effect of these Fmod-dependent events manifests as reduced NF-κB activity and elevated fibroblast apoptosis. This response differs significantly from canonical NF-κB activation, which transpires through IκB kinase-dependent phosphorylation of IκBα and its subsequent ubiquitination and proteosomal degradation (35, 41–44). In fact, our Fmod findings are more aligned with the regulatory events employed in resting cells to govern the balance between IκBα expression and NF-κB activity (43, 45). Indeed, IκBα is rapidly degraded to elicit constitutive NF-κB activity in a variety of cells, including in B and T cells (34, 46), in hepatocytes (38), and as shown here, in fibroblasts. Moreover, our findings established a novel signaling axis that enables Fmod to suppress NF-κB activity via a JNK-dependent mechanism operant in stabilizing IκBα expression. Moreover, we demonstrated the ability of this Fmod signaling axis to negate the pro-survival signals generated by CK2 and its stimulation of NF-κB via calpain-mediated degradation of IκBα. Future studies need to address how altered fibroblast survival impacts stromal homeostasis and its paracrine activity on neighboring epithelial cells.

Along these lines, lysyl oxidases comprise a five member gene family of copper-dependent amine oxidases that function in cross-linking collagens to elastin in the ECM, thereby increasing the tensile strength and structural integrity of tissues during embryonic development and organogenesis, and during the maintenance of normal tissue homeostasis (48, 49). Interestingly, recent findings found that lysyl oxidase expression in Ras-transformed NIH3T3 cells inhibited the activity of NF-κB in part by stabilizing IκBα (50). In addition, FBLN5 interacts physically with the lysyl oxidase family members LOXL1, LOXL2, and LOXL4 (51, 52), which points to a common link between FBLN5, Fmod, and lysyl oxidases in contributing to the suppression of NF-κB activity by increasing the half-life of IκBα. Our findings also suggest that these ECM proteins fulfill both structural and signaling functions within the stromal compartment. The precise interplay between these ECM components in regulating the pathophysiological actions of NF-κB awaits further experimentation.

At present, there exist two major pathways coupled to the turnover of IκBα, namely proteosome- and calpain-dependent degradation of IκBα. Overexpression of the epidermal growth factor receptor family member Her-2/neu induces constitutive NF-κB activity through a PI3K/Akt-dependent pathway that promotes calpain-mediated degradation of IκBα (53). Moreover, it has been reported that IκBα degradation in B cells transpires exclusively via a calcium/calpain-dependent pathway but not via the ubiquitin/proteosome-dependent pathway (35). Finally, CK2-dependent phosphorylation of the PEST domain in IκBα facilitates its destruction by calpain (36). Our study reinforces the importance of calpain in regulating constitutive NF-κB activity in fibroblasts, as well as identifies a novel Fmod-JNK-mediated signaling axis operant in neutralizing the pro-survival functions of CK2 and calpain. Our findings, together with those mentioned above, suggest that these players may represent a general mechanism to fine-tune NF-κB signaling in resting cells and tissues, particularly their ability to adapt to altered survival signals. Up-regulated expression of Fmod and other proteoglycans has been detected during the repair and remodeling of wounded collagen matrices, a response that transpires in the absence of obvious inflammatory reactions (54). Moreover, we previously associated FBLN5 to enhanced wound healing *in vivo* (55), and as such, future studies need to investigate the relative contribution of Fmod to these events and to the initiation and resolution of tissue inflammation and fibrotic responses.

As mentioned previously, Fmod belongs to the LLR family of ECM proteoglycans and glycoproteins typically found in connective tissues, including cartilage, tendon, skin, cornea, and sclera (1). Fmod plays a significant role in collagen assembly and maintenance, and Fmod-deficient mice display abnormally thin type I collagen fibrils in their tendons, which increases their occurrence of arthritis (56). Besides its structural functions, Fmod also transduces signals by interacting with a variety of ECM and secreted molecules. Indeed, recent work demonstrated the ability of Fmod to stimulate the complement cascade through its physical interaction with C1q (57). Interestingly, although other ECM molecules such as laminin and decorin also bind C1q, only Fmod is capable of inducing complement activation and sustained inflammatory reactions. Thus, studies aimed at determining the interplay between Fmod and NF-κB in directing inflammation also appear warranted. Likewise, the regulatory roles of NF-κB in cancer biology, including its coupling to tissue invasion, migration, and metastasis, have been thoroughly established (58, 59). Interestingly, the expression of Fmod transcripts are significantly reduced in metastatic tumors as compared with their non-metastatic counterparts, particularly tumors originating in the breast (60, 61) and prostate (62–64). In addition, Oncomine analyses of Fmod expression showed the expression of this proteoglycan to be reduced at metastatic sites relative to non-metastatic lesions in gastric cancers, head and neck cancers, and sarcomas (data not shown). These analyses also found aberrantly low Fmod expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood (data not shown). Thus, future studies need to investigate the specific contributions of diminished Fmod expression that elicit the acquisition of metastatic phenotypes in human tumors.

Finally, Fmod clearly functions in regulating the dynamics between developing carcinoma and their accompanying stroma (65). In particular, desmoplastic reactions in carcinomas elicit elevated interstitial fluid pressure that diminishes efficient nutrient and gaseous transfer, as well as reduces the delivery of cancer chemotherapeutics (47). The inflammatory environment of developing carcinomas is thought to induce stromal production of Fmod, leading to the acquisition of dense and rigid tissue architectures (65). Future studies clearly need to address the chemotherapeutic potential of augmenting or attenuating Fmod action in developing carcinomas as a novel means to improve the efficacy of cancer chemotherapeutics.

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

Acknowledgments—We thank the members of the Schiemann laboratory for critical reading of the manuscript.

REFERENCES

1. Oldberg, A., Antonsson, P., Lindblom, K., and Heinegård, D. (1989) *EMBO J.* **8,** 2601–2604
2. Hardingham, T. E., and Fosang, A. J. (1992) *FASEB J.* **6,** 861–870
3. Roughley, P. J. (2006) *Eur. Cell Mater.* **12,** 92–101
4. Westergren-Thorsson, G., Antonsson, P., Malmström, A., Heinegård, D., and Oldberg, A. (1991) *Matrix* **11,** 177–183
5. Petri, J. B., Rott, O., Wetzig, T., Herrmann, K., and Haustein, U. F. (1999) *Mol. Cell. Biol. Res. Commun.* **1,** 59–65
6. Hildebrand, A., Romarís, M., Rasmussen, L. M., Heinegård, D., Twardzik, D. R., Border, W. A., and Ruoslahti, E. (1994) *Biochem. J.* **302,** 527–534
7. Westergren-Thorsson, G., Hernnäs, J., Särnstrand, B., Oldberg, A., Heinegård, D., and Malmström, A. (1993) *J. Clin. Invest.* **92,** 632–637
8. Reed, C. C., and Iozzo, R. V. (2002) *Glycoconj. J.* **19,** 249–255
9. Bi, Y., Ehirchiou, D., Kilts, T. M., Inkson, C. A., Embree, M. C., Sonoyama, W., Li, L., Leet, A. I., Seo, B. M., Zhang, L., Shi, S., and Young, M. F. (2007) *Nat. Med.* **13,** 1219–1227
10. Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000) *N. Engl. J. Med.* **342,** 1350–1358
11. Tian, M., and Schiemann, W. P. (2009) *Future Oncol.* **5,** 259–271
12. Schiemann, W. P., Blobe, G. C., Kalume, D. E., Pandey, A., and Lodish, H. F. (2002) *J. Biol. Chem.* **277,** 27367–27377
13. Albig, A. R., and Schiemann, W. P. (2004) *DNA Cell Biol.* **23,** 367–379
14. Albig, A. R., Neil, J. R., and Schiemann, W. P. (2006) *Cancer Res.* **66,** 2621–2629
15. Lee, Y. H., Albig, A. R., Regner, M., Schiemann, B. J., and Schiemann, W. P. (2008) *Carcinogenesis* **29,** 2243–2251
16. Benjamin, M., and Ralphs, J. R. (2004) *Int. Rev. Cytol.* **233,** 1–45
17. Schaefer, L., Gröne, H. J., Raslik, I., Robenek, H., Ugorcakova, J., Budny, S., Schaefer, R. M., and Kresse, H. (2000) *Kidney Int.* **58,** 1557–1568
18. Wendt, M. K., and Schiemann, W. P. (2009) *Breast Cancer Res.* **11,** R68
19. Neil, J. R., Johnson, K. M., Nemenoff, R. A., and Schiemann, W. P. (2008) *Carcinogenesis* **29,** 2227–2235
20. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. (1996) *Nat. Genet.* **14,** 457–460
21. Marton, M. J., DeRisi, J. L., Bennett, H. A., Iyer, V. R., Meyer, M. R., Roberts, C. J., Stoughton, R., Burchard, J., Slade, D., Dai, H., Bassett, D. E., Jr., Hartwell, L. H., Brown, P. O., and Friend, S. H. (1998) *Nat. Med.* **4,** 1293–1301
22. Schiemann, W. P., Graves, L. M., Baumann, H., Morella, K. K., Gearing, D. P., Nielsen, M. D., Krebs, E. G., and Nathanson, N. M. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 5361–5365
23. Neil, J. R., and Schiemann, W. P. (2008) *Cancer Res.* **68,** 1462–1470
24. Birdsey, G. M., Dryden, N. H., Amsellem, V., Gebhardt, F., Sahnan, K., Haskard, D. O., Dejana, E., Mason, J. C., and Randi, A. M. (2008) *Blood* **111,** 3498–3506
25. Droguett, R., Cabello-Verrugio, C., Riquelme, C., and Brandan, E. (2006) *Matrix Biol.* **25,** 332–341
26. Kobayashi, T., Hattori, S., and Shinkai, H. (2003) *Acta Derm. Venereol.* **83,** 105–107
27. Israël, A. (2000) *Trends Cell Biol.* **10,** 129–133
28. Raman, M., Chen, W., and Cobb, M. H. (2007) *Oncogene* **26,** 3100–3112
29. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) *Endocr. Rev.* **22,** 153–183
30. Karin, M. (2006) *Nature* **441,** 431–436
31. Chen, F., Castranova, V., and Shi, X. (2001) *Am. J. Pathol.* **159,** 387–397
32. Liu, J., and Lin, A. (2007) *Oncogene* **26,** 3267–3278
33. Fuchs, S. Y., Adler, V., Pincus, M. R., and Ronai, Z. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10541–10546
34. Ponnappan, S., Cullen, S. J., and Ponnappan, U. (2005) *Immun. Ageing* **2,** 15
35. Miyamoto, S., Seufzer, B. J., and Shumway, S. D. (1998) *Mol. Cell. Biol.* **18,** 19–29
36. Shen, J., Channavajhala, P., Seldin, D. C., and Sonenshein, G. E. (2001) *J. Immunol.* **167,** 4919–4925
37. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996) *Mol. Cell. Biol.* **16,** 1401–1409
38. Cavin, L. G., Romieu-Mourez, R., Panta, G. R., Sun, J., Factor, V. M., Thorgeirsson, S. S., Sonenshein, G. E., and Arsura, M. (2003) *Hepatology* **38,** 1540–1551
39. Lee, C. H., Jeon, Y. T., Kim, S. H., and Song, Y. S. (2007) *Biofactors* **29,** 19–35
40. Baldwin, A. S., Jr. (2001) *J. Clin. Invest.* **107,** 3–6
41. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274,** 784–787
42. Pahl, H. L. (1999) *Physiol. Rev.* **79,** 683–701
43. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9,** 2723–2735
44. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20
45. Ferreiro, D. U., and Komives, E. A. (2010) *Biochemistry* **49,** 1560–1567
46. O'Connor, S., Shumway, S. D., Amanna, I. J., Hayes, C. E., and Miyamoto, S. (2004) *Mol. Cell. Biol.* **24,** 4895–4908
47. Heldin, C. H., Rubin, K., Pietras, K., and Ostman, A. (2004) *Nat. Rev. Cancer* **4,** 806–813
48. Lucero, H. A., and Kagan, H. M. (2006) *Cell. Mol. Life Sci.* **63,** 2304–2316
49. Payne, S. L., Hendrix, M. J., and Kirschmann, D. A. (2006) *J. Cell. Biochem.* **98,** 827–837
50. Jeay, S., Pianetti, S., Kagan, H. M., and Sonenshein, G. E. (2003) *Mol. Cell. Biol.* **23,** 2251–2263
51. Liu, X., Zhao, Y., Gao, J., Pawlyk, B., Starcher, B., Spencer, J. A., Yanagisawa, H., Zuo, J., and Li, T. (2004) *Nat. Genet.* **36,** 178–182
52. Hirai, M., Horiguchi, M., Ohbayashi, T., Kita, T., Chien, K. R., and Nakamura, T. (2007) *EMBO J.* **26,** 3283–3295
53. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J., and Sonenshein, G. E. (2001) *Oncogene* **20,** 1287–1299
54. Provenzano, P. P., Alejandro-Osorio, A. L., Valhmu, W. B., Jensen, K. T., and Vanderby, R., Jr. (2005) *Matrix Biol.* **23,** 543–555
55. Lee, M. J., Roy, N. K., Mogford, J. E., Schiemann, W. P., and Mustoe, T. A. (2004) *J. Am. Coll. Surg.* **199,** 403–410
56. Ameye, L., Aria, D., Jepsen, K., Oldberg, A., Xu, T., and Young, M. F. (2002) *FASEB J.* **16,** 673–680
57. Sjöberg, A., Onnerfjord, P., Mörgelin, M., Heinegård, D., and Blom, A. M. (2005) *J. Biol. Chem.* **280,** 32301–32308
58. Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z. W., Egan, L. J., Kagnoff, M. F., and Karin, M. (2004) *Cell* **118,** 285–296
59. Luo, J. L., Maeda, S., Hsu, L. C., Yagita, H., and Karin, M. (2004) *Cancer Cell* **6,** 297–305
60. Schmidt, M., Böhm, D., von Törne, C., Steiner, E., Puhl, A., Pilch, H., Lehr, H. A., Hengstler, J. G., Kölbl, H., and Gehrmann, M. (2008) *Cancer Res.* **68,** 5405–5413
61. Desmedt, C., Piette, F., Loi, S., Wang, Y., Lallemand, F., Haibe-Kains, B., Viale, G., Delorenzi, M., Zhang, Y., d'Assignies, M. S., Bergh, J., Lidereau, R., Ellis, P., Harris, A. L., Klijn, J. G., Foekens, J. A., Cardoso, F., Piccart, M. J., Buyse, M., and Sotiriou, C. (2007) *Clin. Cancer Res.* **13,** 3207–3214
62. Varambally, S., Yu, J., Laxman, B., Rhodes, D. R., Mehra, R., Tomlins, S. A., Shah, R. B., Chandran, U., Monzon, F. A., Becich, M. J., Wei, J. T., Pienta, K. J., Ghosh, D., Rubin, M. A., and Chinnaiyan, A. M. (2005) *Cancer Cell* **8,** 393–406
63. LaTulippe, E., Satagopan, J., Smith, A., Scher, H., Scardino, P., Reuter, V., and Gerald, W. L. (2002) *Cancer Res.* **62,** 4499–4506
64. Magee, J. A., Araki, T., Patil, S., Ehrig, T., True, L., Humphrey, P. A., Catalona, W. J., Watson, M. A., and Milbrandt, J. (2001) *Cancer Res.* **61,** 5692–5696
65. Oldberg, A., Kalamajski, S., Salnikov, A. V., Stuhr, L., Mörgelin, M., Reed, R. K., Heldin, N. E., and Rubin, K. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 13966–13971

Downloaded from www.jbc.org at Cleveland Health Sciences Library, on March 16, 2011

SUPPLEMENTAL MATERIALS

FIBROMODULIN SUPPRESSES NUCLEAR FACTOR-κB ACTIVITY BY INDUCING THE DELAYED DEGRADATION OF IKBA *VIA* A JNK-DEPENDENT PATHWAY COUPLED TO FIBROBLAST APOPTOSIS

Yong-Hun Lee and William P. Schiemann

From the Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106

Address Correspondence To: William P. Schiemann, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, Room 2131, 2103 Cornell Road, Cleveland, OH 44121. Phone: 216-368-5763. Fax: 216-368-1166. Email: William.Schiemann@case.edu

Supplemental Table S1: Lee, Y-H. and Schiemann, W.P.

SUPPLEMANTAL TABLE S1: **Real-time PCR Primer Sequences**

Gene Target	Application	Sequence (5' to 3')
BB503935	PCR-Sense	5' TCACCCAGCAAAGTAGCACAGAGT
BB503935	PCR-Antisense	5' GGTTCCCCACGCCCACAC
BB533736	PCR-Sense	5' CCAGAGCTGCGGAACGACAT
BB533736	PCR-Antisense	5' GGCTCATATCCCACCCACCTG
BB831146	PCR-Sense	5' ACCTATACCTCAGACCCCGACAGC
BB831146	PCR-Antisense	5' AATGGTAATAAGACGTAGAAAATGCTACC
BC010215	PCR-Sense	5' TGGCTCATTCCTATCTGTGTAACTT
BC010215	PCR-Antisense	5' GCTTTATGCCTGTTTTTGCTTTTGC
BC016492	PCR-Sense	5' GCTGGGCCCTCAAACTAGG
BC016492	PCR-Antisense	5' ACGGTGCGATCCTCTGTGTAAA
BC019150	PCR-Sense	5' CCCCCACCCTGCTCTCCCAACTAA
BC019150	PCR-Antisense	5' CTATCCCCACCCCACCCCCAATCT
BC025502	PCR-Sense	5’ CAATTCCTGCCGCTCCTCTACCAC
BC025502	PCR-Antisense	5’ GCTGCTTCGGCCCCCTACG
M87276	PCR-Sense	5’ TGCCCGCTCCACTCTGCCTTACT
M87276	PCR-Antisense	5’ GCTCCTGCCTCCCCACATCTCA
NM_007729	PCR-Sense	5’ GGTGCTCATTCCCGTCCAT
NM_007729	PCR-Antisense	5’ ATATTTATTGCCATCACAGTCCACAATTG
NM_009640	PCR-Sense	5’ ATTTACTGATTTGTTATGTGGGTTTGA
NM_009640	PCR-Antisense	5’ AAGTTCGAGTTATCTAATTTTTCTGCCC
NM_010516	PCR-Sense	5’ CCCCCTTTCTTGGTTCTACAATTG
NM_010516	PCR-Antisense	5’ TGCCCCAAGGACACTCACAG
NM_015814	PCR-Sense	5’ TGGCAAGGGAATGTGGTAGAGTCA
NM_015814	PCR-Antisense	5’ GATTGTAGCACCCAGAAGAGTAAC
NM_021355	PCR-Sense	5’ CTATGCAGTCCGCCTTATGCCC
NM_021355	PCR-Antisense	5’ AACTGCTTCTTGCCACTCGCTACC
GAPDH	PCR-Sense	5’ CAACTTTGGCATTGTGGAAGGGCTC
GAPDH	PCR-Antisense	5’ GCAGGGATGATGTT CTGGGCAGC

SUPPLEMANTAL TABLE S2: **shRNA Sequences used for Fmod Knockdown**

NAME	Application	Sequence (5' to 3')
Fmod sh1	**Knockdown**	CCGGCCTAGAACACAACAATGTCTACTCGAGTAGACATTGTTGTGTTCTAGGTTTTTG
Fmod sh2	**Knockdown**	CCGGCCGCATGAAGTACGTCTACTTCTCGAGAAGTAGACGTACTTCATGCGGTTTTTG
Fmod sh3	**Knockdown**	CCGGGCGGCCAACCTAACCAATAAACTCGAGTTTATTGGTTAGGTTGGCCGCTTTTTG
Fmod sh4	**Knockdown**	CCGGCACAGCCATGTACTGTGACAACTCGAGTTGTCACAGTACATGGCTGTGTTTTTG
Fmod sh5	**Knockdown**	CCGGTGTCTCACAACAGTCTCACTACTCGAGTAGTGAGACTGTTGTGAGACATTTTTG

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Lee, Y-H. and Schiemann, W.P.

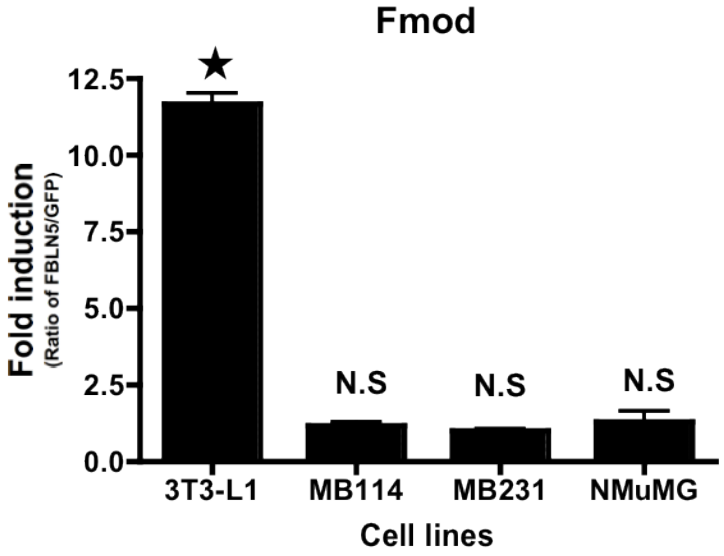


FIGURE S1. **FBLN5 induces Fmod expression in cell-type specific manner**. Real-time PCR analysis for Fmod was performed in various FBLN5-expressing cell lines, including fibroblasts (3T3-L1), endothelial (MB114), and normal (NMuMG) and malignant (MDA-MB-231; MB231) mammary epithelial cells. Fmod expression levels were normalized by calculating the ratios of Fmod expression in FBLN5-expressing cells relative to their corresponding GFP counterparts. Data are the mean (± SE) Fmod expression ratios observed in 3 independent experiments completed in triplicate (⋆, $P < 0.05$).

Figure S2: Lee, Y-H. and Schiemann, W.P.

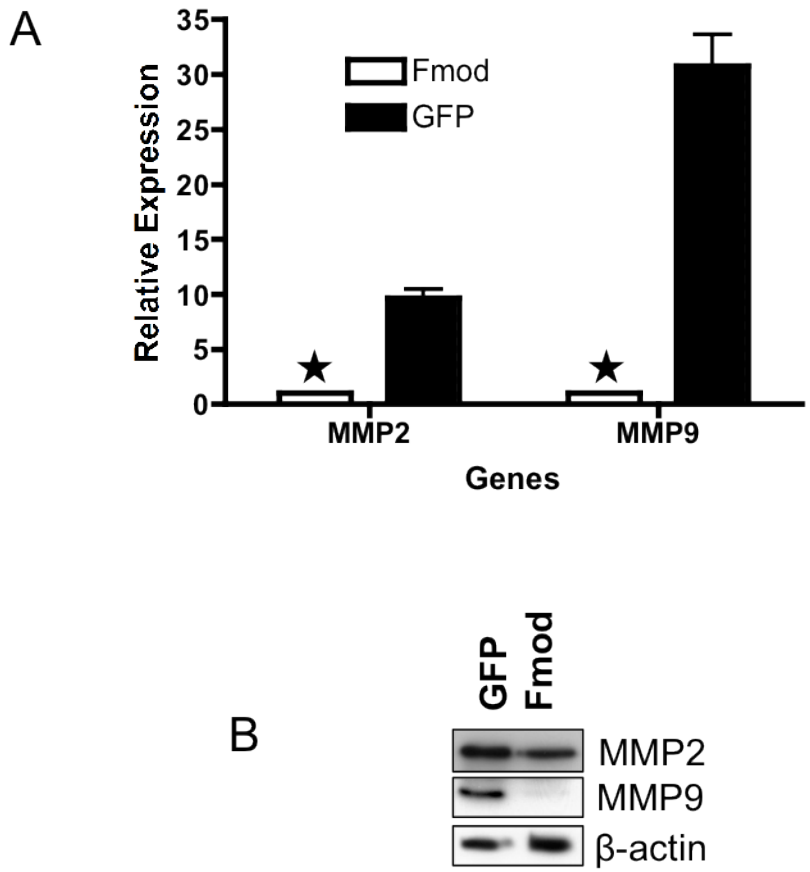


FIGURE S2. **Fmod suppressed NK-κB target genes in fibroblasts.** The expression level of MMP-2 and MMP-9 in parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were monitored by either real-time PCR analysis (*A*) or immunoblot analysis (*B*). Data are the mean (± SE) Fmod expression ratios observed in 3 independent experiments completed in triplicate (⋆, $P < 0.05$). Data are representative images from a single experiment that was performed 4 times.

Figure S3: Lee, Y-H. and Schiemann, W.P.

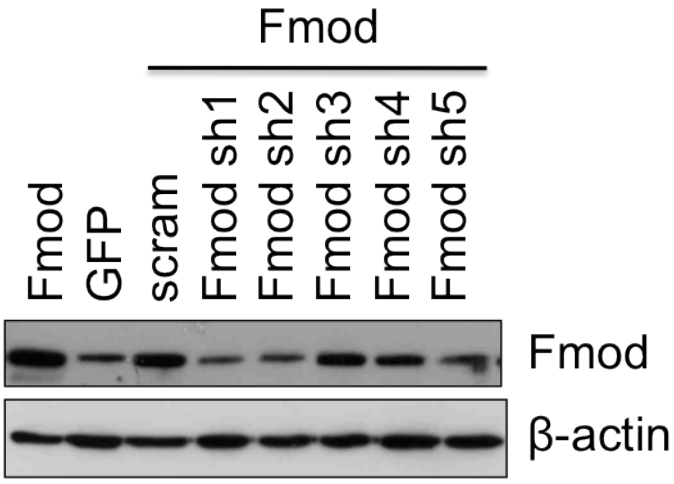


FIGURE S3. **Validation of Fmod knockdown by shRNA targeting of Fmod.** The expression levels of Fmod in Fmod-expressing (Fmod, scram) and Fmod-depleted (Fmod sh1-5) fibroblasts was monitored by immunoblot assay using antibodies against Fmod. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are representative images from a single experiment that was performed 4 times.

Figure S4: Lee, Y-H. and Schiemann, W.P.

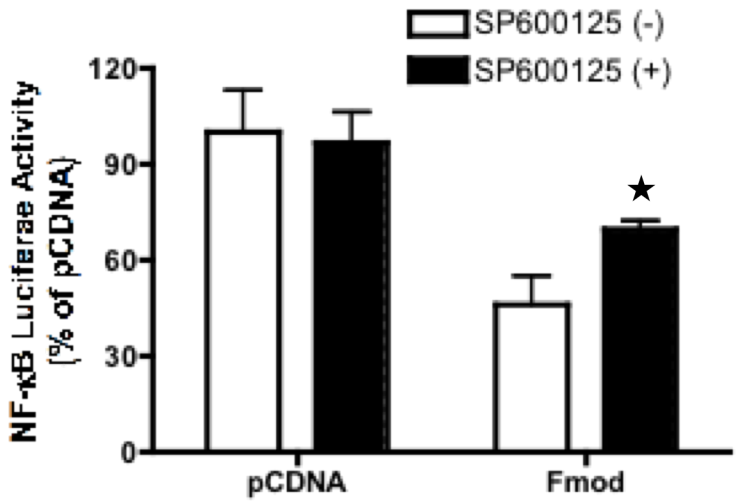


FIGURE S4. **Fmod suppresses NF-κB activity by activating JNK.** Control (*i.e.,* empty pcDNA3.1) or Fmod cDNA were transiently co-transfected with pNF-κB-luciferase and pCMV-β-gal into 3T3-L1 cells, and subsequently were incubated for 24 h in the absence (-) or presence (+) of the JNK inhibitor (SP600125, 25 μM) as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate **(⋆, P** < 0.05).

Figure S5: Lee, Y-H. and Schiemann, W.P.

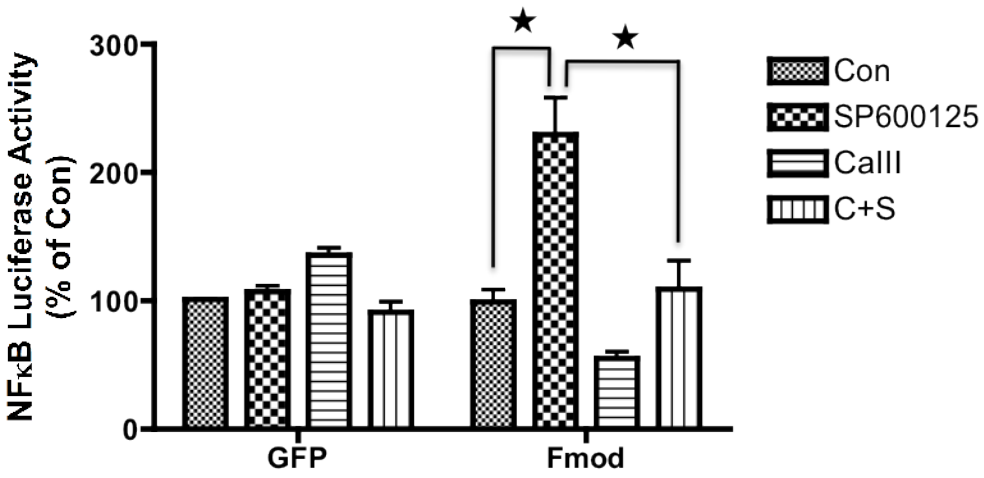


FIGURE S5. **Constitutive degradation of IκBα in fibroblasts is mediated by calpain.** Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with inhibitors to JNK (SP600125, 25 μM), calpain inhibitor III (CaIII, 50 μM), or both compounds as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (⋆, $P < 0.05$).

Figure S6: Lee, Y-H. and Schiemann, W.P.

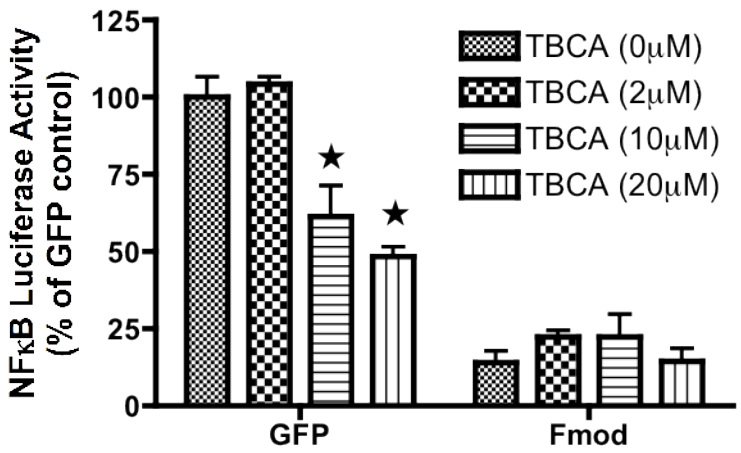


FIGURE S6. **Constitutive degradation of IκBα in fibroblasts is mediated by CK2.** Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the CK2 inhibitor, TBCA as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (⋆, $P < 0.05$).

NEOPLASIA
www.neoplasia.com

Volume 13 Number 5 May 2011 pp. 406–418 406

Lysyl Oxidase Contributes to Mechanotransduction-Mediated Regulation of Transforming Growth Factor-β Signaling in Breast Cancer Cells[1,2]

Molly A. Taylor*, Jay D. Amin†, Dawn A. Kirschmann‡ and William P. Schiemann*,§

*Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA; †Department of Pharmacology, University of Colorado Denver, Aurora, CO, USA; ‡Department of Pediatrics, Northwestern University, Chicago, IL, USA; §Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Abstract

Transforming growth factor-β (TGF-β) regulates all stages of mammary gland development, including the maintenance of tissue homeostasis and the suppression of tumorigenesis in mammary epithelial cells (MECs). Interestingly, mammary tumorigenesis converts TGF-β from a tumor suppressor to a tumor promoter through molecular mechanisms that remain incompletely understood. Changes in integrin signaling and tissue compliance promote the acquisition of malignant phenotypes in MECs in part through the activity of lysyl oxidase (LOX), which regulates desmoplastic reactions and metastasis. TGF-β also regulates the activities of tumor reactive stroma and MEC metastasis. We show here that TGF-β1 stimulated the synthesis and secretion of LOX from normal and malignant MECs *in vitro* and in mammary tumors produced in mice. The ability of TGF-β1 to activate Smad2/3 was unaffected by LOX inactivation in normal MECs, whereas the stimulation of p38 MAPK by TGF-β1 was blunted by inhibiting LOX activity in malignant MECs or by inducing the degradation of hydrogen peroxide in both cell types. Inactivating LOX activity impaired TGF-β1–mediated epithelial-mesenchymal transition and invasion in breast cancer cells. We further show that increasing extracellular matrix rigidity by the addition of type I collagen to three-dimensional organotypic cultures promoted the proliferation of malignant MECs, a cellular reaction that was abrogated by inhibiting the activities of TGF-β1 or LOX, and by degrading hydrogen peroxide. Our findings identify LOX as a potential mediator that couples mechanotransduction to oncogenic signaling by TGF-β1 and suggest that measures capable of inactivating LOX function may prove effective in diminishing breast cancer progression stimulated by TGF-β1.

Neoplasia (2011) 13, 406–418

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates mammary gland development, as well as suppresses mammary tumorigenesis [1,2]. In normal mammary epithelial cells (MECs), TGF-β acts as a tumor suppressor by inducing apoptosis and cell cycle arrest and by stimulating cellular differentiation. However, during breast cancer progression, TGF-β deviates from its role as a tumor suppressor to ultimately acquire tumor promoting functions, including the ability to induce breast cancer cell proliferation, invasion, and metastasis in part through the stimulation of epithelial-mesenchymal transition (EMT) [3,4]. The molecular mechanisms that engender this switch in TGF-β function during tumorigenesis are not well defined. Signaling through Smad2/3 generally is associated

Abbreviations: βAPN, β-aminopropionitrile; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; LOX, lysyl oxidase; MEC, mammary epithelial cell; NMuMG, normal murine mammary gland epithelial cell; TGF-β1, transforming growth factor-β1

Address all correspondence to: William P. Schiemann, PhD, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, Room 2131, 2103 Cornell Road, Cleveland, OH 44106. E-mail: wps20@case.edu

[1]Research support was provided in part by the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), the Department of Defense (BC084651), and the Case Comprehensive Cancer Center to W.P.S. and by the Department of Defense (BC093128) to M.A.T.

[2]This article refers to supplementary materials, which are designated by Figures W1 to W3 and are available online at www.neoplasia.com.

Received 30 July 2010; Revised 17 February 2011; Accepted 23 February 2011

Copyright © 2011 Neoplasia Press, Inc. All rights reserved 1522-8002/11/$25.00
DOI 10.1593/neo.101086

with cytostasis and maintenance of normal epithelial homeostasis. However, TGF-β also signals through non–Smad-mediated pathways, such as ERK1/2 (extracellular-regulated protein kinase1/2), p38 MAPK (p38 mitogen-activated protein kinase), JNK (c-Jun N-terminal protein kinase), PI3K (phosphoinositide-3-kinase), NF-κB (nuclear factor-κB), and Akt [2,3,5]. Indeed, we recently showed that $\alpha_v\beta_3$ integrin interacts with the TGF-β type II receptor (TβR-II), leading to its phosphorylation by Src at Tyr284 and subsequent activation of p38 MAPK [6–8]. Collectively, these events enable TGF-β to stimulate breast cancer growth, invasion, and metastasis. In fact, studies by our group [4,6–9] and others [5] support the notion that inappropriate imbalances between canonical (i.e., Smad2/3–dependent) *versus* noncanonical (i.e., Smad2/3–independent) TGF-β pathways underlie its acquisition of oncogenic function during tumor progression.

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that catalyzes the cross-linking of collagens and elastin in the extracellular matrix (ECM). LOX belongs to a five member gene family consisting of LOX, LOX-like 1 (LOXL1), LOXL2, LOXL3, and LOXL4, all of which play important roles in regulating ECM remodeling and cellular homeostasis [10,11]. In addition, elevated LOX activity is associated with the acquisition of increased ECM tension and stiffness in developing mammary tumors, a reaction that enhances integrin-mediated mechanotransduction coupled to increased breast cancer cell invasion and dissemination from hypoxic primary tumors [12–17]. Clinically, the aberrant expression of LOX, LOXL, and LOXL2 correlates with increased malignancy and invasiveness in human tumors, including those of the breast [16–18]. Along these lines, hydrogen peroxide produced as a byproduct of LOX-mediated collagen and elastin cross-linking stimulates Rac1 activity by promoting the assembly of p130Cas/Crk/Dock180 complexes [19]. Thus, LOX seems critical in governing the transition of tumors from indolent to aggressive disease states through the combined actions of its ECM cross-linking activities, and its production of the potential second messenger, hydrogen peroxide. Thus, chemotherapeutic targeting of LOX may one day improve the clinical course of metastatic breast cancer patients.

Given the parallels between LOX and TGF-β in regulating ECM dynamics and promoting mammary tumorigenesis, we sought to determine the role of LOX in regulating oncogenic TGF-β1 signaling and its coupling to EMT in normal and malignant MECs.

Materials and Methods

Cell Lines and Lentiviral Vectors

Normal murine mammary gland epithelial cell (NMuMG) and metastatic 4T1 cells were cultured as previously described [20], as were human MCF10ACA1a breast cancer cells [21] and human 293T embryonic kidney cells [22]. Lentiviral particles encoding for scrambled (i.e., nonsilencing shRNA) or murine LOX shRNA (Thermo Scientific, Huntsville, AL) were prepared as described previously [20]. The extent of LOX deficiency was monitored by immunoblot analysis using anti-LOX antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). A constitutively active LOX construct, LOX-32 [23], was synthesized by polymerase chain reaction (PCR), amplifying mature and catalytically active human LOX (nucleotides 505-1251) using oligonucleotides containing *Hind* III (N-terminus) and *Xho* I (C-terminus) restriction sites. The resulting PCR product was ligated into corresponding sites in the pSecTag B vector (Invitrogen, Carlsbad, CA), which C-terminally tagged the LOX-32 complementary DNA (cDNA) with Myc- and (His)6-tags and appended the Igκ leader sequence to its N-terminus. The resulting LOX-32 cDNA was sequenced in its entirety on a DNA sequencing machine (3730; Applied Biosystems, Carlsbad, CA).

Immunoblot Analyses

LOX activity was inhibited by pretreating MECs with the irreversible LOX inhibitor, β-aminopropionitrile (βAPN; 300 μM), or with the hydrogen peroxide metabolizer, catalase (400 U/ml). Quiescent NMuMG or 4T1 cells were incubated for varying times in the absence or presence of TGF-β1 (5 ng/ml; R&D Systems, Minneapolis, MN) and, subsequently, were lysed and solubilized on ice in buffer H/Triton X-100 [24]. Clarified whole-cell extracts and conditioned medium collected from these cells before their lysis was resolved through 10% SDS-PAGE gels, transferred electrophoretically to nitrocellulose membranes, and blocked in 5% milk before incubating with the following primary antibodies (dilutions): 1) LOX (1:200; Santa Cruz Biotechnology), 2) E-cadherin (1:500; BD Biosciences, San Jose, CA), 3) phospho-Smad2 (1:1000; Cell Signaling, Danvers, MA), and 4) phospho-p38 MAPK (1:500; Cell Signaling). The resulting immunocomplexes were visualized by enhanced chemiluminescence, and differences in protein loading were monitored by reprobing stripped membranes with anti–β-actin (1:1000; Santa Cruz Biotechnology).

Cell Biological Assays

The effect of antagonizing LOX activity on various TGF-β1–stimulated activities in NMuMG, 4T1, or MCF10ACa1a cells was determined as follows: 1) cell proliferation in three-dimensional organotypic cultures using either ImageJ quantitation or CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's recommendations (Promega, Madison, WI), 2) cell invasion induced by 2% serum using 25,000 cells/well in a modified Boyden chamber coated with Matrigel (1:25 dilution) as described [25], 3) gene expression using 30,000 cells/well in a synthetic p3TP-luciferase reporter gene assay as described [25], and 4) p38 MAPK phosphorylation induced by expression of a constitutively active MKK6 as described [26]. In addition, the ability of TGF-β1 stimulation and LOX inhibition to alter the actin cytoskeleton was monitored using direct TRITC-phalloidin immunofluorescence as described [27]. In some cases, the MECs cells were pretreated for 30 minutes with either βAPN (300 μM) or catalase (400 U/ml) before addition of TGF-β1 (5 ng/ml) for 0 to 48 hours at 37°C.

Lastly, total RNA was isolated from NMuMG and 4T1 cells using the RNeasy Plus Mini Kit according the manufacturer's recommendations (Qiagen, Valencia, CA). Afterward, cDNA were synthesized using iScript cDNA Synthesis System (BioRad, Hercules, CA), and semiquantitative real-time PCR was conducted using iQ SYBR Green (BioRad) as described [28]. In all cases, differences in RNA concentration were controlled by normalizing individual gene signals to their corresponding GAPDH RNA signals. The oligonucleotide primer pairs used were as follows: 1) LOX forward 5′-TGCCAGTGGATTGATATTACAGATGT and reverse 5′-AGCGAATGTCACAGCGTA CAA; 2) E-cadherin forward 5′-CCCTACATACACTCTGGTGGTTCA and reverse 5′-GGCATCATC ATCGGTCACTTTG; 3) N-cadherin forward 5′-CCCCCCAAGTCCAACATTTC and reverse 5′-CGCCG TTTCATCCATACCAC; 4) cytokeratin 19

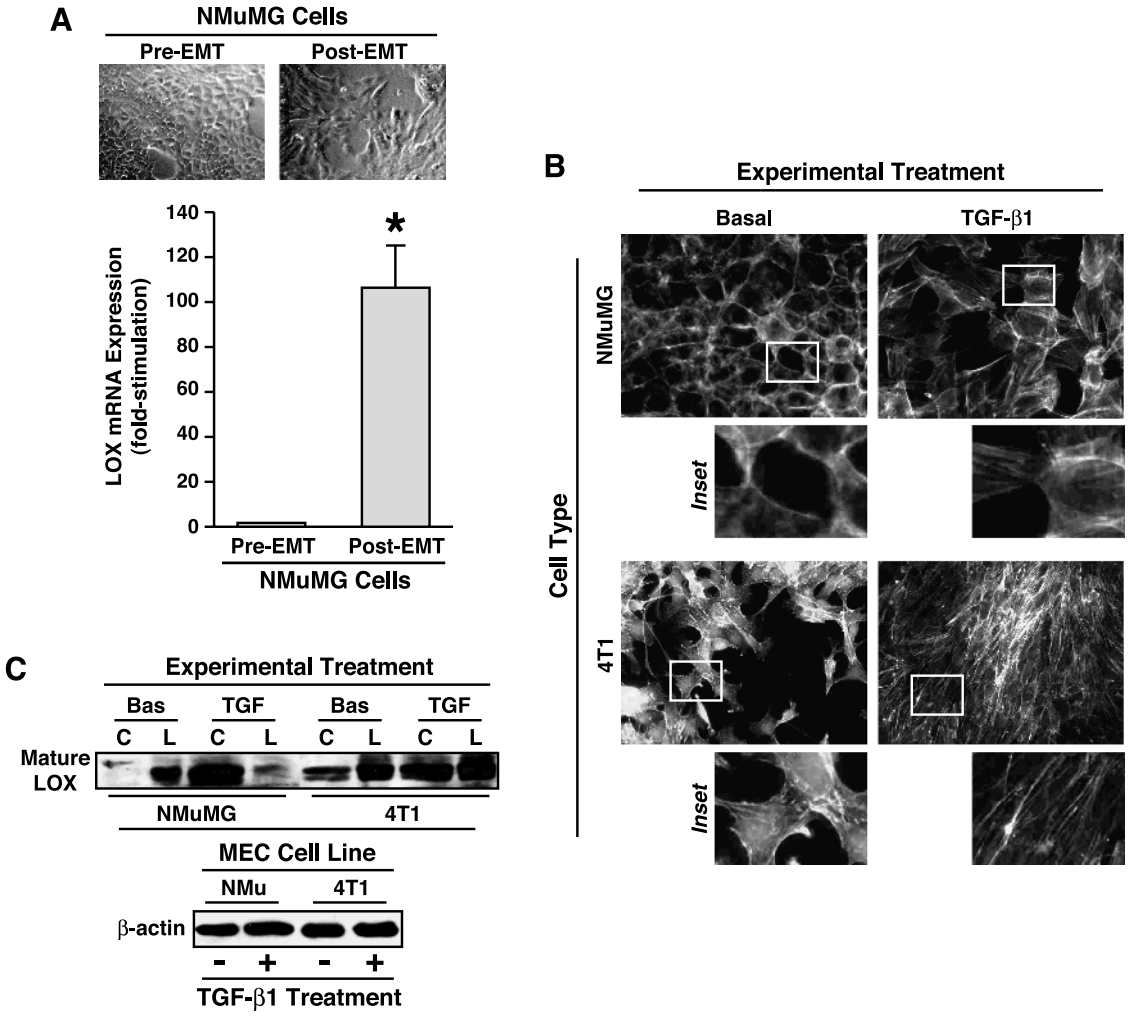


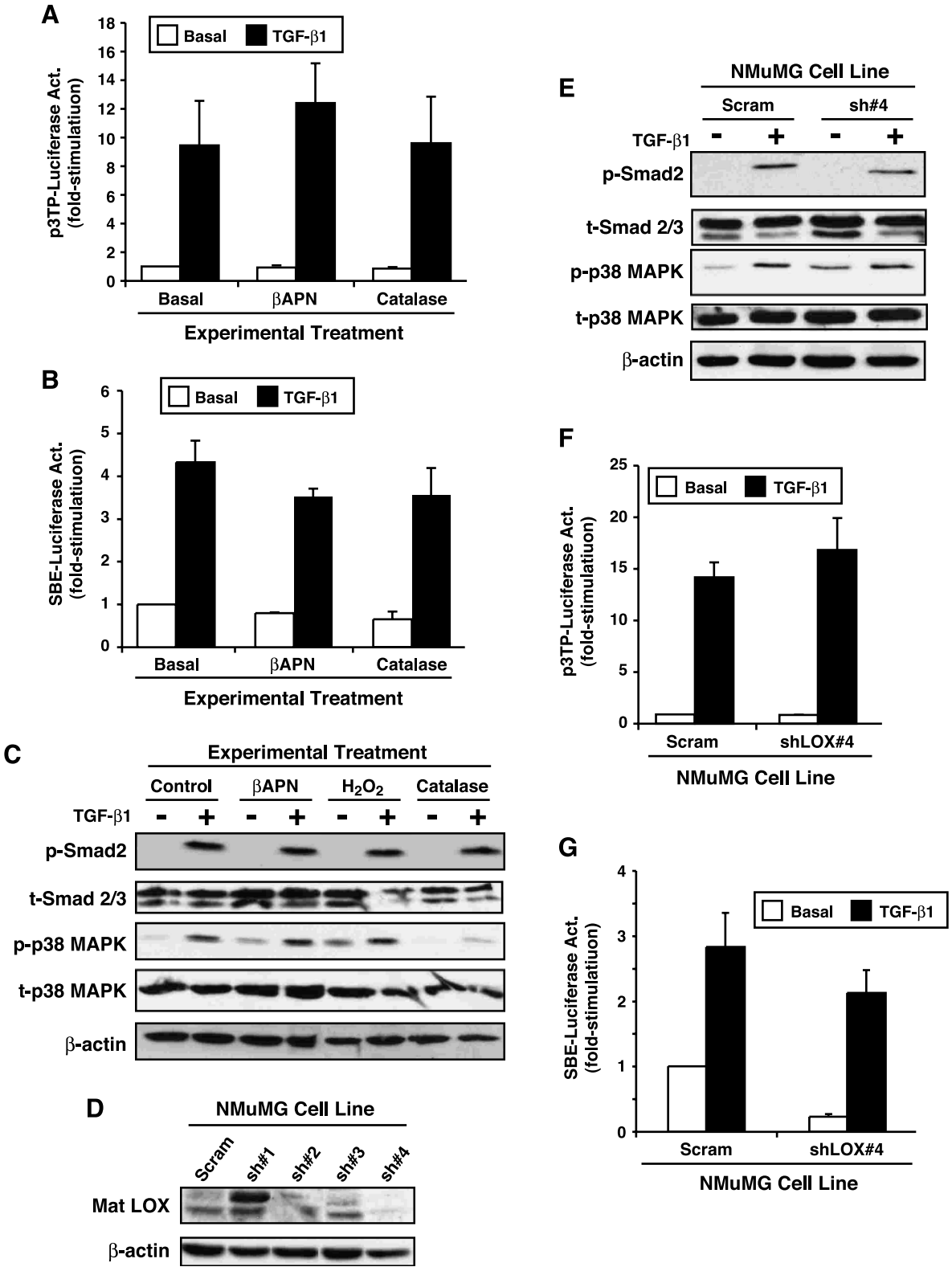
Figure 1. TGF-β1 and EMT stimulate the expression and secretion of LOX in normal and malignant MECs. (A) Shown are representative images of TGF-β1–treated NMuMG cells that have undergone EMT (top panel). TGF-β1 (5 ng/ml) stimulation of EMT in NMuMG cells induced their expression of LOX as determined by semiquantitative real-time PCR. Individual transcript signals were normalized to GAPDH. Data are the mean (±SE; $n = 3$) fold expression of LOX transcripts relative to pre-EMT NMuMG cells. *$P < .05$. (B) NMuMG and 4T1 cells were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 24 hours, at which point alterations in the actin cytoskeleton were monitored by TRITC–phalloidin immunofluorescence. Insets: magnified views of boxed regions. Data are representative images from three independent experiments. (C) TGF-β1 (5 ng/ml) stimulated LOX production and secretion from NMuMG and 4T1 cells as determined by immunoblot analysis conditioned medium (C) or detergent-solubilized whole cell extracts (L) with anti-LOX antibodies. Differences in protein loading were monitored by immunoblot analysis for β-actin. Data are representative images from three independent experiments.

Figure 2. TGF-β1 activates p38 MAPK through a hydrogen peroxide–dependent pathway in normal MECs. NMuMG cells were transiently transfected with the TGF-β1–responsive reporter gene, p3TP-luciferase (A) or pSBE-luciferase (B), and with pCMV–β-gal. Afterward, the transfectants were stimulated with TGF-β1 (5 ng/ml) in the absence or presence of βAPN (300 μM) or catalase (400 U/ml). Luciferase activity was measured and normalized to β-gal. Data are the mean (±SE; $n = 3$). (C) Quiescent NMuMG cells were pretreated with βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β1 (5 ng/ml) for 30 minutes. The phosphorylation and expression levels of Smad2 and p38 MAPK were monitored by immunoblot analysis with phospho-specific antibodies, and differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Shown are representative images from three independent experiments. (D) Four unique shRNA sequences targeting LOX (sh#1–sh#4) were stably expressed in NMuMG cells and differences in LOX expression were analyzed by immunoblot analysis with anti-LOX antibodies to detect mature LOX. Scram indicates scrambled shRNA. β-Actin immunoreactivity is provided as a loading control. (E) Quiescent scrambled (Scram) and LOX-deficient (sh#4) NMuMG cells were stimulated with TGF-β1 (5 ng/ml) for 30 min, at which point the phosphorylation and expression of Smad2 and p38 MAPK were monitored by immunoblot analysis as described in B. Shown are representative images from three independent experiments. Scrambled (Scram) and LOX-deficient (sh#4) NMuMG cells were transiently transfected with p3TP-luciferase (F) or pSBE-luciferase (G) pCMV–β-gal and, subsequently, were stimulated with TGF-β1 (5 ng/ml) as described in A. Data are the mean (±SE; $n = 3$).

forward 5′-TTGGGTCAGGGGGTGTTTTC and reverse 5′-TTCTCATTGCCAGACAGCAGC; 5) vimentin forward 5′-CAAGTCCAAGTTTGCTGACCTCTC and reverse 5′-CTCTTCCATCTCACGCATCTGG; 6) fibronectin III forward 5′-ACAACAACCCCAA GGAGAAG and reverse 5′-GCATCCTCTCTTCTGGTTCTG; and 7) GAPDH forward 5′-CAACTTT GGCATTGTGGAAGGGCTC and reverse 5′-GCAGGGATGATGTTCTGGGCAGC.

LOX Immunohistochemistry

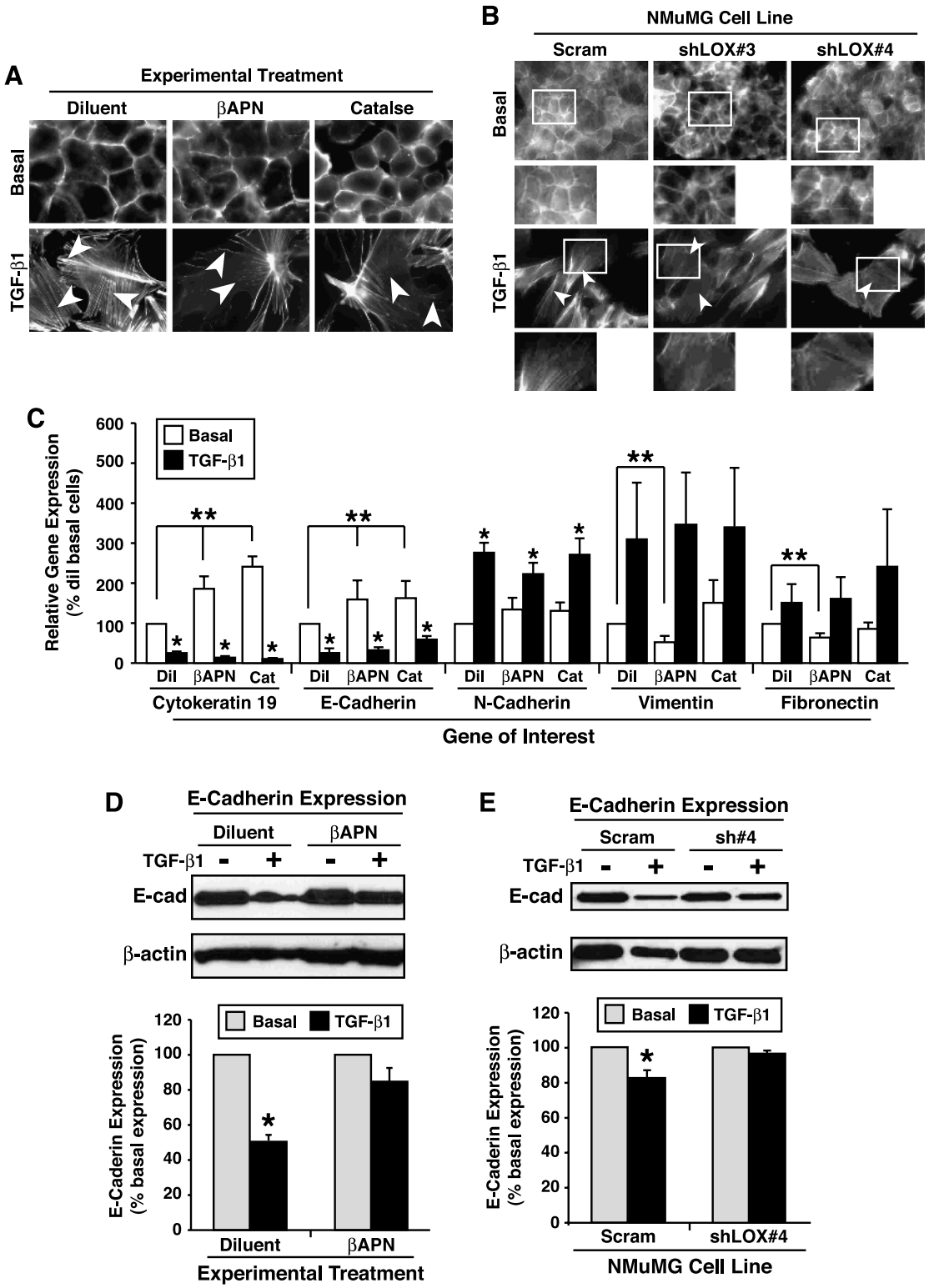
Archival 4T1 tumors that expressed GFP, WT-TGF-β type II receptor (TβR-II), or Y284F–TβR-II [8] were sectioned for histopathologic analysis in the Pathology Core at the University of Colorado Cancer Center. Afterward, LOX immunohistochemistry was performed as described [8] using two independent preparations of anti-LOX antibodies (1:50; Santa Cruz Biotechnology or Payne et al.



[23]). Negative staining controls in all experiments entailed the processing of adjacent samples in the absence of added primary anti-LOX antibodies.

Three-dimensional Organotypic Cultures

Three-dimensional organotypic cultures were performed using the "on-top" method as described [29]. Briefly, 4T1 cells were cultured in either 48-well plates or 8-well chamber slides on Cultrex cushions (100%; Trevigen, Gaithersburg, MD) in complete medium supplemented with 5% Cultrex. ECM rigidity in these organotypic cultures was increased by adding type I collagen (3 mg/ml; BD Biosciences) to Cultrex cushions before their solidification. Where indicated, the MECs were treated with TGF-β1 (5 ng/ml), or the TGF-β type I receptor (TβR-I) antagonist, TβR-I Inhibitor II (Calbiochem, San Diego, CA). Cell growth and acinar formation were monitored by bright-field microscopy. Measuring the secretion of TGF-β1 from



4T1 acinar structures was accomplished using a TGF-β1 ELISA assay (ElisaTech, Aurora, CO) as described [30].

To monitor alterations in E-cadherin localization, 4T1 acinar structures were stained with E-cadherin antibodies (BD Biosciences). Briefly, 4T1 organoids were propagated for 5 days, at which point they were rinsed with PBS supplemented with $CaCl_2$ (0.5 mM) and $MgCl_2$ (0.9 mM) before fixation in 4% paraformaldehyde/PBS. Afterward, the organoids were permeabilized with 0.1% Triton X-100/PBS for 5 minutes, thoroughly washed with PBS, and, subsequently, were blocked in 1% BSA containing 5% goat serum for 1 hour at room temperature before overnight incubation with anti–E-cadherin antibodies (1:250 dilution) at room temperature. The next morning, the organoids were washed and incubated sequentially for 1 hour with biotin-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), followed by Texas Red streptavidin D (Vector, Burlingame, CA). Afterward, the stained organoids were mounted using ProLong Gold Antifade mounting medium that contained DAPI (4′,6-diamidino-2-phenylindole (Invitrogen), and the resulting images were captured on a Leica DM5000 microscope (40×; Leica Microsystems, Bannockburn, IL).

Statistical Analysis

Statistical values were defined using an unpaired Student's *t* test, where $P < .05$ was considered significant.

Results

TGF-β1 and EMT Stimulate the Expression and Secretion of LOX in Normal and Malignant MECs

Normal polarized NMuMG cells undergo EMT in response to TGF-β1 and, in doing so, readily acquire a fibroblastoid-like morphology (Figure 1, *A* and *B*). Interestingly, this same EMT protocol resulted in the significant synthesis of LOX transcripts in post-EMT NMuMG cells compared with their unstimulated counterparts (Figure 1*A*, *bottom panel*). In addition, TGF-β1 also elevated the expression of LOXL1, LOXL2, and LOXL3 in NMuMG cells that underwent EMT (data not shown), suggesting that TGF-β1 functions as a general regulator of LOX family member expression in normal MECs. Because LOX clearly showed the highest induction by TGF-β1 and promotes breast cancer progression [10,13,16,17,23], we restricted our analyses solely to LOX for the remainder of the studies reported herein. The ability of TGF-β1 to stimulate EMT not only was restricted to NMuMG cells but also transpired in metastatic 4T1 cells (Figure 1*B*) [4,7,9,20]. Moreover, we observed TGF-β1 to also stimulate the synthesis and secretion mature LOX (32 kDa) from normal (i.e., NMuMG) and metastatic (i.e., 4T1) MECs as measured by immunoblotting the conditioned medium and whole-cell extracts prepared from these cell types (Figure 1*C*). Collectively, these findings establish TGF-β1 as an inducer of LOX expression, secretion, and proteolytic processing (i.e., an inducer of LOX activation) in normal and malignant MECs. Our results also suggest that upregulated LOX expression may play a role in regulating the malignancy of MECs, particularly their response to TGF-β1.

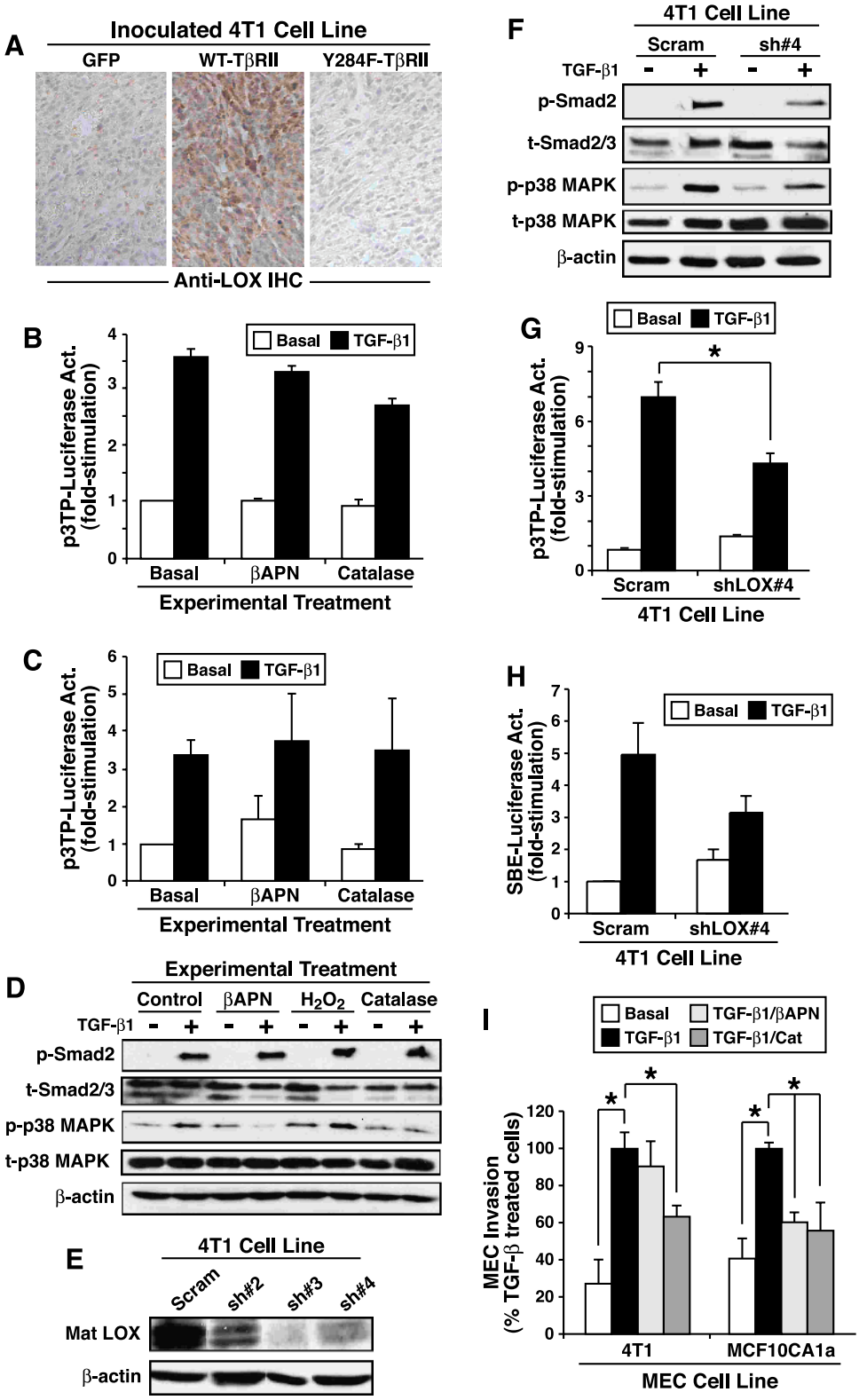
TGF-β1 Regulates EMT in Normal MECs through a LOX- and Hydrogen Peroxide–Dependent Pathway

We [4,6–8,31] and others [32–36] have shown the essential function of canonical (e.g., Smad2/3) and noncanonical (e.g., p38 MAPK) TGF-β1 effectors in mediating its stimulation of EMT in normal and malignant MECs. To determine the impact of LOX on TGF-β1–mediated activation of Smad2/3 and p38 MAPK, we first treated normal NMuMG cells with the irreversible competitive LOX inhibitor, βAPN, which has been shown to specifically inhibit the catalytic activity of LOX [10,11,37] and several LOXL family members [11,18]. In complementary approaches, we also administered catalase to these cells to degrade the LOX second messenger, hydrogen peroxide [17], or stably transduced them with shRNA against LOX to deplete its expression. Figure 2 shows that these experimental conditions failed to alter the transcriptional activity and phosphorylation of Smad2/3 stimulated by TGF-β1, as did depleting LOX expression in these MECs by their transduction with shRNA against LOX (Figures 2, *A*–*G*, and W1). Interestingly, catalase administration inhibited TGF-β1 stimulation of p38 MAPK in NMuMG cells (Figure 2*C*), thereby implicating hydrogen peroxide as a mediator of p38 MAPK activation in NMuMG cells. In stark contrast, we observed both βAPN administration and LOX deficiency to elevate p38 MAPK phosphorylation in resting NMuMG cells (Figure 2, *C* and *E*). These findings suggest that disrupting LOX expression and activity may elicit a stress response in normal MECs. Alternatively, these results may point toward the activities of other LOXL family members in mediating the ability of TGF-β1 to stimulate p38 MAPK in LOX-deficient MECs.

In light of the above results and those linking TGF-β1–driven EMT to elevated LOX expression (Figure 1), we also examined the role of LOX in mediating EMT stimulated by TGF-β1. As such, administration of either βAPN or catalase (Figure 3*A*) or transduction with shRNA against LOX (Figure 3*B*) attenuated the formation of stress

Figure 3. TGF-β1 regulates EMT in normal MECs through a LOX- and hydrogen peroxide–dependent pathway. (A) NMuMG cells were incubated in the absence or presence of either βAPN (300 μM) or catalase (400 U/ml) while undergoing EMT stimulated by TGF-β1 (5 ng/ml). Arrowheads show strong actin fibers localized to focal adhesions in diluent-treated cells stimulated with TGF-β1 and, conversely, stunted actin fibers in cells treated with βAPN or catalase. (B) Parental (scram) or LOX-deficient (shLOX#3 and shLOX#4) NMuMG cells were stimulated by TGF-β1 (5 ng/ml) to induce EMT. Arrowheads show strong actin fibers localized to focal adhesions in Scram cells stimulated with TGF-β1 and, conversely, the presence of stunted actin fibers in LOX-deficient cells. Shown are representative images from three independent experiments. (C) NMuMG cells were stimulated to undergo EMT by TGF-β1 (5 ng/ml) in the absence (i.e., diluent; Dil) or presence of either βAPN (300 μM) or catalase (400 U/ml; Cat). Altered expression of cytokeratin 19, E-cadherin, N-cadherin, vimentin, or fibronectin mRNA was determined by semiquantitative real-time PCR. Individual transcript signals were normalized to GAPDH. Data are the mean (±SE; $n = 3$) transcript levels normalized to corresponding unstimulated controls. *$P < .05$. **$P < .05$. (D and E) Altered E-cadherin (E-cad) expression was monitored by immunoblot analysis detergent-solubilized whole-cell extracts with anti–E-cadherin antibodies. Protein loading was controlled with anti–β-actin antibodies. Shown are representative images from two independent experiments. Accompanying graphs show the densitometric mean (±SE; $n = 2$) relative to corresponding basal cells. *$P < .05$.

fibers stimulated by TGF-β1, suggesting that targeting LOX activity can diminish EMT stimulated by TGF-β1. Accordingly, TGF-β1 induced NMuMG cells to acquire an EMT phenotype that included attenuated expression of the epithelial markers cytokeratin 19 and E-cadherin and augmented expression of the mesenchymal cell markers, N-cadherin, vimentin, and fibronectin (Figure 3*C*). Pharmacological targeting of LOX family members using βAPN or administration of catalase both significantly stimulated the expression of cytokeratin-19 and E-cadherin compared with their diluent treated counterparts, suggesting that the production of hydrogen peroxide by LOX family members selectively suppressed the expression of epithelial markers, and perhaps, sensitized cells to undergo EMT in response to TGF-β1. Similarly, βAPN treatment led to a significant reduction in vimentin and fibronectin expression compared with diluent-treated controls (Figure 3*C*).



Interestingly, LOX deficiency failed to fully recapitulate these responses (data not shown), suggesting that other βAPN-sensitive LOXL members may play a role during EMT induced by TGF-β1. Along these lines, βAPN treatment or LOX deficiency significantly inhibited the ability of TGF-β1 to downregulate E-cadherin protein expression in transitioning NMuMG cells (Figure 3, *D* and *E*). Thus, these findings suggest that LOX and its LOXL relatives sensitize transitioning MECs to complete the EMT program when stimulated by TGF-β1.

LOX Regulates Breast Cancer Cell p38 MAPK Activation and Invasion Stimulated by TGF-β1

Previous findings by our group demonstrated that β_3-integrin interacts physically with TβR-II leading to its phosphorylation on Y284 by Src and the subsequent activation of p38 MAPK that drives the pulmonary metastasis of 4T1 tumors [6–8]. Activation of this oncogenic TGF-β signaling cascade is amplified by overexpression of wild-type (WT) TβR-II in 4T1 cells and, more importantly, completely inactivated by their overexpression of Y284F–TβR-II mutants [8]. Histopathologic analysis of sections obtained from these same tumors revealed that LOX expression was upregulated significantly and specifically in 4T1 tumors that possessed enhanced oncogenic TGF-β signaling (i.e., WT–TβR-II–expressing 4T1 tumors) but not in 4T1 tumors engineered to express either GFP (i.e., parental controls) or Src-resistant Y284F–TβR-II mutants (Figure 4*A*). Thus, LOX expression was upregulated in late-stage mammary tumors in response to oncogenic TGF-β signaling. Given the importance of p38 MAPK in driving breast cancer progression stimulated by TGF-β, we administered βAPN or catalase to 4T1 cells before their stimulation with TGF-β1. Similar to NMuMG cells (Figure 2), both experimental treatments failed to alter the coupling of TGF-β1 to Smad2/3 in 4T1 cells (Figures 4, *B–D*, and W1). However, unlike NMuMG cells, administration of either βAPN or catalase significantly reduced the coupling of TGF-β1 to p38 MAPK in 4T1 cells (Figure 4*D*), suggesting that LOX and hydrogen peroxide may play an expanded role in regulating TGF-β1 signaling in metastatic MECs compared with their normal counterparts. In addition, transient overexpression of constitutively active LOX-32 in these cells increased basal p38 MAPK activation but failed to further augment p38 MAPK activation by TGF-β1 (Figure W2). Even more surprisingly, depleting 4T1 cells of LOX expression (Figure 4*E*) not only attenuated TGF-β1 stimulation of p38 MAPK (Figure 4*F*) but also significantly reduced Smad2/3 transcriptional activity and phosphorylation of Smad2 induced by TGF-β1 (Figure 4, *E–H*). This reduction in Smad2/3 phosphorylation and transcriptional activity cannot be attributed to enhanced activation of p38 MAPK, which can phosphorylate the linker region of Smad2/3 [38]. For instance, Figure W3 shows that overexpression of constitutively active MKK6, which significantly enhanced p38 MAPK activation, had no effect on the coupling of TGF-β1 to Smad2/3 phosphorylation and reporter gene expression in 4T1 cells. Functionally, administration of catalase significantly inhibited 4T1 cell invasion stimulated by TGF-β1, whereas inclusion of either βAPN or catalase significantly antagonized the ability of TGF-β1 to induce the invasion of human MCF10ACA1a cells (Figure 4*I*), which were previously established as a model for metastatic progression regulated by TGF-β [21]. Similar to βAPN treatment, LOX deficiency failed to affect the extent of 4T1 cell invasion stimulated by TGF-β1 (data not shown). These findings establish TGF-β1 as an inducer of LOX expression *in vivo* and suggest that upregulated expression LOX or another LOXL family member and/or hydrogen peroxide production may regulate the malignancy of MECs in response to TGF-β1, including its ability to induce breast cancer cell invasion.

Mechanotransduction Induces Autocrine TGF-β1 Signaling Coupled to MEC Proliferation

Tissue compliance and ECM rigidity play vital roles in mediating cellular organization during embryogenesis and in maintaining tissue homeostasis in adult tissues [12]. Moreover, increased tissue tension resulting from desmoplastic and fibrotic stromal reactions is associated with mammary tumorigenesis and its progression to metastasis, activities that have also been attributed to LOX-dependent cross-linking of collagen to elastin during neoplastic progression [12,14,15,39]. At present, the role of tissue rigidity and mechanotransduction in regulating the behaviors of MECs to TGF-β remains unknown. As such, we compared the response of 4T1 cells to TGF-β1 when propagated under compliant and rigid culture conditions. Figure 5*A* shows that in traditional two-dimensional cultures, which are extremely rigid

Figure 4. LOX regulates breast cancer cell p38 MAPK activation and invasion stimulated by TGF-β1. (A) Elevated oncogenic TGF-β1 signaling (i.e., WT–TβR-II expression) greatly accelerates the growth and pulmonary metastasis of 4T1 tumors in mice [8]. LOX immunohistochemistry performed on these same tumor slices showed that TGF-β1 signaling significantly induced the expression of LOX expression in WT–TβR-II–expressing 4T1 tumors compared with their GFP- or Y284F–TβR-II–expressing counterparts. Data are representative images from two independent experiments. WT indicates wild-type. 4T1 cells were transiently transfected with p3TP-luciferase (B) or pSBE-luciferase (C) and pCMV–β-gal and, subsequently, were stimulated with TGF-β1 (5 ng/ml) in the absence or presence of βAPN (300 μM) or catalase (400 U/ml). Luciferase activity was measured and normalized to β-gal. Data are the mean (±SE; $n = 3$). (D) Quiescent 4T1 cells were pretreated with βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β1 (5 ng/ml) for 30 minutes. The phosphorylation and expression of Smad2 and p38 MAPK was monitored by immunoblot analysis, and differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Shown are representative images from three independent experiments. (E) Three unique shRNA sequences targeting LOX (sh#2–sh#4) were stably expressed in 4T1 cells, and differences in LOX expression were analyzed by immunoblot analysis with anti-LOX antibodies. Scram indicates scrambled shRNA. β-Actin immunoreactivity is provided as a loading control. (F) Quiescent scrambled (Scram) and LOX-deficient (sh#4) 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 30 min, at which point the phosphorylation status and expression of Smad2 and p38 MAPK were monitored by immunoblot analysis as described in D. Shown are representative images from three independent experiments. Scrambled (Scram) and LOX-deficient (sh#4) 4T1 cells were transiently transfected with p3TP-luciferase (G) or pSBE-luciferase (H) and pCMV–β-gal and, subsequently, were stimulated with TGF-β1 (5 ng/ml) as described in A. Data are the mean (±SE; $n = 3$). (I) 4T1 or MCF10ACA1a (CA1a) cells were incubated in the absence or presence of either βAPN (300 μM) or catalase (400 U/ml) while undergoing invasion through synthetic basement membranes in response to TGF-β1 (5 ng/ml). Data are the mean (±SE; $n = 3$) invasion relative to that stimulated by TGF-β1. *$P < .05$.

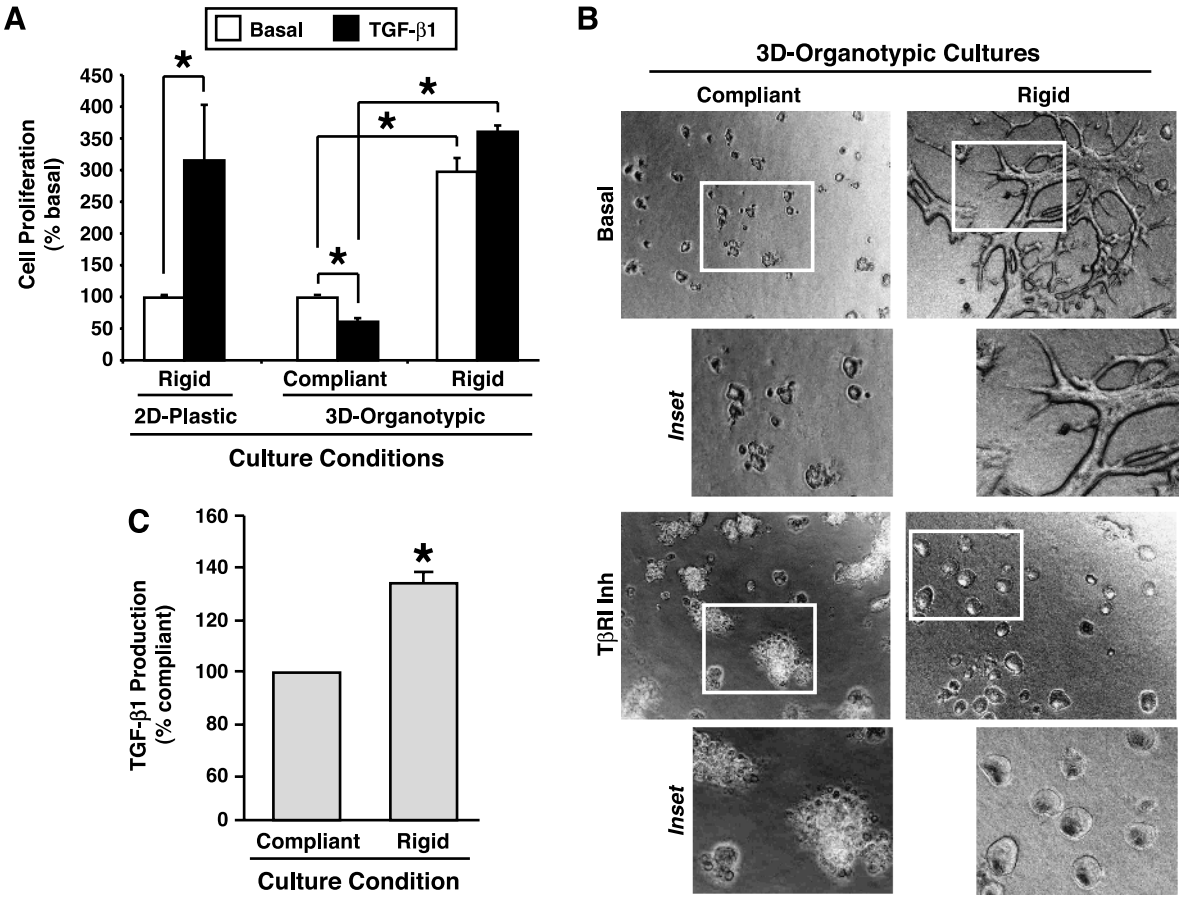


Figure 5. Mechanotransduction induces autocrine TGF-β1 signaling coupled to MEC proliferation. (A) 4T1 cells were in incubated the absence or presence of TGF-β1 (5 ng/ml) in two-dimensional tissue culture plastic or in three-dimensional organotypic cultures supplemented without (i.e., compliant) or with type I collagen (3 mg/ml; rigid). Bright-field images were captured and used to quantitate cell proliferation through ImageJ. Data are the mean (±SE; $n = 3$) proliferation relative to basal 4T1 cells. *$P < .05$. (B) Inhibition of TGF-β1 signaling by administration of the TβR-I inhibitor (100 ng/ml) enhanced the growth of 4T1 cells in compliant three-dimensional organotypic cultures, but inhibited their growth in rigid (3 mg/ml type I collagen) three-dimensional organotypic cultures. Insets: magnified views of boxed regions. Data are representative images from three independent experiments. (C) Conditioned medium harvested from compliant or rigid three-dimensional organotypic cultures was acidified to activate total TGF-β1. After sample neutralization, TGF-β1 concentrations were determined by ELISA analysis. Data are the mean (±SE; $n = 3$) TGF-β1 concentrations relative to those measured in compliant cultures. *$P < .05$.

[12], TGF-β1 readily promoted the proliferation of 4T1 cells. In stark contrast, propagating 4T1 cells in compliant three-dimensional organotypic cultures was sufficient to restore the cytostatic activities of TGF-β1 in these malignant MECs, which normally fail to undergo growth arrest in response to TGF-β1 (Figure 5*A*) [4,7–9,28,40–43]. Importantly, supplementing these three-dimensional organotypic cultures with type I collagen to initiate mechanotransduction uncoupled TGF-β1 from the regulation of cell cycle progression in 4T1 cells (Figure 5*A*). Thus, ECM tension and rigidity clearly alter how MECs respond to the cytostatic activities of TGF-β1 [20]. Along these lines, we also inhibited TGF-β1 signaling in 4T1 cells by treating them with a small-molecule TβR-I antagonist, TβR-I Inhibitor II [28,40–43], and subsequently monitored alterations in their growth and morphology. As shown in Figure 5*B*, 4T1 organoids propagated in compliant cultures formed abnormal acinar structures, which underwent dramatic expansion and branching in response to increased ECM rigidity. Interestingly, the growth and branching of 4T1 organoids elicited by mechanotransduction were abrogated by inactivating TGF-β1 signaling, which restored the formation of spherical acinar structures (Figure 5*B*). Consistent with the ability of compliant microenvironments to reinstate cytostatic signaling by TGF-β1 (Figure 5*A*), inactivation of TGF-β1 signaling in these cultures by treating them with the TβR-I antagonist was sufficient to stimulate the growth and expansion of 4T1 organoids (Figure 5*B*). Thus, autocrine TGF-β1 signaling seems to play a prominent role in regulating MEC response to TGF-β1 under compliant and rigid ECM conditions. Accordingly, 4T1 (Figure 5*C*) and MCF-7 (data not shown) organoids propagated under rigid ECM conditions produced significantly more TGF-β1 compared with their counterparts propagated under compliant ECM conditions. Taken together, these findings show for the first time that exposing late-stage breast cancer cells to compliant ECM signals reinstates the cytostatic activities TGF-β1 and, in effect, partially reestablishes the tumor suppressing functions of TGF-β1 in malignant MECs.

Mechanotransduction Induces MEC Proliferation and E-cadherin Redistribution in a LOX-Dependent Manner

To examine the specific contributions of LOX activity in regulating MEC response to TGF-β, we again cultured 4T1 cells under compliant or rigid ECM conditions with or without added βAPN or catalase. Figure 6*A* shows that inhibiting LOX activity or degrading hydrogen

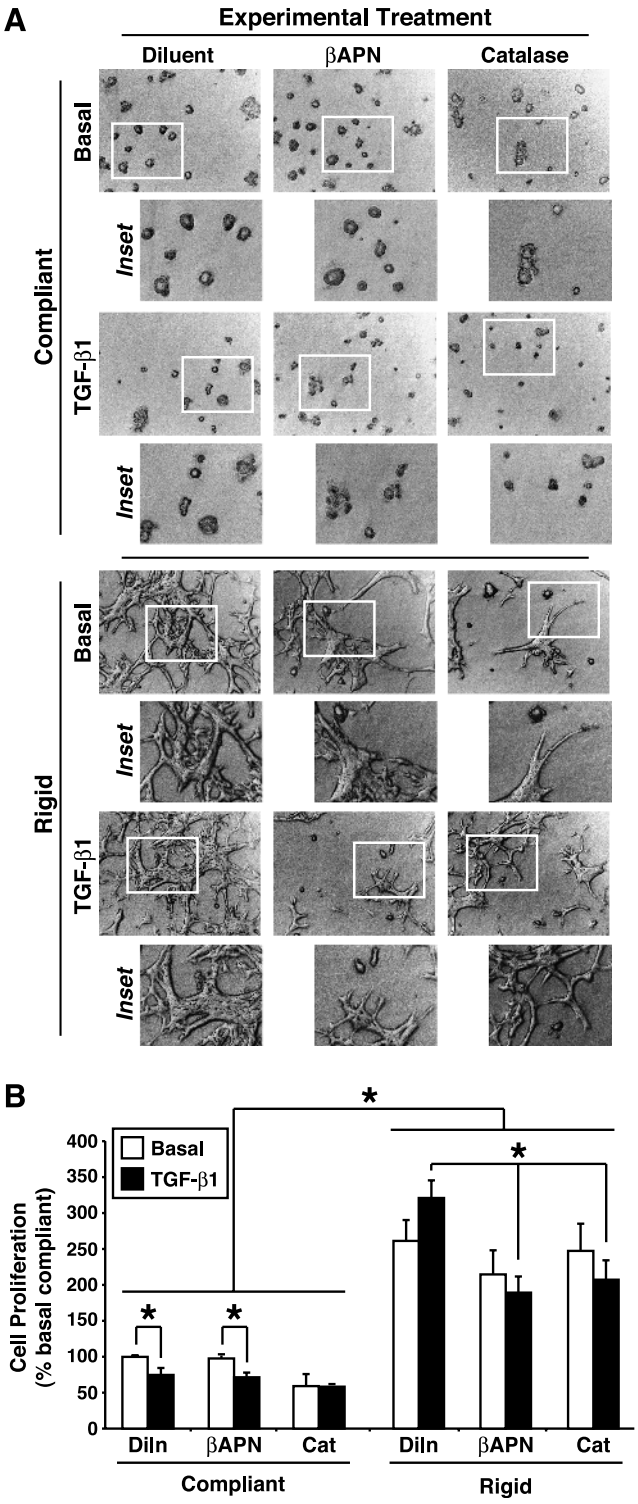


Figure 6. Mechanotransduction induces MEC proliferation in a LOX-dependent manner. (A) LOX antagonism using βAPN (300 μM) or catalase (400 U/ml) inhibited the ability of TGF-β1 to stimulate 4T1 cell growth in rigid three-dimensional organotypic cultures. Insets: magnified views of boxed regions. Shown are representative images from three independent experiments. (B) Accompanying data are the mean (±SE; $n = 3$) proliferation relative to the growth of basal cells in compliant cultures. *$P < .05$.

peroxide both significantly reduced the growth of 4T1 cells stimulated by rigid ECM. Consistent with the effect of compliant ECM to restore cytostasis mediated by TGF-β1, these same experimental treatments elicited little-to-no effect on the ability of 4T1 cells to undergo growth arrest in response to TGF-β1 (Figure 6, *A* and *B*). Similarly, LOX deficiency (Figure 4*E*) abrogated the ability of TGF-β1 to stimulate the growth of 4T1 organoids in rigid ECM (Figure 7*A*) and, instead, partially reestablished the cytostatic activities of TGF-β1 in metastatic MECs.

Finally, given the ability of LOX activity to suppress E-cadherin expression (Figure 3) and given the ability of E-cadherin expression to suppress the uncontrolled growth of cancer cells [44], we hypothesized that LOX deficiency may suppress the growth of 4T1 organoids by upregulating and stabilizing E-cadherin expression at the plasma membrane. Accordingly, E-cadherin expression was readily detected and localized to the cell surface in parental and LOX-deficient 4T1 organoids propagated under rigid culture conditions (Figure 7*B*). Importantly, treating these same organoids with TGF-β1 resulted in the complete loss of E-cadherin from the plasma membrane in parental and scrambled shRNA-expressing 4T1 organoids, a reaction that was not recapitulated in their LOX-deficient counterparts (Figure 7*B*). Collectively, these findings suggest that LOX expression and activity may be essential in linking the oncogenic activities of TGF-β1 and mechanotransduction in breast cancer cells, presumably by regulating the expression and localization of E-cadherin.

Discussion

Breast cancer is the second leading cause of cancer death in women. Although the 5-year survival rate for women with localized disease is high at 98%, this number drops precipitously to only 27% once the primary tumor has metastasized [45]. For this reason, it is vitally important for science and medicine to enhance their understanding of the processes that underlie breast cancer invasion and metastasis. It has long been established that breast cancer development reflects a loss-of-tissue organization and differentiation, factors that have more recently been associated with increases in LOX expression and activity [46,47]. We show here that TGF-β and EMT both induce the expression and secretion of LOX from normal and malignant MECs and that mammary tumors engineered to house elevated TGF-β signaling produced more LOX than did their parental counterparts, which correlated with the ability of TGF-β to stimulate mammary tumor growth and pulmonary metastasis [8]. Equally important, we provide the first evidence that the manner in which MECs respond to TGF-β can be regulated by tissue rigidity, which elicits dramatic changes in MEC acinar morphology and growth in a LOX- and hydrogen peroxide–dependent manner. Although the exact mechanisms whereby tissue tension regulates TGF-β1 function remain to be elucidated fully, it is tempting to speculate that enhanced ECM rigidity may promote the inappropriate clustering of TGF-β receptors with integrins and other growth factor receptors, thereby inducing amplified coupling of TGF-β to its noncanonical effectors [48,49]. Accordingly, we demonstrated that antagonizing LOX activity led to the diminished ability of TGF-β to stimulate MEC invasion and EMT. Furthermore, we show that antagonizing LOX activity partially uncoupled TGF-β1 from p38 MAPK activation in metastatic 4T1 cells, whereas only catalase administration facilitated this event in normal NMuMG cells, suggesting that the roles of LOX and hydrogen peroxide depend on the pathophysiology of MECs.

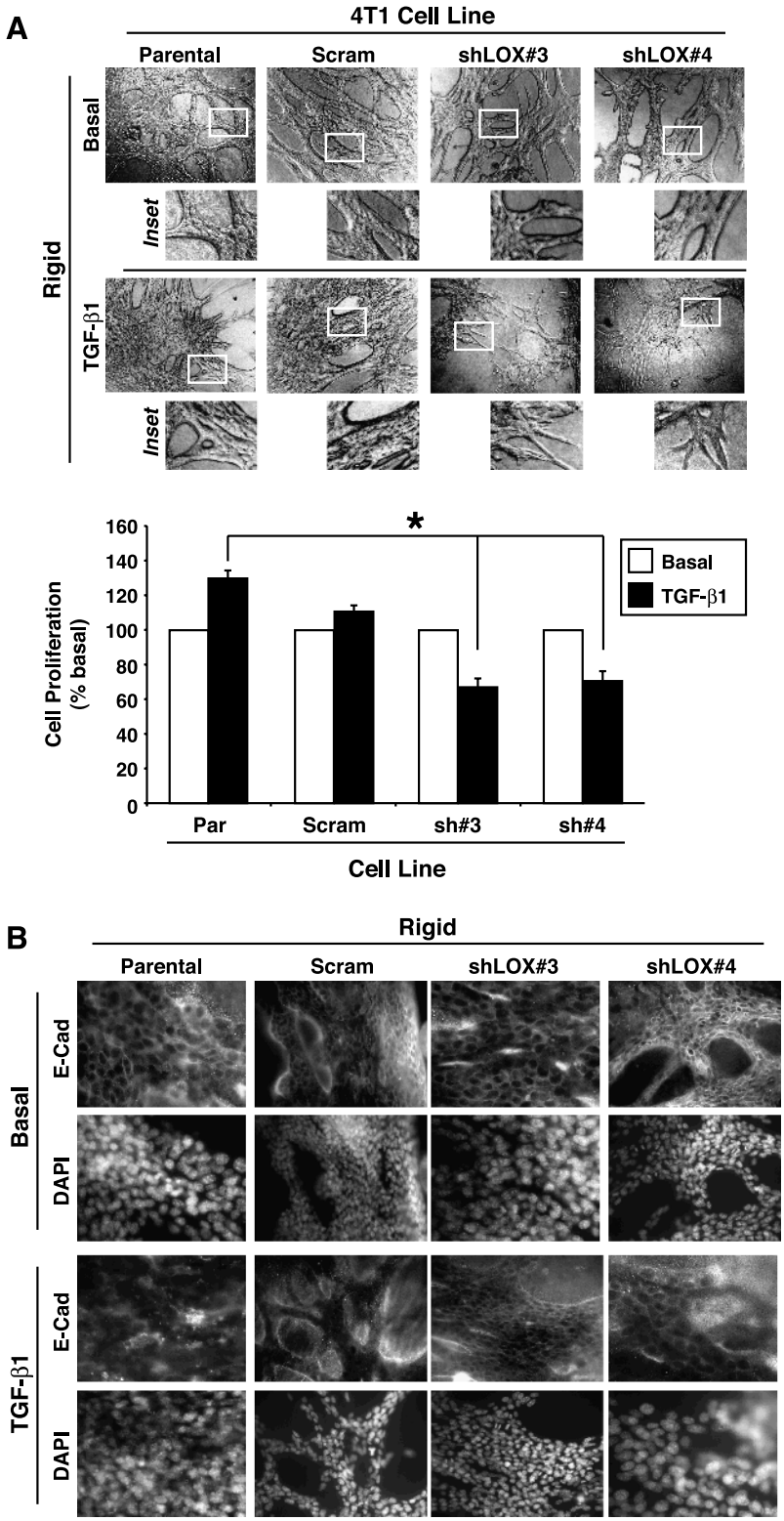


Figure 7. LOX deficiency suppresses mechanotransduction and TGF-β1 stimulation of MEC proliferation by restoring cell surface E-cadherin expression. (A) Parental, scrambled (Scram), or LOX-deficient (sh#3 and sh#4) 4T1 cells were propagated in rigid three-dimensional organotypic cultures in the absence or presence of TGF-β1 (5 ng/ml) as indicated. Bright-field images were captured (top panel) and used to quantitate cell proliferation through ImageJ (bottom panel). Insets: magnified views of boxed regions. Data are the mean (±SE; $n = 3$) proliferation relative to basal 4T1 cells. *$P < .05$. (B) Parental, scrambled (Scram), or LOX-deficient (sh#3 and sh#4) 4T1 cells were propagated in rigid three-dimensional organotypic cultures as described in A and, subsequently, were processed to visualize the expression and localization of E-cadherin by immunofluorescence. Corresponding nuclei were detected by inclusion of DAPI as indicated. Data are representative images from three independent experiments.

It is important to note that although our current findings support an extracellular role of LOX in mediating the oncogenic activities of TGF-β (i.e., catalase neutralizes hydrogen peroxide), we cannot exclude the possibility that LOX may promote oncogenic TGF-β signaling by acting intracellularly. Indeed, mature LOX has been detected not only in the ECM but also in the cytoplasm (Figure 1) and nucleus of malignant cells [10,11]; however, the identification of specific molecules capable of interacting with and/or being targeted by LOX in either intracellular compartment remains to be elucidated fully [11]. Along these lines, LOX was shown to activate Src and promote cell adhesion through a hydrogen peroxide–dependent mechanism [23]. We show here that administering catalase to degrade hydrogen peroxide prevented TGF-β from stimulating MEC proliferation, EMT, and invasion (Figures 3–7) and from fully activating p38 MAPK (Figure 4). Thus, hydrogen peroxide may function as a novel "second messenger" for TGF-β in normal and malignant MECs. Collectively, our findings suggest that LOX may play an important role in initiating the conversion of TGF-β function from a suppressor to a promoter of mammary tumorigenesis. At present, the specific players targeted by LOX and hydrogen peroxide that affect TGF-β signaling remain an active and important topic for future experimentation. In addition, it is unclear what overlapping functions other LOX family members may play. Importantly, LOXL2 is not inhibited by βAPN treatment and may compensate for loss of LOX function [11,50], and in fact, both LOX and LOXL2 mediate the ability of HIF-1α to suppress E-cadherin expression [51]. Thus, future studies need to dissect the relative contribution of individual LOX family members to the initiation of oncogenic TGF-β signaling and its coupling to canonical and noncanonical effectors.

LOX plays a critical role during the formation of premetastatic niches by stimulating collagen cross-linking and fibronectin synthesis, leading to the recruitment of bone marrow–derived cells to metastatic niches [13]. TGF-β has also been implicated in the recruitment of immature bone marrow–derived cells to drive breast cancer metastasis [52], which suggests a potential link between TGF-β and LOX in regulating the formation of premetastatic niches. Interestingly, the use of copper chelators in preclinical and phase 2 clinical trials has shown some success in diminishing metastatic burden [53], findings that are potentially important because LOX activity is absolutely dependent on copper as one of its two cofactors (the other being lysyl tyrosyl quinone [11]). Thus, it is plausible that the clinical success of copper chelators to reduce tumor metastasis lies in their ability to inhibit LOX activity and, consequently, perhaps to alleviate the oncogenic activities of TGF-β as well. Along these lines, tumor hypoxia predicts for poor prognosis and decreased survival of breast cancer patients, which is linked to hypoxia-induced expression of LOX and the generation of metastatic niches in breast cancer patients [13,16,54]. These findings, together with those presented herein, support the idea that LOX dictates how malignant MECs respond to the varied activities of TGF-β, and as such, identify LOX as a novel participant in oncogenic TGF-β1 signaling in late-stage mammary tumors. Thus, chemotherapeutic targeting of LOX may offer new inroads to alleviate breast cancer progression stimulated by TGF-β.

Lastly, the ability of LOX to cross-link collagen to elastin results in increased tissue tension and ECM rigidity [12,15,39,55]. More recently, ECM rigidity has been shown to play an important role in breast cancer development, particularly their acquisition of invasive and metastatic phenotypes [12,39,47,56]. The ability of normal and malignant MECs to sense ECM stiffness transpires through integrins and other mechanotransducers, which in turn activate Src, FAK, and the GTPases, Rho, Rac, and Cdc42 [46,47]. Importantly, we identified an oncogenic TGF-β signaling axis comprised in part of $\alpha_v\beta_3$ integrin, FAK, and Src that induces mammary tumor growth, invasion, and metastasis in mice [4,8,9], as well as stimulates significant LOX expression in these same mammary tumors (Figure 4). We speculate that tumor-initiated MECs evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-β. The continued growth of the developing neoplasm enhances ECM rigidity by upregulating TGF-β production (Figure 5) and LOX expression (Figure 1), which may lead to the inappropriate formation of integrin–TβR-II complexes [6–8]. Once formed, these complexes are also likely to interact with other growth factor receptors that presumably amplify the activation of noncanonical effectors by TGF-β [57]. Ultimately, these adverse events culminate in the ability of TGF-β to induce the acquisition of EMT, stem-like, and metastatic phenotypes in malignant MECs, leading to their metastasis at distant locales. Moreover, extending our findings to encompass sites of micrometastases [13], which are predicted to possess compliant ECM tension, leads us to propose that the cytostatic activities of TGF-β may be partially reinstated at these newly seeded sites, perhaps contributing to tumor dormancy. Over time, this vicious microenvironmental cycle is repeated, leading to disease recurrence and poor clinical outcomes in breast cancer patients harboring metastatic disease. The basic tenets of this model are supported by the findings presented herein (Figures 5–7), and as such, this model should serve as a launching point for future studies aimed at identifying the individual effectors operant in regulating ECM tension and TGF-β1 function in distinct breast cancer subtypes.

Acknowledgments

Members of the Schiemann Laboratory are thanked for critical reading of the article.

References

[1] Chang CF, Westbrook R, Ma J, and Cao D (2007). Transforming growth factor-β signaling in breast cancer. *Front Biosci* **12**, 4393–4401.

[2] Tian M and Schiemann WP (2009). The TGF-β paradox in human cancer: an update. *Future Oncol* **5**, 259–271.

[3] Galliher AJ, Neil JR, and Schiemann WP (2006). Role of transforming growth factor-β in cancer progression. *Future Oncol* **2**, 743–763.

[4] Wendt MK and Schiemann WP (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. *Breast Cancer Res* **11**, R68.

[5] Moustakas A and Heldin CH (2005). Non-Smad TGF-β signals. *J Cell Sci* **118**, 3573–3584.

[6] Galliher AJ and Schiemann WP (2006). β_3 integrin and Src facilitate transforming growth factor-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* **8**, R42.

[7] Galliher AJ and Schiemann WP (2007). Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer Res* **67**, 3752–3758.

[8] Galliher-Beckley AJ and Schiemann WP (2008). Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. *Carcinogenesis* **29**, 244–251.

[9] Wendt MK, Smith JA, and Schiemann WP (2009). $p130^{Cas}$ is required for mammary tumor growth and TGF-β–mediated metastasis through regulation of Smad2/3 activity. *J Biol Chem* **284**, 31145–31156.

[10] Payne SL, Hendrix MJ, and Kirschmann DA (2007). Paradoxical roles for lysyl oxidases in cancer—a prospect. *J Cell Biochem* **101**, 1338–1354.

[11] Kagan HM and Li W (2003). Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* **88**, 660–672.

[12] Butcher DT, Alliston T, and Weaver VM (2009). A tense situation: forcing tumour progression. *Nat Rev Cancer* **9**, 108–122.

[13] Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT, and Giaccia AJ (2009). Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* **15**, 35–44.

[14] Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, et al. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell* **8**, 241–254.

[15] Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, et al. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**, 891–906.

[16] Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, and Giaccia AJ (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* **440**, 1222–1226.

[17] Kirschmann DA, Seftor EA, Fong SF, Nieva DR, Sullivan CM, Edwards EM, Sommer P, Csiszar K, and Hendrix MJ (2002). A molecular role for lysyl oxidase in breast cancer invasion. *Cancer Res* **62**, 4478–4483.

[18] Barry-Hamilton V, Spangler R, Marshall D, McCauley S, Rodriguez HM, Oyasu M, Mikels A, Vaysberg M, Ghermazien H, Wai C, et al. (2010). Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. *Nat Med* **16**, 1009–1017.

[19] Payne SL, Hendrix MJ, and Kirschmann DA (2006). Lysyl oxidase regulates actin filament formation through the p130(Cas)/Crk/DOCK180 signaling complex. *J Cell Biochem* **98**, 827–837.

[20] Allington TM, Galliher-Beckley AJ, and Schiemann WP (2009). Activated Abl kinase inhibits oncogenic transforming growth factor-β signaling and tumorigenesis in mammary tumors. *FASEB J* **23**, 4231–4243.

[21] Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, and Wakefield LM (2003). TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* **112**, 1116–1124.

[22] Sokol JP and Schiemann WP (2004). Cystatin C antagonizes transforming growth factor β signaling in normal and cancer cells. *Mol Cancer Res* **2**, 183–195.

[23] Payne SL, Fogelgren B, Hess AR, Seftor EA, Wiley EL, Fong SF, Csiszar K, Hendrix MJ, and Kirschmann DA (2005). Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide–mediated mechanism. *Cancer Res* **65**, 11429–11436.

[24] Schiemann WP, Pfeifer WM, Levi E, Kadin ME, and Lodish HF (1999). A deletion in the gene for transforming growth factor β type I receptor abolishes growth regulation by transforming growth factor β in a cutaneous T-cell lymphoma. *Blood* **94**, 2854–2861.

[25] Schiemann WP, Blobe GC, Kalume DE, Pandey A, and Lodish HF (2002). Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-β and affects protein kinase cascades. *J Biol Chem* **277**, 27367–27377.

[26] Albig AR and Schiemann WP (2005). Identification and characterization of regulator of G protein signaling 4 (RGS4) as a novel inhibitor of tubulogenesis: RGS4 inhibits mitogen-activated protein kinases and vascular endothelial growth factor signaling. *Molecular Biol Cell* **16**, 609–625.

[27] Sokol JP, Neil JR, Schiemann BJ, and Schiemann WP (2005). The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by transforming growth factor-β. *Breast Cancer Res* **7**, R844–R853.

[28] Neil JR, Johnson KM, Nemenoff RA, and Schiemann WP (2008). COX-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanism. *Carcinogenesis* **29**, 2227–2235.

[29] Lee GY, Kenny PA, Lee EH, and Bissell MJ (2007). Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* **4**, 359–365.

[30] Schiemann BJ, Neil JR, and Schiemann WP (2003). SPARC inhibits epithelial cell proliferation in part through stimulation of the transforming growth factor-β–signaling system. *Mol Biol Cell* **14**, 3977–3988.

[31] Wendt MK, Allington TM, and Schiemann WP (2009). Mechanisms of the epithelial-mesenchymal transition by TGF-β. *Future Oncol* **5**, 1145–1168.

[32] Bakin AV, Rinehart C, Tomlinson AK, and Arteaga CL (2002). p38 mitogen–activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci* **115**, 3193–3206.

[33] Bhowmick NA, Zent R, Ghiassi M, McDonnell M, and Moses HL (2001). Integrin β_1 signaling is necessary for transforming growth factor-β activation of p38MAPK and epithelial plasticity. *J Biol Chem* **276**, 46707–46713.

[34] Valcourt U, Kowanetz M, Niimi H, Heldin CH, and Moustakas A (2005). TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol Biol Cell* **16**, 1987–2002.

[35] Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, and Chapman HA (2009). Integrin $\alpha3\beta_1$-dependent β-catenin phosphorylation links epithelial Smad signaling to cell contacts. *J Cell Biol* **184**, 309–322.

[36] Saika S, Kono-Saika S, Ohnishi Y, Sato M, Muragaki Y, Ooshima A, Flanders KC, Yoo J, Anzano M, Liu CY, et al. (2004). Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. *Am J Pathol* **164**, 651–663.

[37] Postovit LM, Abbott DE, Payne SL, Wheaton WW, Margaryan NV, Sullivan R, Jansen MK, Csiszar K, Hendrix MJ, and Kirschmann DA (2008). Hypoxia/reoxygenation: a dynamic regulator of lysyl oxidase–facilitated breast cancer migration. *J Cell Biochem* **103**, 1369–1378.

[38] Wrighton KH, Lin X, and Feng XH (2009). Phospho-control of TGF-β superfamily signaling. *Cell Res* **19**, 8–20.

[39] Erler JT and Weaver VM (2009). Three-dimensional context regulation of metastasis. *Clin Exp Metastasis* **26**, 35–49.

[40] Neil JR and Schiemann WP (2008). Altered TAB1:IκB kinase interaction promotes transforming growth factor β–mediated nuclear factor-κB activation during breast cancer progression. *Cancer Res* **68**, 1462–1470.

[41] Tian M and Schiemann WP (2009). PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. *FASEB J* **24**, 1105–1116.

[42] Tian M and Schiemann WP (2009). Preclinical efficacy of cystatin C to target the oncogenic activity of transforming growth factor β in breast cancer. *Transl Oncol* **2**, 174–183.

[43] Neil JR, Tian M, and Schiemann WP (2009). x-linked inhibitor of apoptosis protein and its E3 ligase activity promote transforming growth factor-β–mediated nuclear factor-κB activation during breast cancer progression. *J Biol Chem* **284**, 21209–21217.

[44] Wells A, Yates C, and Shepard CR (2008). E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* **25**, 621–628.

[45] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, and Thun MJ (2008). Cancer statistics, 2008. *CA Cancer J Clin* **58**, 71–96.

[46] Kass L, Erler JT, Dembo M, and Weaver VM (2007). Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis. *Int J Biochem Cell Biol* **39**, 1987–1994.

[47] Paszek MJ and Weaver VM (2004). The tension mounts: mechanics meets morphogenesis and malignancy. *J Mammary Gland Biol Neoplasia* **9**, 325–342.

[48] Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, and Bissell MJ (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* by integrin blocking antibodies. *J Cell Biol* **137**, 231–245.

[49] Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, Lupu R, and Bissell MJ (1998). Reciprocal interactions between β_1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc Natl Acad Sci USA* **95**, 14821–14826.

[50] Kim YM, Kim EC, and Kim Y (2011). The human lysyl oxidase-like 2 protein functions as an amine oxidase toward collagen and elastin. *Mol Biol Rep* **38**, 145–149.

[51] Schietke R, Warnecke C, Wacker I, Schodel J, Mole DR, Campean V, Amann K, Goppelt-Struebe M, Behrens J, Eckardt KU, et al. (2010). The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. *J Biol Chem* **285**, 6658–6669.

[52] Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, et al. (2008). Abrogation of TGFβ signaling in mammary carcinomas recruits Gr-1$^+$CD11b$^+$ myeloid cells that promote metastasis. *Cancer Cell* **13**, 23–35.

[53] Goodman VL, Brewer GJ, and Merajver SD (2004). Copper deficiency as an anti-cancer strategy. *Endocr Relat Cancer* **11**, 255–263.

[54] Erler JT and Giaccia AJ (2006). Lysyl oxidase mediates hypoxic control of metastasis. *Cancer Res* **66**, 10238–10241.

[55] Lopez JI, Mouw JK, and Weaver VM (2008). Biomechanical regulation of cell orientation and fate. *Oncogene* **27**, 6981–6993.

[56] Kumar S and Weaver VM (2009). Mechanics, malignancy, and metastasis: the force journey of a tumor cell. *Cancer Metastasis Rev* **28**, 113–127.

[57] Wendt MK, Smith JA, and Schiemann WP (2010). Transforming growth factor-β–induced epithelial-mesenchymal transition facilitates epidermal growth factor–dependent breast cancer progression. *Oncogene* **29**, 6485–6498.

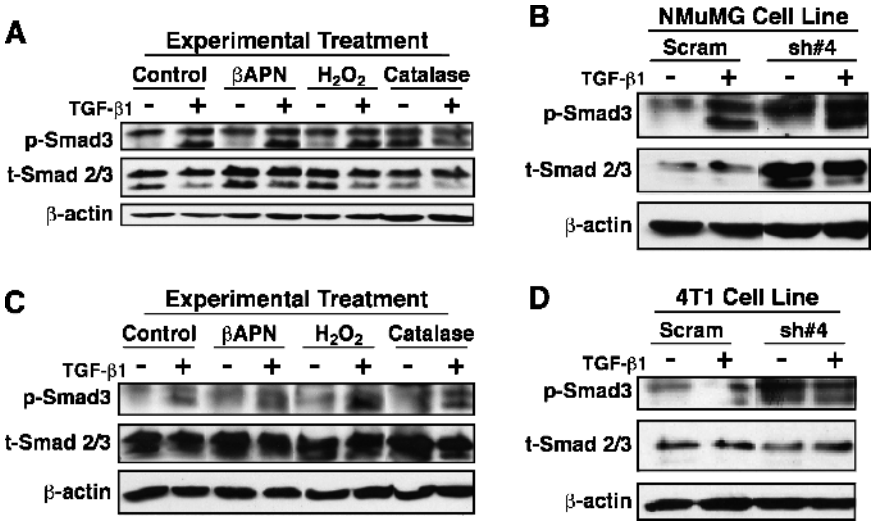


Figure W1. Pharmacologic and genetic targeting of LOX fail to alter TGF-β1 stimulation of Smad3 phosphorylation in normal and malignant MECs. Quiescent NMuMG (A) and 4T1 (C) cells were pretreated with βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated. Afterward, these MECs and their LOX-deficient (sh#4) counterparts (B, D) were stimulated with TGF-β1 (5 ng/ml) for 30 minutes before monitoring changes in the expression (t-Smad2/3) and phosphorylation (p-Smad3) of Smad3 by immunoblot analysis. Differences in protein loading were assessed by reprobing stripped membranes with β-actin antibodies. Data are representative images from three independent experiments. Scram indicates scrambled shRNA.

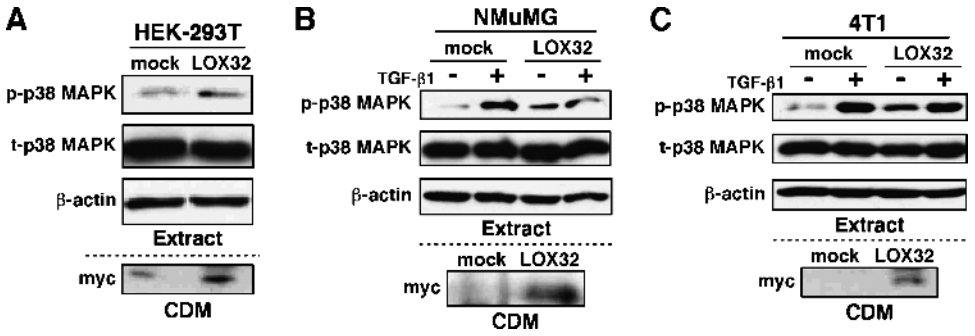


Figure W2. Expression of constitutively active LOX stimulates p38 MAPK in normal and malignant MECs. Human 293T (A), NMuMG (B), or 4T1 (C) cells were transiently transfected with constitutively active LOX (LOX32). Thirty-six hours after transfection, the conditioned medium (CDM) was tumbled with Ni^{2+}-agarose beads to capture recombinant LOX32 proteins (A, C) or was precipitated with trichloroacetic/deoxycholate (B). Recombinant LOX32 protein expression was visualized by immunoblot analysis with anti-Myc antibodies. After collecting CDM, the cells were incubated for 4 hours in serum-free medium before stimulation with TGF-β1 (5 ng/ml) for 30 minutes at 37°C as indicated. The phosphorylation status of p38 MAPK was assessed by immunoblot analysis with phospho-specific p38 MAPK (p-p38 MAPK) antibodies, whereas differences in protein loading were monitored by reprobing stripped membranes with antibodies against p38 MAPK (t-p38 MAPK) and β-actin. Data are representative images from two independent experiments.

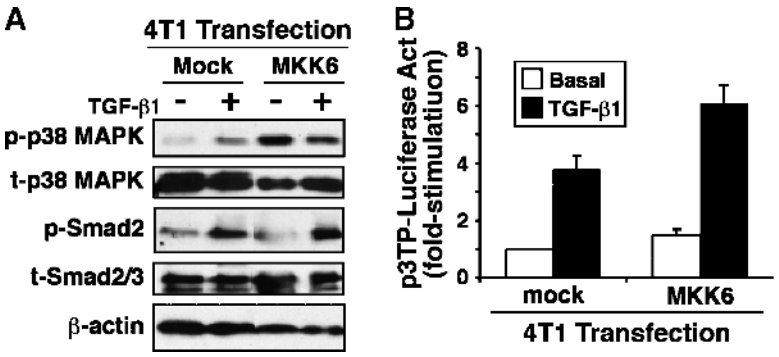


Figure W3. Expression of constitutively active MKK6 activates p38 MAPK but has no effect on the coupling of TGF-β to Smad2/3 in malignant MECs. 4T1 cells were transiently transfected overnight with p3TP-luciferase and pCMV–β-gal, together with either empty vector (mock) or constitutively active MKK6 as indicated. Afterward, the cells were stimulated with TGF-β1 (5 ng/ml) for 24 hours, at which point cell extracts were prepared for p38 MAPK and Smad2 immunoblot analysis (A) or luciferase and β-gal assays (B). Data are representative images or the resulting luciferase activity (mean ± SE) from three independent experiments.

J Mammary Gland Biol Neoplasia (2011) 16:127–146
DOI 10.1007/s10911-011-9207-3

Noncanonical TGF-β Signaling During Mammary Tumorigenesis

Jenny G. Parvani · Molly A. Taylor · William P. Schiemann

Received: 5 March 2011 / Accepted: 8 March 2011 / Published online: 31 March 2011
© Springer Science+Business Media, LLC 2011

Abstract Breast cancer is a heterogeneous disease comprised of at least five major tumor subtypes that coalesce as the second leading cause of cancer death in women in the United States. Although metastasis clearly represents the most lethal characteristic of breast cancer, our understanding of the molecular mechanisms that govern this event remains inadequate. Clinically, ~30% of breast cancer patients diagnosed with early-stage disease undergo metastatic progression, an event that *(a)* severely limits treatment options, *(b)* typically results in chemoresistance and low response rates, and *(c)* greatly contributes to aggressive relapses and dismal survival rates. Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that regulates all phases of postnatal mammary gland development, including branching morphogenesis, lactation, and involution. TGF-β also plays a prominent role in suppressing mammary tumorigenesis by preventing mammary epithelial cell (MEC) proliferation, or by inducing MEC apoptosis. Genetic and epigenetic events that transpire during mammary tumorigenesis conspire to circumvent the tumor suppressing activities of TGF-β, thereby permitting late-stage breast cancer cells to acquire invasive and metastatic phenotypes in response to TGF-β. Metastatic progression stimulated by TGF-β also relies on its ability to induce epithelial-mesenchymal transition (EMT) and the expansion of chemoresistant breast cancer stem cells. Precisely how this metamorphosis in TGF-β function comes about remains incompletely understood; however, recent findings indicate that the initiation of oncogenic TGF-β activity is contingent upon imbalances between its canonical and noncanonical signaling systems. Here we review the molecular and cellular contributions of noncanonical TGF-β effectors to mammary tumorigenesis and metastatic progression.

Keywords Epithelial-mesenchymal transition · Metastasis · Signal transduction · Transforming growth factor-β · Tumor microenvironment

Abbreviations

AKT/PKB	protein kinase B
BMDC	bone marrow-derived cell
CSC	cancer stem cell
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
HGF	hepatocyte growth factor
hnRNP E1	heterogeneous nuclear ribronucleoprotein E1
ILEI	interleukin-like EMT inducer
JNK	c-Jun N-terminal kinase
MAP kinase	mitogen-activated protein kinase
MEC	mammary epithelial cell
MET	mesenchymal-epithelial transition
miR	microRNA
MMP	matrix metalloproteinase
MSP	macrophage-stimulating protein

Jenny G. Parvani and Molly A. Taylor were equal contributors to this work.

J. G. Parvani · M. A. Taylor
Case Comprehensive Cancer Center,
Case Western Reserve University,
Wolstein Research Building, Room 2404, 2103 Cornell Road,
Cleveland, OH 44106, USA

W. P. Schiemann (✉)
Case Comprehensive Cancer Center,
Case Western Reserve University,
Wolstein Research Building, Room 2131, 2103 Cornell Road,
Cleveland, OH 44106, USA
e-mail: William.Schiemann@case.edu

MTA3	metastasis associated protein 3
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PI3K	phosphoinositide-3-kinase
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptor
TGF-β	transforming growth factor-β
TRAF6	TNF receptor-associated factor 6
uPA	urokinase plasminogen activator
uPAR	uPA receptor
ZO-1	zonula occluden-1

Introduction

Breast cancer and its associated metastases are the second leading cause of cancer death in women, accounting for more than 40,000 deaths and 211,000 new invasive cancer cases annually in the United States [1]. Metastasis is devastating and underlies the deaths of ~90% of patients with solid tumors [2, 3], including those originating in the breast [4, 5]. Indeed, the 5 year survival rate of breast cancer patients diagnosed with localized disease is 98%, a figure that drops precipitously to 23% in patients diagnosed with evidence of metastatic disease [6]. The dissemination of breast cancer cells, which occurs most frequently to the brain, liver, bone, and lung [7], involves a complex cascade of gene expression and repression that coalesce in an orderly manner to engender distinct stages of the metastatic process, including *(a)* local invasion through the basement membrane; *(b)* intravasation into the vasculature to traverse the circulation; and *(c)* extravasation, infiltration, and eventual colonization of secondary organ sites [2, 8–11]. Clonal selection theory paints metastasis as the ultimate developmental rung achieved by evolving breast cancer; however, recent evidence indicates that many breast cancers disseminate long before their primary tumors become symptomatic, and in fact, 33% of women diagnosed with small mammary tumors (4 mm) already harbor disseminated breast cancer cells in their bone marrow [12–14]. Moreover, these micrometastases can remain dormant for years before reemerging as incurable secondary tumors that are surprisingly insensitive to neoadjuvant chemotherapies that originally attacked the primary tumor [15, 16]. These clinical correlates point to the presence of a molecular bifurcation in the signaling events that underlie metastatic outgrowth versus those operant in promoting primary tumor development. Presently, science and medicine lack sufficient knowledge to synthesize novel pharmaceuticals capable of specifically targeting and alleviating metastatic progression in cancers of the breast. Although genomic approaches have offered some molecular insights into how genetically distinct breast cancer subtypes may be identified and treated, these analyses have yet to provide information related to *(a)* "how and when" metastasis transpires during mammary tumorigenesis; *(b)* the overall metastatic potential of distinct breast cancer subtypes; and *(c)* the impact of conventional chemotherapies and treatment regimens to influence, either positively or negatively, breast cancer metastasis and disease recurrence. Thus, metastasis may in fact represent the last unknown frontier to be interrogated by science and medicine.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that plays essential roles during the branching morphogenesis, lactation, and involution phases of post-natal mammary glands [17, 18]. TGF-β is also a powerful tumor suppressor that governs essentially every aspect of the pathophysiology of mammary epithelial cells (MECs), including their ability to proliferate, migrate, differentiate, and survive [5, 19, 20]. During mammary tumorigenesis, genetic and epigenetic events undermine the tumor suppressive functions of TGF-β, thereby enhancing the development and progression of evolving breast cancers. Mammary tumorigenesis also elicits dramatic alterations in the architecture of breast cancers and their accompanying microenvironments (e.g., desmoplastic and fibrotic reactions), which further inactivate the tumor suppressing activities of TGF-β [19, 21, 22]. Even more remarkably, these abnormal events coalesce to confer TGF-β the ability to stimulate the invasion and metastasis of late-stage breast cancer cells. This peculiar conversion in TGF-β function is known as the "TGF-β Paradox," which underlies the lethality of TGF-β in metastatic breast cancer cells [19, 23, 24]. An emerging explanation for the dichotomous functions of TGF-β may reflect its ability to generate cancer stem cells via its stimulation of epithelial-mesenchymal transition (EMT), an event essential for the initiation of oncogenic TGF-β signaling in breast cancer cells [5, 19]. Along these lines, breast cancer resistance to chemotherapies is clearly associated with the acquisition of EMT [25–28], particularly that driven by TGF-β [29, 30], and with alterations in the tumor microenvironment [31]. Precisely how TGF-β participates in these inappropriate events remains an active area of scientific research; however, recent findings suggest that imbalances between canonical and noncanonical TGF-β signaling inputs manifest the "TGF-β Paradox" and the acquisition of oncogenic activity by TGF-β.

Here we review recent findings that directly impact our understanding of the role of noncanonical TGF-β signaling systems in regulating its oncogenic activities and ability to promote metastatic progression and EMT in developing mammary carcinomas.

TGF-β Signaling Systems

Canonical TGF-β Signaling

Canonical TGF-β signaling refers to messages transduced by the activation of the latent transcription factors, Smad2 and Smad3, and as such, canonical TGF-β signaling is synonymous with Smad-dependent TGF-β signaling. All intracellular signals stimulated by TGF-β commence upon its binding to three high-affinity transmembrane receptors, namely TGF-β receptors type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). Mammals express three genetically unique TGF-β cytokines (i.e., TGF-βs 1–3) whose mature and biologically active forms are ~97% identical and exhibit virtually indistinguishable actions in vitro [32, 33]. Interestingly, individual TGF-β ligands are expressed in a spatiotemporal manner during embryogenesis and tissue morphogenesis, which contributes to the array of diverse and nonredundant phenotypes displayed by mice lacking distinct TGF-β isoforms [34]. Once synthesized, TGF-β ligands are secreted into the extracellular matrix (ECM) as inactive latent complexes whose conversion to active forms transpires through several mechanisms, including proteolysis, reactive oxygen species, acidic microenvironments, and binding to integrins [35, 36]. Following their activation, only TGF-βs 1 and 3 can bind directly to TβR-II, while that of TGF-β2 must first bind TβR-III to facilitate presentation to TβR-II. Although TβR-III lacks intrinsic enzymatic activity, TβR-I and TβR-II both possess Ser/Thr protein kinases in their cytoplasmic domains that serve to initiate downstream signaling [19, 37]. Indeed, ligand engagement of TβR-II leads to the recruitment, phosphorylation, and activation of TβR-I, which subsequently binds, phosphorylates, and stimulates Smad2/3 [19, 38]. Once activated, Smad2/3 rapidly interact physically with the common Smad, Smad4, thereby exposing cryptic nuclear localization sequences that permit these heterotrimeric complexes to accumulate in the nucleus. Upon gaining entry into the nucleus, Smad2/3/4 complexes function in conjunction with a host of transcription factors, enhancers, and repressors that collectively bring about dramatic alterations in gene expression in a cell- and context-specific manner [Fig. 1, *left panel;*[19, 38]]. In addition, the amplitude and duration of Smad2/3 responses are impacted by their interaction with a variety of adapter molecules, including SARA [39], Hgs [40], PCTA [41], and Dab2 [42, 43]. Interestingly, altered expression of SARA and Dab2 have both been linked to the ability of TGF-β to regulate the plasticity of MECs [44–47], suggesting that imbalances in Smad2/3 signaling contribute to oncogenic TGF-β signaling. Along these lines, the focal adhesion adapter protein, p130Cas, can bind and promote the degradation of Smad3, leading to diminished cytostatic activity by TGF-β [48]. We showed that mammary tumorigenesis upregulates the expression of p130Cas, an event that enhances TGF-β stimulation of metastatic progression in part via amplifying the coupling of TGF-β to its noncanonical effectors relative to signals derived from their Smad2/3 counterparts [49]. The activation of Smad2/3 by TβR-I is governed by the inhibitory Smad, Smad7, whose binding to TβR-I occludes its ability to phosphorylate Smad2/3 [50–52], as well as recruits the E3 ubiquitin ligase, Smurf1/2, to promote TGF-β receptor ubiquitination, internalization, and degradation [53, 54]. In addition, the functions of Smad7 are positively regulated by its binding to STRAP [55], but negatively regulated by its interactions with AMSH2 [56] and Arkadia [57]. Finally, canonical TGF-β signaling can be terminated through several distinct mechanisms, including *(a)* dephosphorylation of Smad2/3 by the nuclear phosphatase PPM1A [58]; *(b)* degradation of Smad2/3 following their ubiquitination by Smurf1, Smurf2, and SCF/Roc1 [59–62]; and *(c)* reversible monoubiquitination of Smad4 which governs its binding to activated Smad2/3 [63].

The essential function of Smad2/3 and canonical TGF-β signaling in suppressing tumorigenesis, including those occurring in the breast, is well documented in the scientific literature (*see* [38, 64, 65]). Unfortunately, it remains unclear as to the extent to which canonical TGF-β signaling collaborates with its noncanonical counterparts in mediating the oncogenic activities of TGF-β in late-stage breast cancer. For instance, both canonical and noncanonical TGF-β signaling are essential for TGF-β stimulation of EMT in MECs [5, 66–69]. Moreover, cross-talk between Smad2/3 and the noncanonical TGF-β effector, Ras, is sufficient in driving EMT and metastasis [70, 71]. We have shown that sustained EMT induced by TGF-β selectively diminishes Smad3 expression following the activation of several noncanonical TGF-β effectors, including p130Cas [49], GSK-3β[72], and a NF-κB:Cox-2:PGE2:EP2 signaling axis [72–75]. Yet how these individual events coalesce during distinct stages of metastatic progression remain purely speculative, as is the nature of these signaling collaborations to the behaviors of normal MECs. The molecular readouts of Smad2/3 signaling are also regulated by the phosphorylation of their proline-rich linker domains by a host of Ser/Thr protein kinases. Indeed, activated Ras promotes ERK1/2-mediated phosphorylation of Smad2/3 linker domains, leading to a reduction in their ability to undergo nuclear translocation [76]. However, ERK1/2-mediated phosphorylation of the MH1 domain of Smad2/3 enhances its transcriptional activity [77], suggesting that the location of the phospho-accepting residue has dramatic effects on Smad2/3 function. Likewise, stimulating JNK or its upstream activator, MEKK1, both enhance Smad2/3 signaling in response to TGF-β [78, 79], as does that mediated by casein kinase I-ε[80]. In stark contrast, the phosphorylation of Smad2/3 by PKC [81], calmodulin kinase

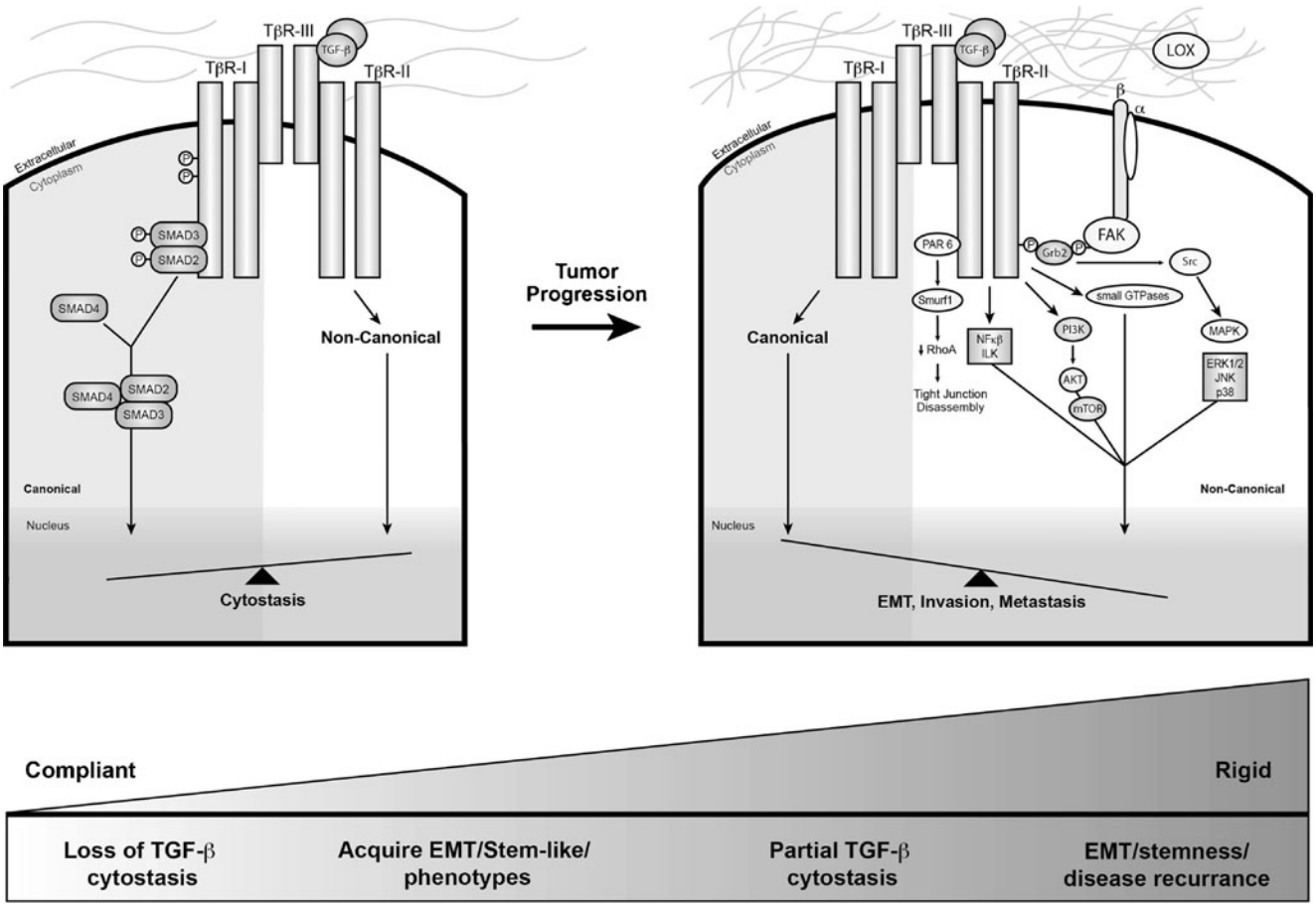


Figure 1 Schematic depicting the canonical and noncanonical TGF-β signaling naling systems activated during mammary tumorigenesis. Transmembrane signaling by TGF-β is stimulated upon its binding and activation of the Ser/Thr protein kinase receptors, TβR-I and TβR-II. The physical interaction of TGF-β with either TβR-III or TβR-II facilitates the recruitment and transphosphorylation of TβR-I, resulting in its activation and subsequent phosphorylation of the receptor-activated Smads, Smad2 and Smad3. Once activated, Smad2/3 form heterocomplexes with Smad4 and translocate to the nucleus to regulate the expression of TGF-β-responsive genes in concert with an ever expanding list of transcriptional coactivators and repressors. This branch of the bifurcated TGF-β signaling system represents the "canonical" or "Smad2/3-dependent" TGF-β pathway, which is the predominant pathway coupled to cytostasis and activated by TGF-β in normal MECs (*left panel*). Alternatively, TGF-β also activates a variety of "noncanonical" or "Smad2/3-independent" effectors, including Par6, NF-κB, ILK, FAK, Src, Rho-family GTPases, MAP kinases, and the PI3K:AKT:mTOR signaling axes (*right panel*). During EMT and mammary tumorigenesis, the balance between canonical and noncanonical TGF-β signaling systems becomes distorted and favors coupling to noncanonical effector systems, an event that manifests the "TGF-β Paradox" and the initiation of EMT, invasion, and metastasis. These events are further exacerbated by elevated deposition and eventual cross-linking of ECM molecules within tumor microenvironments (LOX, *right panel*), an event that promotes tumor rigidity and the activation of mechanotransduction pathways operant in amplifying noncanonical TGF-β signaling and its oncogenic activities in mammary tumors. See text for additional details.

II [82], and GSK-3 [83] all suppress the intracellular communications propagated by canonical TGF-β signaling. Collectively, these studies highlight the complexities associated with TGF-β signaling and the need to further dissect the molecular interactions between Smad2/3 and their noncanonical counterparts in mediating the biology and pathology of TGF-β in normal and malignant MECs.

Noncanonical TGF-β Signaling Systems

In addition to its ability to activate Smad2/3, TGF-β also regulates the pathophysiology of MECs by stimulating an ever expanding array of noncanonical signaling molecules (i.e., Smad2/3-independent), whose activation and biological activities span all cellular compartments. Recent studies by our group [49, 72–75, 84–91] and others [92, 93] support the idea that inappropriate imbalances between canonical and noncanonical TGF-β signaling pathways manifest the "TGF-β Paradox" and the acquisition of oncogenic signaling by TGF-β in developing and progressing mammary tumors (Fig. 1, *right panel*). In the succeeding sections, we highlight prominent noncanonical effectors activated by TGF-β and discuss their role in mediating oncogenic TGF-β signaling in cancers of the breast.

Integrin and Focal Adhesion Signaling

Integrins are heterodimeric transmembrane receptors that specialize in linking the ECM to the cytoskeletal apparatus within normal and malignant cells [94]. Interestingly, individual tissues bear a unique "fingerprint" through their expression of unique and distinguishing ECM signatures [95], which are readily recognized and complemented by an equally diverse array of integrin expression. Importantly, carcinoma cells undergoing neoplastic development can alter their integrin expression profiles to reflect changes within the tumor microenvironment, as well as those necessary for metastatic progression [94]. Indeed, integrin activation and signaling is essential to cell proliferation, survival, migration, and invasion, and to the development of chemoresistance in breast cancer cells [96]. These integrin-mediated functions are surprisingly reminiscent of those attributed to TGF-β during its stimulation of metastatic progression of mammary tumors, and as such, these observations suggest the potential involvement of integrins in oncogenic TGF-β signaling. Accordingly, integrins αvβ6 and αvβ8 function in binding and activating latent TGF-β complexes, a mechanism that involves matrix metalloproteinase (MMP)-14 and the proteolytic release of active TGF-β to affect the behaviors of neighboring cells [97, 98]. Furthermore, TGF-β stimulates the expression of αvβ3 integrin, which confers migratory and invasive phenotypes to normal and malignant MECs, as well as mediates pulmonary metastasis induced by TGF-β in late-stage breast cancer cells [85–87]. Mechanistically, β3 integrin interacts physically with TβR-II and promotes its phosphorylation on Tyr284 by Src. Following its phosphorylation, Tyr284 serves as a docking site for ShcA and Grb2, leading to the amplified activation of p38 MAPK and the acquisition of EMT phenotypes by MECs [85–87]. Importantly, pharmacologic or genetic manipulations to inactivate this noncanonical signaling axis are sufficient in blocking oncogenic TGF-β signaling and its stimulation of EMT and metastasis in malignant MECs [85–87]. Along these lines, administration of TβR-I inhibitors to mice prevents the metastasis of breast cancer cells to the bone and lungs in part via diminished expression of αvβ3 integrin [99, 100]. Subsequent studies established focal adhesion kinase (FAK) as the adapter molecule operant in bridging the formation of β3 integrin:TβR-II complexes [88], and as an essential mediator for TGF-β to *(a)* stimulate EMT and metastasis in breast cancer cells [49, 88], *(b)* induce macrophage infiltration into developing mammary tumors [88]; and *(c)* facilitate EGFR-dependent metastatic progression in malignant MECs [89]. In the presence of degraded ECM fragments, the scaffolding functions of FAK enable the formation of complexes comprised of β1 integrins and TGF-β receptors, which initiate oncogenic TGF-β signaling in a ligand-independent manner [101]. Besides FAK, we also identified p130Cas as a molecular fulcrum that governs the balance between canonical and noncanonical TGF-β signaling during mammary tumorigenesis. Indeed, the phosphorylation and activation of p130Cas enables this adapter molecule to form heteromeric complexes with TβR-I and Smad3, resulting in the degradation of Smad3 and the initiation of metastatic progression stimulated by TGF-β [48, 49]. Elevated expression of p130Cas occurs frequently during the progression of mammary tumors and is associated with the development of resistance to adriamycin and tamoxifen [102, 103], and with the increased malignancy of ErbB2 tumors due to enhanced signaling via Src, FAK, Rac1, and MMP-9 [104, 105]. Finally, signaling downstream of β1 integrin also mediates TGF-β stimulation of p38 MAPK, JNK, and Dab2 expression, all of which are essential for the acquisition of EMT phenotypes in MECs [46, 106]. Along these lines, we recently discovered a novel interaction between β1 and β3 integrins in regulating oncogenic TGF-β, such that pharmacologic or genetic inactivation of β1 integrin engenders a dramatic compensatory upregulation of β3 integrin expression sufficient to restore oncogenic TGF-β signaling in metastatic MECs (J.G. Parvani and W.P. Schiemann, *unpublished observation*). Thus, metastatic breast cancers may have evolved the means necessary to evade single agent integrin-based therapies through "integrin-switching" [107, 108], and if so, future studies need to identify the collection of integrins operant in mediating the oncogenic activities of TGF-β.

PI3K, AKT, and mTOR

Oncogenic TGF-β signaling is also associated with the activation of phosphoinositide-3-kinase (PI3K) and its downstream target, AKT/PKB, which collectively serve in enhancing breast cancer proliferation, survival, and motility [109]. In addition, activation of the PI3K:AKT signaling axis also enables TGF-β to induce EMT and metastatic progression in malignant MECs, doing so via either the direct coupling of TGF-β receptors to the PI3K machinery [110] or indirectly through the ability of TGF-β to transactivate the receptors for EGF [111] and PDGF [112]. In fact, dual activation of the receptors for TGF-β and EGF can produce a hyper-EMT response related to the stimulation of PI3K/AKT and ERK1/2. Interestingly, antagonizing PI3K/AKT activity pharmacologically has no effect on the morphological features of EMT, but is sufficient in preventing elevated cell motility and invasion associated with EMT phenotypes [113]. These findings suggest that the morphologic and motile phenotypes of EMT may in fact be distinct physiological entities, each coupled to

unique branches of the noncanonical TGF-β signaling system. Accordingly, inactivating mTOR (mammalian target of rapamycin) also failed to alter the morphological features of EMT stimulated by TGF-β, but did prevent its ability to induce the invasion of post-EMT MECs [114]. The complexities of connecting PI3K:AKT signaling to the oncogenic activities of TGF-β are further highlighted by the ability of AKT to interact physically with Smad3 and prevent its nuclear translocation in response to TGF-β, thereby diminishing the cytostatic activities of TGF-β by enhancing cell survival [115, 116]. Finally, recent studies have established AKT2 as an essential mediator of EMT stimulated by TGF-β [45, 47]. For instance, heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) strongly binds to structural elements in the 3'-UTRs of Dab2 and interleukin-like EMT inducer (ILEI) transcripts, thereby repressing their translation and subsequent induction of EMT in polarized MECs. In response to TGF-β, AKT2 readily phosphorylates and inactivates hnRNP E1, leading to its release from Dab2 and ILEI mRNA and the initiation of EMT and metastatic progression in MECs [45, 47]. Thus, the ability of TGF-β to regulate the translation and elongation of transcripts associated with the EMT process may represent a unique clinical target to alleviate breast cancer metastasis by overriding noncanonical TGF-β signaling inputs coupled to PI3K:AKT:mTOR activation.

Rho-family GTPases

Recent studies have implicated members of the Rho GTPase family (i.e., RhoA/B/C, Rac1, and Cdc42) in mediating the initiation of oncogenic TGF-β signaling [69, 117, 118]. Indeed, these small plasma membrane-associated GTP-binding proteins collaborate with integrins and other receptor systems to regulate a wide array of cellular functions, including alterations in cell adhesion, morphology, and motility reflecting the generation of filopodia (e.g., Cdc42), lamellipodia (e.g., Rac1), and actin stress fibers (e.g., RhoA) [119, 120]. Thus, these findings implicate Rho-family GTPases as potential participants in all stages of the metastatic cascade. Accordingly, TGF-β stimulation of EMT requires RhoA activation, as well as that of its downstream effector, $p160^{ROCK}$ [121]. Additionally, the phosphorylation of Par6 by TβR-II results in the ubiquitination and degradation of RhoA [122, 123], presumably leading to upregulated Snail expression that promotes the E-cadherin deficiencies associated with EMT phenotypes [124]. On a similar note, the ability of TGF-β to suppress RhoC expression also elicits the dissolution of junctional complexes by inducing the proteosomal degradation of E-cadherin [124]. More recently, TGF-β has been observed to induce the phosphorylation of CdGAP, a RhoGAP that only targets Rac1 and Cdc42, leading to elevated cell motility and invasion in ErbB2-positive breast cancers [125]. Independent of its ability to bind ligand, TβR-III bound to β-arrestin2 elicits constitutive activation of Cdc42, which inhibits directional migration of ovarian and breast cancer cells [126]. Finally, RhoA expression is differentially regulated by TGF-β-responsive microRNAs (miRs), such that TGF-β stimulation of miR-155 expression promotes EMT by degrading RhoA [127], while that of miR-31 suppresses breast cancer metastasis in part by reducing RhoA expression [128, 129]. Taken together, these findings highlight the functional complexities associated with the expression and activation of Rho-family GTPases during metastatic progression stimulated by TGF-β.

MAP Kinases

A major mechanism whereby TGF-β induces EMT and metastatic progression is through the stimulation of members of the MAP kinase family of dual-specificity protein kinases, which includes ERK1/2 (extracellular signal-related kinase 1 and 2), JNK (c-Jun N-terminal kinase, and p38 MAPK [106, 130–132]. Indeed, TGF-β stimulation of EMT and its accompanying delocalization of E-cadherin and ZO-1 from the plasma membrane requires ERK1/2 activation, a cellular reaction abrogated by administration of MEK1/2 inhibitors [132]. Likewise, rendering MECs deficient in Dab2 expression prevents TGF-β from stimulating JNK, and from promoting MEC migration and fibronectin expression during EMT [46]. Elevated tumor expression of type I collagen has been shown to induce JNK activation [133]. As such, pharmacological inhibition of either JNK or PI3K activity abrogates the ability of type I collagen to promote the migration and metastasis of breast cancer cells [134]. The activation of p38 MAPK by TGF-β in normal and malignant MECs requires these cells to express either β1 [106] or β3 integrins [85–87]. As mentioned previously, we defined a novel signaling axis comprised of αvβ3 integrin:Src:FAK:p130Cas:TβR-II:Grb2 that is critical for TGF-β stimulation of p38 MAPK, EMT, and pulmonary metastasis of breast cancer cells [49, 85–88]. In addition, this signaling axis also confers oncogenic activity to EGF, including its ability to facilitate p38 MAPK activation and metastatic progression in post-EMT populations of breast cancer cells [89]. Recently, an alternative mechanism operant in activating JNK and p38 MAPK by TGF-β has been elucidated. Indeed, following their activation by cytokine, TGF-β receptors interact physically with TRAF6 (TNF receptor-associated factor 6), thereby enabling this E3 ligase to ubiquitinate and activate TAK1 and its eventual stimulation of JNK and p38 MAPK [135, 136]. Importantly, depleting MECs of TRAF6 expression fails to affect canonical TGF-β signaling;

however, this same cellular condition prevents TGF-β from activating JNK and p38 MAPK, as well as from stimulating EMT [135, 136]. Collectively, these studies highlight the importance of MAP kinases in mediating the acquisition of oncogenic signaling by TGF-β, leading to the hypothesis that chemotherapeutic targeting of MAP kinase pathways may reinstate the cytostatic function of TGF-β by normalizing the inherent balance between its canonical and noncanonical signaling systems.

Nuclear Factor-κB

NF-κB is an essential mediator of inflammation associated with the growth, survival, invasion and angiogenesis of developing neoplasms [137]. TGF-β typically represses NF-κB activity in normal cells by inducing the expression of IκBα [138, 139], or by preventing the degradation of IκBα via the formation of TβR-III:β-arrestin2 complexes [140]. In stark contrast, mammary tumorigenesis paradoxically converts TGF-β from an inhibitor to a stimulator of NF-κB activity. In doing so, TGF-β acquires the ability to form TβR-I:xIAP:TAB1:TAK1:IKKβ complexes in malignant MECs, as well as in their normal counterparts undergoing EMT in response to TGF-β [72–74]. Uncoupling TGF-β from NF-κB activation dramatically inhibits *(a)* mammary tumor development in mice in part via regulation of the innate immune system [73], and *(b)* the acquisition of EMT and invasive phenotypes in normal and malignant MECs [72–74, 141]. Along these lines, the coupling of TGF-β to NF-κB facilitates Ras-transformed breast cancer cells to undergo EMT and colonize the lung [142], as well as promotes the formation of an autocrine Cox-2:PGE2:EP2 signaling cascade essential for metastatic progression and EMT induced by TGF-β [72, 75]. Future studies need to assess the relative contribution of NF-κB in the acquisition of chemoresistant phenotypes displayed by late-stage mammary tumors, particularly those subjected to the oncogenic activities of TGF-β.

Lysyl Oxidase (LOX)

Lysyl oxidase (LOX) belongs to a five member gene family of copper-dependent amine oxidases (LOX, LOXL1, LOXL2, LOXL3, and LOXL4) that function in cross-linking collagen and elastin in the ECM [143, 144]. The cross-linking activity of LOX leads to increased ECM tension and rigidity in developing mammary tumors, which enhances integrin-mediated mechanotransduction coupled to the induction of breast cancer invasion and metastasis [145, 146]. Elevated LOX expression is also associated with hypoxia-induced metastasis of breast cancer cells in mice, and with increased metastatic burden and poor survival in breast cancer patients [147, 148]. Interestingly, the hydrogen peroxide produced as a byproduct of LOX activity leads to Rac1 activation through the assembly of p130Cas/Crk/Dock180 complexes [149]. Along these lines, we recently observed LOX expression and secretion to be stimulated by TGF-β in normal and malignant MECs, and in 4T1 mammary tumors produced in mice [90]. Additionally, antagonizing the expression and activity of LOX impairs the ability of TGF-β to induce EMT and invasion, as well as partially uncouples TGF-β from the activation of p38 MAPK in metastatic cells [90]. Even more remarkably, we demonstrated that exposing late-stage breast cancer cells to compliant microenvironments is sufficient in reinstating the cytostatic function of TGF-β, a reaction that is readily reversed by rendering these same 3D-organotypic cultures mechanically rigid by the inclusion of type I collagen. Importantly, inhibiting the activities of TGF-β or LOX, or degrading hydrogen peroxide in rigid cultures abrogates oncogenic TGF-β signaling [90], thereby implicating LOX as an important mediator of breast cancer progression stimulated by TGF-β. Finally, the formation of premetastatic niches has been linked to LOX and its ability to stimulate collagen cross-linking and fibronectin production, which coalesce to recruit bone marrow-derived cells (BMDCs) to future sites of metastasis [147]. TGF-β also induces BMDC recruitment to premetastatic niches [150], suggesting a clinically important link between TGF-β and its stimulation of LOX expression in mediating the establishment of premetastatic niches. Future studies need to demonstrate the validity of this supposition, as well as determine the chemotherapeutic effectiveness of preventing LOX expression or activation as a novel means to alleviate oncogenic TGF-β signaling in cancers of the breast.

Noncanonical TGF-β Signaling and MEC Plasticity

EMT

The ability of TGF-β to promote metastatic progression is strongly linked to EMT, which represents a transdifferentiation process that enables immotile, polarized MECs to acquire highly motile, apolar fibroblastoid-like phenotypes [5, 69, 151]. For instance, MECs undergoing EMT exhibit several unique features, including *(a)* the loss of cell polarity due to down regulated expression of epithelial cell markers (e.g., E-cadherin, ZO-1, and β4 integrin); *(b)* cytoskeletal architecture reorganization and intracellular organelle redistribution; *(c)* upregulated expression of fibroblastoid markers (e.g., vimentin, N-cadherin, α-smooth muscle actin); and *(d)* elevated invasion factors (e.g., MMP-9, fibronectin) [5, 69, 151, 152]. Generally speaking, these steps underlie the pathophysiology of EMT, which were recently categorized into three distinct sub-

types: *(a)* ***Type 1 EMT***, which refers to the plasticity exhibited by epithelial cells during embryogenesis and tissue morphogenesis; *(b)* ***Type 2 EMT***, which refers to the plasticity exhibited by epithelial cells during tissue regeneration and fibrotic reactions; and *(c)* ***Type 3 EMT***, which refers to the plasticity exhibited by carcinoma cells during metastatic progression [153]. Indeed, Type 3 EMT has been observed to confer carcinoma cells a selective invasive advantage to exit both the primary tumor [88] and the circulation at sites of dissemination [89, 154]. At first blush, this classification scheme acknowledges that the extent to which an EMT reaction transpires likely reflects the overall health and well-being of the epithelium and its immediate microenvironment. Unfortunately, the vast majority of EMT studies employ "cell-centric" approaches to assess the functional consequences of EMT in normal and malignant cells, and as such, the contributions of the microenvironment in regulating epithelial plasticity remains a critical and underexplored question. Readers desiring in-depth summaries pertaining to the molecular mechanisms whereby TGF-β promotes EMT are directed to several recent reviews [5, 69, 151, 152].

Junctional Dissolution

Tight Junctions

Tight Junctions are localized to lateral cell membranes where they coalesce to form a permeability seal that physically restricts solutes from entering intercellular spaces [155, 156]. Structurally, tight junctions are formed by the actions of the transmembrane proteins, claudins, occludins, and JAMs (**J**unctional **A**dhesion **M**olecules), which require ZO-1, -2, and -3 scaffolding proteins for linkage to the actin cytoskeleton [157, 158]. Once formed, tight junctions and their constituents play essential roles in regulating the architecture and homeostasis of MECs, and in preventing the initiation of EMT and tumorigenesis [159]. TGF-β is a master regulator of the expression and localization of several tight junction components, particularly during EMT. For example, phosphorylation of Par6 in response to TGF-β promotes the ubiquitination and degradation of RhoA, resulting in the dissolution of tight junctions during EMT stimulated by TGF-β [122, 123]. By stimulating the formation of Snail1:Smad3:Smad4 complexes in MECs, TGF-β downregulates the expression of the tight junction proteins CAR (coxsackie and adenovirus receptor), occludin, and claudin-3, as well as that of the adherens junction protein, E-cadherin [160, 161]. As mentioned previously, TGF-β delocalizes ZO-1 away from tight junctions by activating PI3K and ERK1/2 [110, 132], while co-administration of TGF-β and EGF reduces claudin-1 and occludin expression concomitantly with a loss of epithelial barrier function [162]. Somewhat surprisingly, the loss of claudin-1 expression requires Smad4 and β-catenin activity, but was independent of TGF-β and its receptors [163]. Finally, recent studies implicate a role of occludin in localizing TGF-β receptors to basolateral [164] or lateral [165] cell surfaces, a sorting reaction that requires the cytoplasmic domains of these receptors [165]. Conversely, the secretion of TGF-β ligands is localized specifically to the apical surface in non-transformed cells and to the basolateral surface of transformed cells [165, 166]. Collectively, these studies highlight the importance of tight junctions in regulating MEC homeostasis, as well as their potential to coordinate the responses of MECs to TGF-β in a spatiotemporal manner.

Adherens Junctions

E-cadherin is the primary molecule responsible for forming adherens junctions in polarized MECs [167, 168], and the loss of E-cadherin expression in neoplastic MECs leads to their acquisition of EMT, invasive, and metastatic phenotypes that correlate with poor clinical outcomes in breast cancer patients [169–171]. TGF-β transcriptionally silences E-cadherin expression in malignant MECs by activating a variety of EMT-responsive transcription factors, including Snail, ZEB1/2, or Twist [172]. Interestingly, as MECs transdifferentiate and acquire mesenchymal phenotypes they typically undergo "cadherin switching" whereby epithelial E-cadherin is replaced by the upregulated expression of mesenchymal N-cadherin or cadherin-11 [167, 173]. Adherens junctions are stabilized by the binding of cadherins to the actin cytoskeleton via their interaction with α- and β-catenins and plakoglobin [167, 168]. These events are readily disrupted by TGF-β and its targeting of α- and β-catenins for Tyr-phosphorylation, an event that reduces cell-cell adhesion and alters the actin cytoskeleton [174–176]. The ability of TGF-β to downregulate E-cadherin-based cell-cell adhesion depends upon the formation of Smad2:β-catenin complexes [174, 175], and upon the integration of PI3K and PTEN signaling inputs [177]. Future studies need to define the precise role that "cadherin switching" plays during metastatic progression stimulated by TGF-β, as well as delineate the relative contributions of canonical and noncanonical TGF-β signaling inputs in mediating these events.

Microenvironments and MEC Plasticity

The biology of TGF-β can largely be divided into two broad categories: regulation of cell cycling versus regulation of cell microenvironments (i.e., extracellular matrix (ECM) remodeling, angiogenesis, fibroblast activation, and immune cell infiltration). Thus, in addition to inducing cytostasis in MECs,

TGF-β also governs the behaviors of adjacent fibroblasts, adipocytes, and endothelial cells, as well as their synthesis of paracrine factors and ECM molecules that collectively suppress carcinoma development. During mammary tumorigenesis, the tumor microenvironment readily develops a variety of cellular and structural abnormalities that have a profound impact on TGF-β signaling. For example, targeted inactivation of the TGF-β signaling system in fibroblasts [178] or T cells [179] both elicit carcinoma development due to disruptions of tumor suppressing paracrine signaling networks. Likewise, conditional deletion of TβR-II in mammary gland fibroblasts greatly enhances their proliferative potential and elicits abnormal ductal development [180]. Additionally, TβR-II-deficient mammary fibroblasts promote the growth and invasion of breast cancer cells in part via upregulated expression of TGF-α, MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor) [22, 178, 180, 181]. Similar inactivation of TβR-II in breast cancer cells is observed to promote their metastatic progression through the initiation of two inappropriate paracrine signaling axes-namely, SDF-1:CXCR4 and CXCL5:CXCR2 [150]. The net-effect of these events result in the recruitment of immature $GR1^{+}CD11b^{+}$ myeloid cells that drive breast cancer metastasis by inhibiting host tumor immunosurveillance, and by inducing MMP expression [150]. Collectively, these findings touch upon the importance of the microenvironment and paracrine signaling networks in dictating the pathophysiological activities of TGF-β in the mammary gland. Readers desiring more in-depth analyses and descriptions of the role of fibroblasts in regulating mammary development and tumorigenesis are directed the accompanying review by Moses and colleagues. In the succeeding sections, we highlight the role of fibronectin and collagen in promoting metastatic progression stimulated by TGF-β.

Fibronectin

TGF-β has long been recognized as a principle player operant in stimulating fibronectin expression and incorporation into the ECM [182]. Importantly, elevated fibronectin expression enables dormant MEC micrometastases to reinitiate cell proliferation in a β1 integrin-dependent manner [183]. Along these lines, upregulated fibronectin expression in conjunction with that of activated Ras significantly alters MEC integrin expression profiles in a manner reminiscent of "cadherin switching," such that epithelial α6β4 integrins are replaced by mesenchymal α5β1 integrins. Collectively, these events enhance TGF-β stimulation of EMT and its induction of MEC motility and survival signaling [184]. Interestingly, the ability of TGF-β to bestow anchorage-independent properties to responsive cells can be recapitulated by fibronectin administration, a reaction dependent upon integrin signaling [182]. Fibronectin production is also associated with the formation of the "premetastatic niche," which recruits BDMCs to provide a permissive metastatic microenvironment for the arrival of disseminated breast cancer cells [147, 185]. Finally, a recent study established the importance of TGF-β secreted by mammary tumors to assemble a fibronectin matrix that is three times more mechanically rigid than matrices assembled by normal MECs [186]. Collectively, these findings implicate fibronectin as an essential mediator of metastatic progression stimulated by TGF-β, particularly its ability to respond to signaling inputs derived from integrins and mechanotransduction.

Collagen

In addition to its stimulation of fibronectin expression, TGF-β also has a longstanding relationship with collagen [182], whose elevated expression in mammary tumors associates with increased tumor occurrence and metastasis, as well as with poor clinical outcomes [187, 188]. Increased collagen deposition also promotes ECM rigidity, which contributes greatly to the development and progression of mammary tumors [145, 146]. Interestingly, TGF-β stimulates basal-like breast cancer cells to express Endo180, which is a cell surface receptor that promotes the *(a)* growth and motility of highly invasive breast cancer cells, and *(b)* internalization and intracellular degradation of collagen [189]. Recently, collagen was observed to activate TGF-β receptors in a ligand-independent manner, leading to the dual stimulation of canonical and noncanonical TGF-β effectors in breast cancer cells [101]. Collectively, these findings highlight the intimate relationship between collagen and TGF-β in promoting disease progression in breast cancer patients, and suggest that measures capable of neutralizing upregulated collagen expression and activity may offer new inroads to alleviate oncogenic TGF-β signaling.

Novel Modes of MEC Plasticity Regulated by TGF-β

microRNAs

microRNAs are small, noncoding RNAs that *(a)* typically range from 20 to 25 base pairs in length, and *(b)* play essential roles in suppressing gene expression through their ability to bind to the 3'-UTRs of target mRNAs, which induces either their degradation or translational repression [190]. Interestingly, the majority of miRs identified to date localize to fragile genomic regions associated with cancer [191], leading to the notion that microRNA-based expression signatures may be developed as diagnostic platforms

for cancer patients. Accordingly, microRNA expression profiling studies have demonstrated the ability of microRNA signatures to readily distinguish normal tissues from their tumorigenic counterparts, as well as to stage and classify human mammary tumors [192–194]. Along these lines, differential microRNA expression can function in either suppressing or promoting mammary tumorigenesis, and in governing specific steps of the metastatic cascade, including the induction of MEC migration, invasion, and EMT [195–198]. The pathophysiological processes regulated by miRs are highly reminiscent of those controlled by TGF-β, and as such, it is not surprising to learn that microRNAs are active participants in regulating MEC response to TGF-β. For instance, TGF-β stimulation of EMT down regulates the expression of the miR-200 family of miRs, which normally suppresses the expression of the EMT-responsive transcription factors, ZEB1 and ZEB2/SIP1 [199]. Consequently, elevated ZEB1 and ZEB2 expression initiate EMT in part by repressing E-cadherin expression in breast cancer cells [199]. EMT induced by TGF-β has also been linked to its stimulation of miR-21 expression [200], which enhances the migration and invasion of breast cancers by down regulating the expression of tropomyosin [197, 201]. Clinically, high miR-21 expression in early-stage breast cancers is associated with decreased disease-free survival, as well as with significantly elevated expression of TGF-β [202]. Canonical TGF-β signaling was recently observed to regulate miR-21 activity by enhancing the processing of its pri-miR-21 transcripts to their pre-miR-21 counterparts, a post-translational mechanism supported by the formation of Smad2/3:DROSHA complexes [203]. Finally, canonical TGF-β signaling regulates the differential expression of 28 miRs in MECs undergoing EMT in response to TGF-β. Of these EMT-related miRs, the expression of miR-155 is essential in mediating MEC motility and tight junction dissolution due to a loss of RhoA expression in transitioning MECs [127]. Collectively, these studies demonstrate the role of TGF-β in directing microRNA expression in normal and malignant MECs, doing so via employment of intricate transcriptional and post-translational mechanisms. Future studies need to comprehensively map the microRNAome governed by TGF-β, as well as the extent to which targeting microRNA expression can abrogate metastatic progression stimulated by TGF-β in developing mammary tumors.

Acquisition of Stemness

Heterogeneity in cell morphology, gene signatures, and sensitivity to chemotherapeutics are all hallmarks of various human cancers, including those of the breast [204–207]. The phenotypic changes associated with oncogenic Type 3 EMT can give rise to the generation and expansion of cancer-initiating cells that possess stem cell-like characteristics [29, 208], including the ability to undergo self-renewal and asymmetrical cell division [204, 205, 209]. At present, the origins of cancer stem cells (CSCs) remain controversial; however, recent findings do suggest that CSCs may arise through their ability to commandeer a core set of transcription factors during the malignant transformation of normal stem cells, or during the dedifferentiation of bulk tumor cells ([Fig. 2; [210–214]). Transcription factors underlying cellular reprogramming coupled to pluripotency include Nanog, Oct4, Sox2, Klf4, and c-Myc [215], all of which are regulated by TGF-β [216–219].

With respect to breast cancer, recent studies have identified a small CSC population that possesses stem cell-like properties and exhibits a $CD44^{high}/CD24^{low}$ antigenic phenotype [209]. Interestingly, terminally differentiated luminal MECs readily express ER-α and CD24, which contrasts sharply with their mammary progenitor-like counterparts that express CD44, but not ER-α [30]. When stimulated by TGF-β or in response to constitutive expression of either Twist or Snail, MECs undergo an EMT program that generates a $CD44^{high}/CD24^{low}$population of cells that possess stem cell-like and mammosphere forming features [29]. The loss of E-cadherin expression that transpires during EMT reinforces these events by permitting the nuclear translocation of β-catenin and its stimulation of CD44 expression [220]. Moreover, pharmacological inhibition of TGF-β signaling in breast CSCs suppresses their tumorigenicity in part by restoring E-cadherin expression via mesenchymal-epithelial transitions [30]. Interestingly, genomic analysis of primary breast tumors identified clonal genetic differences existing between $CD44^{high}/CD24^{low}$ and $CD44^{low}/CD24^{high}$ breast cancer cells, suggesting that these malignant MEC populations undergo independent evolutionary routes during mammary tumorigenesis. The importance of TGF-β signaling, particularly that mediated by the stem cell markers and noncanonical TGF-β effectors β1 (CD29) and β3 (CD61) integrins, in regulating CSC evolution is supported by the finding that TβR-II is readily expressed in $CD44^{high}/CD24^{low}$ populations as compared to their $CD44^{low}/CD24^{high}$counterparts, which transcriptionally silences this TGF-β receptor [30]. Future studies need to establish the role of "stemness" in manifesting the "TGF-β Paradox" and its initiation of oncogenic TGF-β signaling during mammary tumorigenesis.

Unanswered Questions and Future Directions

Mammary Tumor Heterogeneity and Metastatic Progression

Mammary tumor microenvironments play an essential role governing the growth, metastasis, and chemosensitivity of

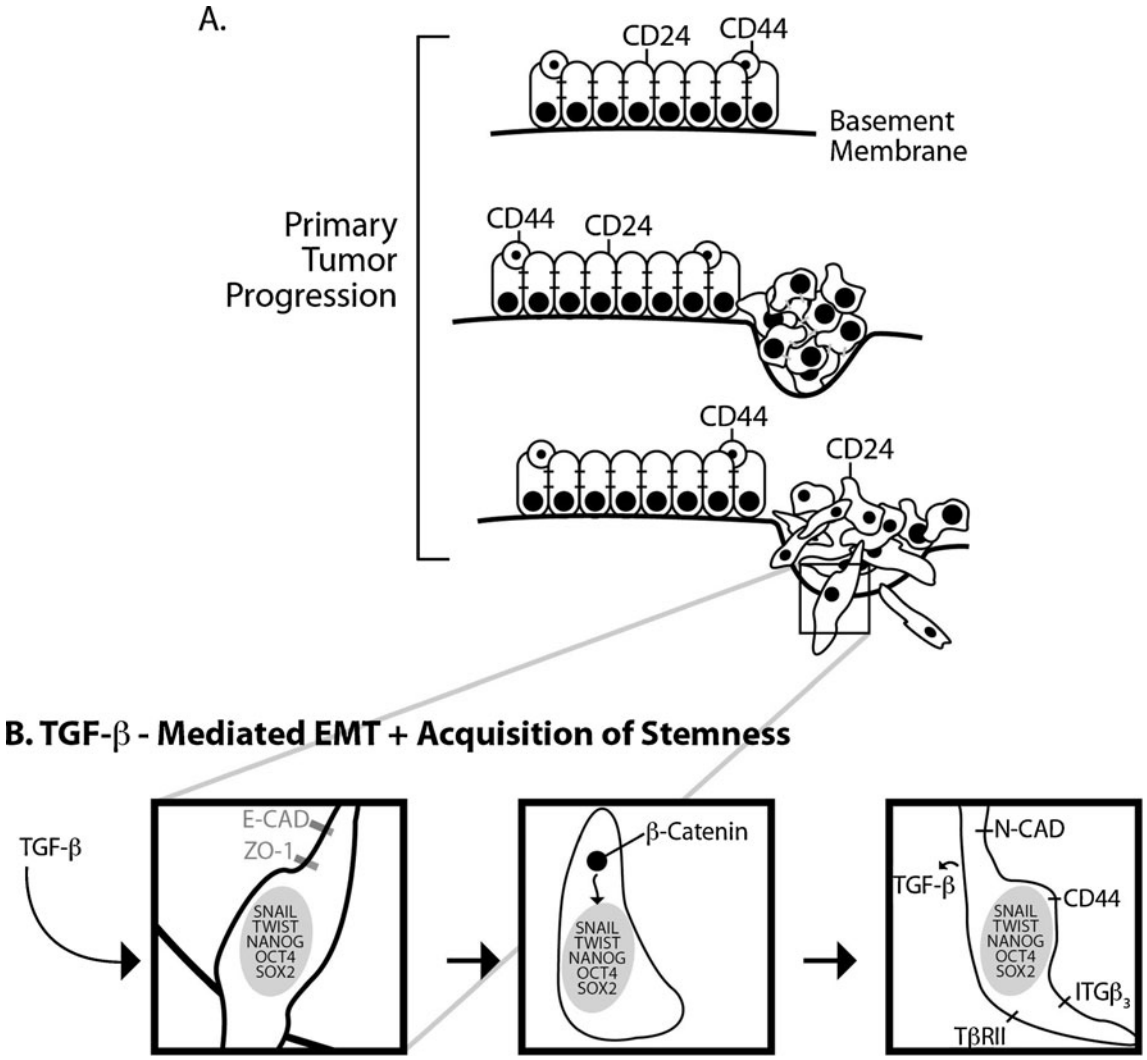


Figure 2 The exodus of invasive breast cancer cells from the primary tumor may require EMT and its induction of acquired stemness. **a** Normal mammary epithelium is comprised of CD24+ luminal cells and CD44+ basal cells (*top panel*). Tumor initiation in either cell type may confer a proliferative advantage and deregulated growth *(middle panel)*. Disease progression ultimately results in the local invasion of breast cancer cells, followed by their dissemination into the circulation *(bottom panel)*. **b** Metastatic progression stimulated by TGF-β is coupled to EMT and its ability to *(a)* induce the expression of EMT- and developmentally-responsive transcription factors *(left panel)*; *(b)* promote the dissolution of adherens and tight junctional complexes, as well as the downregulation of epithelial phenotypic markers *(middle panel)*; and *(c)* stimulate the upregulation of mesenchymal and stem-like phenotypic markers, including CD44.

developing breast cancers [22, 181], as does the relative heterogeneity and clonality displayed by individual carcinoma cells housed within evolving mammary tumors [221]. The ability of MECs to undergo "integrin switching" during EMT and metastatic progression stimulated by TGF-β implicates integrins as master regulators in coordinating the interactions between reactive stroma and malignant MECs [85–87, 184]. Moreover, changes in integrin expression not only dictate the coupling of TGF-β to its canonical and noncanonical signaling systems [49, 85–89], but also govern the ability of malignant MECs to respond and interact with components in their microenvironments, including reactive fibroblasts, adipocytes, lymphocytes, and endothelial cells [22, 181]. Collectively, these dynamic interactions coalesce in aiding MECs to successfully navigate the metastatic cascade and establish secondary lesions at distant organ sites. Simultaneous stimulation of αvβ3 integrin (by vitronectin) and TGF-β receptors is essential in mediating the acquisition of EMT and invasive phenotypes by normal and malignant MECs, and in promoting metastatic progression in triple-negative breast cancer cells [49, 85–88]. Thus, differential vitronectin expression within tumor microenvironments and throughout the periphery may dictate the preferential metastasis of breast cancer cells to the liver, lung, brain, and bone based on integrin expression profiles [7]. The importance of β3 integrin to these events is underscored by our recent observation that rendering metastatic MECs deficient in

β1 integrin actually enhances, not suppresses, metastatic progression at earlier stages of tumor development (J.P. Parvani and W.P. Schiemann, *unpublished observation*). Interestingly, targeted inactivation of β3 integrin expression in these same cells abrogates TGF-β stimulation of pulmonary metastasis [87]. Thus, while MECs possess considerable plasticity related to their integrin expression profiles, it appears that only a select repertoire of integrins may in fact couple TGF-β to metastatic progression in mammary tumors. Future studies need to establish the repertoire of integrins operant in mediating canonical TGF-β signaling and its cytostatic function versus those integrins operant in mediating oncogenic TGF-β signaling and its aberrantly amplified activation of noncanonical TGF-β effectors.

Investigating the role of integrins during cell:cell or cell:ECM interactions is complicated by the intratumoral heterogeneity of carcinoma cells within primary mammary tumors, of which only a minute fraction are actually endowed with the abilities to survive the metastatic cascade (Fig. 3; [204, 222]). Thus, analyzing the interactions of bulk tumors with their surrounding ECM and microenvironment may be misleading and unrepresentative of the reactions necessary in driving the egress of carcinoma cells out of these dysregulated microenvironments. Along these lines, the extent to which symbiotic relationships between epithelial-like and mesenchymal-like MECs cooperate in mediating completion of the metastatic cascade remain largely unexplored. For instance, in studying several human and murine isogeneic breast cancer progression series [222–224], we repeatedly identify weakly tumorigenic derivatives that are highly invasive as compared to their more malignant counterparts that are surprisingly noninvasive (M.K Wendt and W.P. Schiemann, *unpublished observation*). Thus, do weakly tumorigenic and highly invasive MECs generate a readily available route for their weakly invasive counterparts to escape the confines of the primary tumor? And if so, do these "worker cells" traverse the circulation in tandem with their metastatic "queen cells" to facilitate their efficient exodus from the vasculature at suitable metastatic niches? And finally, what role does TGF-β and its noncanonical effectors play in regulating these collective events (Fig. 3). Although future studies need to address these questions, several recent studies do support a collective and collaborative effort in facilitating breast cancer metastasis. First, epithelial-like (non-EMT and noninvasive) and mesenchymal-like (EMT and invasive) MECs were singularly unable to colonize the lungs of mice; however, engrafting both lines simultaneously resulted in robust pulmonary metastasis [225], suggesting that both MEC populations cooperate to complete the metastatic cascade. Along these lines, indolent breast micrometastases can be reactivated by BMDCs mobilized into the circulation by systemic instigation derived from signals (e.g., osteopontin) originating from the primary mammary tumor [226]. Finally, aggressive circulating breast cancer cells have been observed to "self-seed" and infiltrate established tumors, thereby accelerating metastatic progression in recipient mammary tumors [227]. Clearly, future studies need to expand these findings and determine the extent to which these events are either regulated by TGF-β or participate in manifesting the "TGF-β Paradox."

Smad2/3 Signaling and Metastasis: Prominent Players or Innocent Bystanders?

Although Smads 2 and 3 are often referred to as being a single entity in the scientific literature, recent findings have established distinct roles for Smad2 versus Smad3 in mediating the pathophysiology of TGF-β. For instance, targeted deletion of Smad2 elicits embryonic lethality [228, 229], while Smad3-deficient mice are viable and are highly susceptible to inflammation-induced colon tumorigenesis [230–232]. Along these lines, the gene expression profiles coupled specifically to Smad2 activation are readily distinguishable from those coupled to the activation of Smad3 [233–235], findings that offer unique insights into how TGF-β modulates cell growth, motility, and survival [236]. With respect to cancers of the breast, experimental inactivation of Smad2 augments breast cancer metastasis to bone, while similar inactivation of Smad3 attenuates this metastatic response by reducing tumor angiogenesis and VEGF expression [237]. Likewise, rendering early-stage breast cancer cells deficient in Smad2/3 signaling enhances their malignancy, while similar manipulations in their late-stage counterparts prevents these breast cancer cells from colonizing the lungs [238, 239]. Interestingly, a TβR-I mutant unable to activate Smad2/3 fails to support the ability of breast cancer cells to colonize the lung [240], suggesting that canonical and noncanonical TGF-β signaling inputs coalesce in facilitating metastatic outgrowth. At present, the precise molecular mechanisms that enable these disparate signaling systems to collaborate in promoting metastatic progression by TGF-β remains unclear. Addressing this question is critical to unraveling the mysteries of the "TGF-β Paradox." For instance, a recent study demonstrated that the administration of bisphosphonates was only effective in suppressing osteolytic bone lesions and canonical TGF-β signaling at early stages of the metastatic process, not in fully established lesions [241]. These findings indicate that canonical TGF-β signaling is differentially regulated during specific stages of the metastatic cascade. Accordingly, transient activation of Smad2/3 by TGF-β converted the migration of breast cancer cells from cohesive to single cell programs [242, 243]. Quite intriguingly, reinitiation of proliferation programs

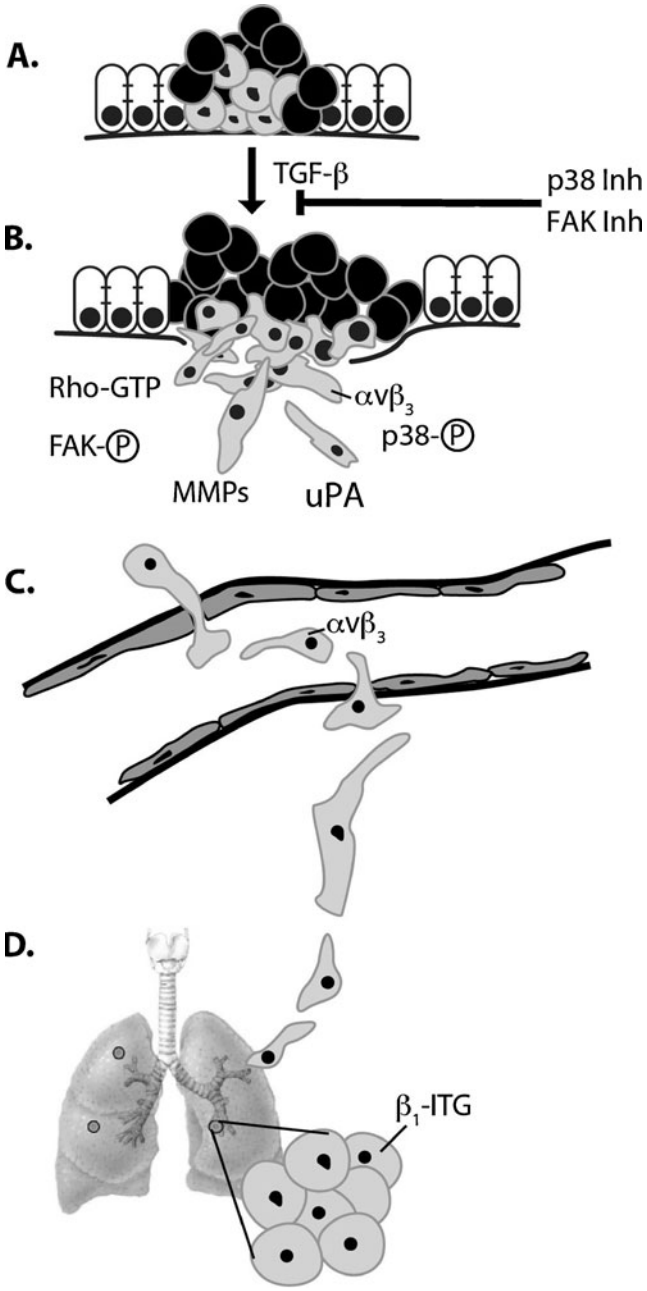


Figure 3 Cooperation between distinct subpopulations of breast cancer cells may underlie local invasion and metastasis. **a** Carcinoma cell heterogeneity is a hallmark of primary mammary tumors and represented as "gray" and "black" breast cancer cells. **b** TGF-β signaling within the primary tumor microenvironment induces a subpopulation of breast cancer cells to undergo EMT and local invasion (*gray* cells), doing so via the activation of αvβ3 integrin, FAK, p38 MAPK, and Rho GTPases and their coupling to the production of MMPs and uPA. The efficiency of these events may be enhanced and bolstered by the cooperation of nonmetastatic cells (black cells) to produce "prometastatic" microenvironments. **c** Once liberated from the primary tumor, metastatic breast cancer cells intravasate the endothelium to gain access to the vasculature for systemic dissemination. Surviving transport through the circulatory system is essential for the metastatic cascade and may be linked to upregulated expression of αvβ3 integrin, which is also essential for disseminated breast cancer cells to extravasate the vasculature. **d** Colonization and eventual outgrowth of the metastatic lesion is associated with β1 integrin expression.

necessary for pulmonary metastatic outgrowth of these breast cancer cells required them to first inactivate Smad2/3 signaling [242, 243]. Thus, it is tempting to speculate that altered elastic moduli govern the coupling of TGF-β to Smad2/3. In support of this supposition, we recently determined that canonical TGF-β signaling is selectively silenced in *(a)* compliant 3D-organotypic cultures relative to rigid tissue culture plastic, and *(b)* pulmonary metastases relative to their site of origin (M.A. Taylor, M.K. Wendt and W.P. Schiemann, *unpublished observation*). Taken together, these intriguing findings demonstrate the plasticity present in the TGF-β signaling system as carcinoma cells undergo EMT and metastatic outgrowth, presumably reflecting a shift from canonical (i.e., Smad2/3-based) to noncanonical (i.e., non-Smad2/3-based) signaling that originates from altered mechanotransduction and integrin signaling. Future studies need to define which stages of the metastatic process are dependent upon TGF-β signaling, as well as delineate which branches of the TGF-β signaling system engender these deadly events.

The Future of TGF-β Targeted Chemotherapies

The challenge in designing pharmaceuticals effective in targeting TGF-β signaling lies in the ability of these agents to circumvent the principles of the "TGF-β Paradox." For instance, an ideal anti-TGF-β agent would be expected to specifically inactivate the oncogenic activities of TGF-β, while simultaneously preserving and/or enhancing its tumor suppressing functions. Currently, all chemotherapeutics developed against the TGF-β pathway universally function as pan-TGF-β antagonists, and as such, the preclinical and clinical use of these drugs has been shown to enhance the tumorigenicity of early-stage cancers as predicted by the "TGF-β Paradox" [24]. We previously hypothesized that the specific targeting of noncanonical TGF-β effectors might provide a novel means to restore its cytostatic function in developing mammary tumors [24]. In support of this supposition, we recently discovered three novel noncanonical TGF-β signaling axes that drive EMT and metastatic progression by TGF-β: *(a)* αvβ3 integrin:FAK:p130Cas:Src:pY284-TβR-II:Grb2:p38 MAPK [49, 85–88]; *(b)*TAB1:TAK1:IKKβ:xIAP:NF-κB [73, 74]; and *(c)* Cox-2:PGE2:EP2 [72, 75]. Importantly, preventing TGF-β from activating any of these pathways is sufficient in alleviating its metastatic behavior, as well as in partially reinstating its cytostatic function in breast cancer cells. Future studies need to gauge the extent to which these events can be translated into clinical settings to prevent breast cancer metastasis and disease recurrence.

We also envision two additional approaches to offer new opportunities to selectively inhibit noncanonical TGF-β signaling. First, the activation of mechanotransduction by ECM rigidity clearly plays an important role in promoting breast cancer development and progression [145, 147, 244–246], including that stimulated by TGF-β [90]. These findings suggest that pharmacological targeting of the tumor microenvironment and its accompanying desmoplastic reactions may provide novel avenues to treat metastatic progression stimulated by TGF-β. Indeed, co-administration of LOX and integrin inhibitors may simultaneously abrogate the oncogenic activities of TGF-β and mechanotransduction in developing mammary tumors. Along these lines, the use of iRGD peptides directed at αv integrins to deliver chemotherapeutics has also proven effective in enhancing drug penetration into breast cancers [247]. Thus, employment of iRGD peptides for the delivery of anti-TGF-β agents to mammary tumors may facilitate the specific inactivation of TGF-β signaling at the invasive front, thereby reducing metastatic burden. Second, the development of microRNA-based therapies may permit the fine-tuning of TGF-β behavior by targeting the MicroRNAome in mammary tumors. Indeed, tumor progression has been inhibited in a variety of preclinical models by overexpressing tumor suppressive miRs [248–250], or by neutralizing the activities of oncogenic miRs [251–254]. Given the role of microRNAs in mediating EMT and TGF-β signaling, it stands to reason that identifying the MicroRNAome regulated by TGF-β during its induction of metastatic progression may offer new inroads to enhance the overall survival of breast cancer patients.

Acknowledgements We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651), while M.A.T. was supported by the Department of Defense (BC093128).

Financial Support W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), and the Department of Defense (BC084651), while M.A.T. was supported by the Department of Defense (BC093128).

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin. 2010;60(5):277–300.
2. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009;9(4):274–84.
3. Nguyen DX, Massague J. Genetic determinants of cancer metastasis. Nat Rev Genet. 2007;8(5):341–52.
4. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell. 2008;14(6):818–29.
5. Taylor MA, Parvani JG, Schiemann WP. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia. 2010;15(2):169–90.
6. Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminous R, Waldron W, et al. SEER Cancer Statistics Review 1975–1007. National Cancer Institute. 2010.
7. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol. 2010;28(20):3271–7.
8. Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med. 2008;359(26):2814–23.
9. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002;2(8):563–72.
10. Weinstat-Saslow D, Steeg PS. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. FASEB J. 1994;8(6):401–7.
11. Scheel C, Onder T, Karnoub A, Weinberg RA. Adaptation versus selection: the origins of metastatic behavior. Cancer Res. 2007;67(24):11476–9.
12. Talmadge JE. Clonal selection of metastasis within the life history of a tumor. Cancer Res. 2007;67(24):11471–5.
13. Talmadge JE, Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 2010;70(14):5649–69.
14. Spratt JS, Spratt JA. Growth rates. In: Donegan WL editor. Cancer of the Breast. Spratt, J.S.; 2002. p. 443–476.
15. Gupta GP, Massague J. Cancer metastasis: building a framework. Cell. 2006;127(4):679–95.
16. Li F, Tiede B, Massague J, Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. Cell Res. 2007;17(1):3–14.
17. Serra R, Crowley MR. TGF-β in mammary gland development and breast cancer. Breast Dis. 2003;18:61–73.
18. Serra R, Crowley MR. Mouse models of transforming growth factor β impact in breast development and cancer. Endocr Relat Cancer. 2005;12(4):749–60.
19. Tian M, Schiemann WP. The TGF-β paradox in human cancer: an update. Future Oncol. 2009;5(2):259–71.
20. Barcellos-Hoff MH, Akhurst RJ. Transforming growth factor-β in breast cancer: too much, too late. Breast Cancer Res. 2009;11(1):202.
21. Tian M, Neil JR, Schiemann WP. Transforming growth factor-β and the hallmarks of cancer. Cell Signal. 2010; PMID: 20940046.
22. Bierie B, Moses HL. Tumour microenvironment: TGFβ: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer. 2006;6(7):506–20.
23. Rahimi RA, Leof EB. TGF-β signaling: a tale of two responses. J Cell Biochem. 2007;102(3):593–608.
24. Schiemann WP. Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? Expert Rev Anticancer Ther. 2007;7(5):609–11.
25. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell. 2010;141(1):69–80.
26. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA. 2010;107(35):15449–54.
27. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA. 2009;106(33):13820–5.

28. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med. 2009;15(1):68–74.
29. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704–15.
30. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell. 2007;11(3):259–73.
31. Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer. 2007;7(2):139–47.
32. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. N Engl J Med. 2000;342 (18):1350–8.
33. Massague J. TGF-β signal transduction. Annu Rev Biochem. 1998;67:753–91.
34. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocr Rev. 2002;23(6):787–823.
35. Nishimura SL. Integrin-mediated transforming growth factor-β activation, a potential therapeutic target in fibrogenic disorders. Am J Pathol. 2009;175(4):1362–70.
36. Rifkin DB. Latent transforming growth factor-β (TGF-β) binding proteins: orchestrators of TGF-β availability. J Biol Chem. 2005;280(9):7409–12.
37. Massague J, Gomis RR. The logic of TGFβ signaling. FEBS Lett. 2006;580(12):2811–20.
38. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev. 2005;19(23):2783–810.
39. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFβreceptor. Cell. 1998;95(6):779–91.
40. Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol. 2000;20(24):9346–55.
41. Faresse N, Colland F, Ferrand N, Prunier C, Bourgeade MF, Atfi A. Identification of PCTA, a TGIF antagonist that promotes PML function in TGF-β signalling. EMBO J. 2008;27 (13):1804–15.
42. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the transforming growth factor β receptors to the Smad pathway. EMBO J. 2001;20(11):2789–801.
43. Penheiter SG, Singh RD, Repellin CE, Wilkes MC, Edens M, Howe PH, et al. Type II transforming growth factor-β receptor recycling is dependent upon the clathrin adaptor protein Dab2. Mol Biol Cell. 2010;21(22):4009–19.
44. Runyan CE, Hayashida T, Hubchak S, Curley JF, Schnaper HW. Role of SARA (SMAD anchor for receptor activation) in maintenance of epithelial cell phenotype. J Biol Chem. 2009;284(37):25181–9.
45. Chaudhury A, Hussey GS, Ray PS, Jin G, Fox PL, Howe PH. TGF-β-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. Nat Cell Biol. 2010;12(3):286–93.
46. Hocevar BA, Prunier C, Howe PH. Disabled-2 (Dab2) mediates transforming growth factor β (TGFβ)-stimulated fibronectin synthesis through TGFβ-activated kinase 1 and activation of the JNK pathway. J Biol Chem. 2005;280(27):25920–7.
47. Hussey GS, Chaudhury A, Dawson AE, Lindner DJ, Knudsen CR, Wilce MC, et al. Identification of an mRNP complex regulating tumorigenesis at the translational elongation step. Mol Cell. 2011;41(4):419–31.
48. Kim W, Seok Kang Y, Soo Kim J, Shin NY, Hanks SK, Song WK. The integrin-coupled signaling adaptor p130Cas suppresses Smad3 function in transforming growth factor-β signaling. Mol Biol Cell. 2008;19(5):2135–46.
49. Wendt MK, Smith JA, Schiemann WP. p130Cas is required for mammary tumor growth and transforming growth factor-β-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem. 2009;284(49):34145–56.
50. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, et al. The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell. 1997;89:1165–73.
51. Nakao A, Afrakht M, Moren A, Nakayama T, Christian JL, Heuchel R, et al. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature. 1997;389:631–5.
52. Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL, et al. Physical and functional interaction of murine and xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-β receptors. J Biol Chem. 1998;273:25364–70.
53. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem. 2001;276(16):12477–80.
54. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. Mol Cell. 2000;6(6):1365–75.
55. Datta PK, Moses HL. STRAP and Smad7 synergize in the inhibition of transforming growth factor β signaling. Mol Cell Biol. 2000;20(9):3157–67.
56. Ibarrola N, Kratchmarova I, Nakajima D, Schiemann WP, Moustakas A, Pandey A, et al. Cloning of a novel signaling molecule, AMSH-2, that potentiates transforming growth factor β signaling. BMC Cell Biol. 2004;5:2.
57. Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, et al. Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7. EMBO J. 2003;22 (24):6458–70.
58. Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, et al. PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. Cell. 2006;125(5):915–28.
59. Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-β signaling. J Biol Chem. 2000;275 (47):36818–22.
60. Lo RS, Massague J. Ubiquitin-dependent degradation of TGF-β-activated Smad2. Nat Cell Biol. 1999;1(8):472–8.
61. Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, et al. Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol Biol Cell. 2001;12(5):1431–43.
62. Izzi L, Attisano L. Regulation of the TGFβ signalling pathway by ubiquitin-mediated degradation. Oncogene. 2004;23 (11):2071–8.
63. Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFβ signaling, controls Smad4 monoubiquitination. Cell. 2009;136(1):123–35.
64. Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. Nat Genet. 2001;29(2):117–29.
65. Massague J. TGFβ in cancer. Cell. 2008;134(2):215–30.
66. Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-β family proteins in development and disease. Nat Cell Biol. 2007;9(9):1000–4.
67. Piek E, Moustakas A, Kurisaki A, Heldin CH, ten Dijke P. TGF-β type I receptor/ALK-5 and Smad proteins mediate epithelial to

mesenchymal transdifferentiation in NMuMG breast epithelial cells. J Cell Sci. 1999;112:4557–68.
68. Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell. 2005;16(4):1987–2002.
69. Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol. 2009;5(8):1145–68.
70. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-Ras and Smad2 levels. Nat Cell Biol. 2002;4(7):487–94.
71. Lehmann K, Janda E, Pierreux CE, Rytomaa M, Schulze A, McMahon M, et al. Raf induces TGFβ production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. Genes Dev. 2000;14(20):2610–22.
72. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis. 2008;29(11):2227–35.
73. Neil JR, Schiemann WP. Altered TAB1:IκB kinase interaction promotes transforming growth factor β-mediated nuclear factor-κB activation during breast cancer progression. Cancer Res. 2008;68(5):1462–70.
74. Neil JR, Tian M, Schiemann WP. x-linked inhibitor of apoptosis protein and its E3 ligase activity promote transforming growth factor-β-mediated nuclear factor-κB activation during breast cancer progression. J Biol Chem. 2009;284(32):21209–17.
75. Tian M, Schiemann WP. PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J. 2010;24(4):1105–16.
76. Kretzschmar M, Doody J, Timokhina I, Massague J. A mechanism of repression of TGFβ/ Smad signaling by oncogenic Ras. Genes Dev. 1999;13(7):804–16.
77. Funaba M, Zimmerman CM, Mathews LS. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J Biol Chem. 2002;277(44):41361–8.
78. Brown JD, DiChiara MR, Anderson KR, Gimbrone Jr MA, Topper JN. MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. J Biol Chem. 1999;274(13):8797–805.
79. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J Biol Chem. 1999;274(52):37413–20.
80. Waddell DS, Liberati NT, Guo X, Frederick JP, Wang XF. Casein kinase Iepsilon plays a functional role in the transforming growth factor-β signaling pathway. J Biol Chem. 2004;279(28):29236–46.
81. Yakymovych I, Ten Dijke P, Heldin CH, Souchelnytskyi S. Regulation of Smad signaling by protein kinase C. FASEB J. 2001;15(3):553–5.
82. Wicks SJ, Lui S, Abdel-Wahab N, Mason RM, Chantry A. Inactivation of smad-transforming growth factor β signaling by Ca(2+)-calmodulin-dependent protein kinase II. Mol Cell Biol. 2000;20(21):8103–11.
83. Guo X, Ramirez A, Waddell DS, Li Z, Liu X, Wang XF. Axin and GSK3βcontrol Smad3 protein stability and modulate TGF-βsignaling. Genes Dev. 2008;22(1):106–20.
84. Allington TM, Galliher-Beckley AJ, Schiemann WP. Activated Abl kinase inhibits oncogenic transforming growth factor-β signaling and tumorigenesis in mammary tumors. FASEB J. 2009;23(12):4231–43.
85. Galliher AJ, Schiemann WP. β3 integrin and Src facilitate transforming growth factor-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res. 2006;8(4):R42.
86. Galliher AJ, Schiemann WP. Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res. 2007;67(8):3752–8.
87. Galliher-Beckley AJ, Schiemann WP. Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis. 2008;29(2):244–51.
88. Wendt MK, Schiemann WP. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. Breast Cancer Res. 2009;11(5):R68.
89. Wendt MK, Smith JA, Schiemann WP. Transforming growth factor-β-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene. 2010;29(49):6485–98.
90. Taylor MA, Amin J, Kirschmann DA, Schiemann WP. Lysyl oxidase and hydrogen peroxide promote oncogenic signaling by transforming growth factor-βin mammary epithelial cells. Neoplasia. 2011;13:in press.
91. Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP. Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-βin mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis. 2008;29 (12):2243–51.
92. Moustakas A, Heldin CH. Non-Smad TGF-βsignals. J Cell Sci. 2005;118(Pt 16):3573–84.
93. Kang JS, Liu C, Derynck R. New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol. 2009;19(8):385–94.
94. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer. 2010;10(1):9–22.
95. Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. Dev Cell. 2010;18 (6):884–901.
96. Zutter MM. Integrin-mediated adhesion: tipping the balance between chemosensitivity and chemoresistance. Adv Exp Med Biol. 2007;608:87–100.
97. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, et al. The integrin αvβ6 binds and activates latent TGFβ1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 1999;96(3):319–28.
98. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, et al. The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. J Cell Biol. 2002;157(3):493–507.
99. Sloan EK, Pouliot N, Stanley KL, Chia J, Moseley JM, Hards DK, et al. Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. Breast Cancer Res. 2006;8(2):R20.
100. Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, et al. Inhibition of pulmonary and skeletal metastasis by a transforming growth factor-β type I receptor kinase inhibitor. Cancer Res. 2006;66(13):6714–21.
101. Garamszegi N, Garamszegi SP, Samavarchi-Tehrani P, Walford E, Schneiderbauer MM, Wrana JL, et al. Extracellular matrix-induced transforming growth factor-β receptor signaling dynamics. Oncogene. 2010;29(16):2368–80.
102. van der Flier S, Chan CM, Brinkman A, Smid M, Johnston SR, Dorssers LC, et al. BCAR1/p130Cas expression in untreated and acquired tamoxifen-resistant human breast carcinomas. Int J Cancer. 2000;89(5):465–8.
103. Ta HQ, Thomas KS, Schrecengost RS, Bouton AH. A novel association between p130Cas and resistance to the chemotherapeutic drug adriamycin in human breast cancer cells. Cancer Res. 2008;68(21):8796–804.

104. Cabodi S, Tinnirello A, Bisaro B, Tornillo G, del Pilar Camacho-Leal M, Forni G, et al. p130Cas is an essential transducer element in ErbB2 transformation. FASEB J. 2010;24(10):3796–808.
105. Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, et al. p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis. Cancer Res. 2006;66(9):4672–80.
106. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin β1 signaling is necessary for transforming growth factor-β activation of p38MAPK and epithelial plasticity. J Biol Chem. 2001;276(50):46707–13.
107. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med. 1999;222(2):124–38.
108. Weaver VM, Lelievre S, Lakins JN, Chrenek MA, Jones JC, Giancotti F, et al. β4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell. 2002;2(3):205–16.
109. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009;9(8):550–62.
110. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000;275(47):36803–10.
111. Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. Oncogene. 2005;24(28):4580–7.
112. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest. 2006;116(6):1561–70.
113. Uttamsingh S, Bao X, Nguyen KT, Bhanot M, Gong J, Chan JL, et al. Synergistic effect between EGF and TGF-β1 in inducing oncogenic properties of intestinal epithelial cells. Oncogene. 2008;27(18):2626–34.
114. Lamouille S, Derynck R. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J Cell Biol. 2007;178(3):437–51.
115. Conery AR, Cao Y, Thompson EA, Townsend Jr CM, Ko TC, Luo K. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-β induced apoptosis. Nat Cell Biol. 2004;6(4):366–72.
116. Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-β signalling through a direct interaction with Smad3. Nat Cell Biol. 2004;6(4):358–65.
117. Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res. 2009;19(2):156–72.
118. Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol. 2009;21(2):166–76.
119. Hall A. Rho GTPases and the control of cell behaviour. Biochem Soc Trans. 2005;33(Pt 5):891–5.
120. Hall A. The cytoskeleton and cancer. Cancer Metastasis Rev. 2009;28(1–2):5–14.
121. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell. 2001;12(1):27–36.
122. Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, et al. A role for the TGFβ-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci USA. 2009;106(33):14028–33.
123. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science. 2005;307(5715):1603–9.
124. Hutchison N, Hendry BM, Sharpe CC. Rho isoforms have distinct and specific functions in the process of epithelial to mesenchymal transition in renal proximal tubular cells. Cell Signal. 2009;21(10):1522–31.
125. He Y, Northey JJ, Primeau M, Machado RD, Trembath R, Siegel PM, et al. CdGAP is required for transforming growth factor β- and Neu/ErbB-2-induced breast cancer cell motility and invasion. Oncogene. 2010;30(9):1032–45.
126. Mythreye K, Blobe GC. The type III TGF-β receptor regulates epithelial and cancer cell migration through β-arrestin2-mediated activation of Cdc42. Proc Natl Acad Sci USA. 2009;106(20):8221–6.
127. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, et al. MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol. 2008;28(22):6773–84.
128. Valastyan S, Benaich N, Chang A, Reinhardt F, Weinberg RA. Concomitant suppression of three target genes can explain the impact of a microRNA on metastasis. Genes Dev. 2009;23(22):2592–7.
129. Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, Wang ZC, et al. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell. 2009;137(6):1032–46.
130. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor β-mediated signaling. J Biol Chem. 1997;272(3):1429–32.
131. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci. 2002;115(Pt 15):3193–206.
132. Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. Neoplasia. 2004;6(5):603–10.
133. Shintani Y, Hollingsworth MA, Wheelock MJ, Johnson KR. Collagen I promotes metastasis in pancreatic cancer by activating c-Jun NH(2)-terminal kinase 1 and up-regulating N-cadherin expression. Cancer Res. 2006;66(24):11745–53.
134. Shintani Y, Wheelock MJ, Johnson KR. Phosphoinositide-3 kinase-Rac1-c-Jun NH2-terminal kinase signaling mediates collagen I-induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells. Mol Biol Cell. 2006;17(7):2963–75.
135. Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, et al. The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol. 2008;10(10):1199–207.
136. Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol Cell. 2008;31(6):918–24.
137. Karin M. Nuclear factor-κB in cancer development and progression. Nature. 2006;441(7092):431–6.
138. Sovak MA, Arsura M, Zanieski G, Kavanagh KT, Sonenshein GE. The inhibitory effects of transforming growth factor β1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-κB/Rel expression. Cell Growth Differ. 1999;10(8):537–44.
139. Arsura M, Wu M, Sonenshein GE. TGF β1 inhibits NF-κB/Rel activity inducing apoptosis of B cells: transcriptional activation of IκBα. Immunity. 1996;5(1):31–40.

140. You HJ, How T, Blobe GC. The type III transforming growth factor-β receptor negatively regulates nuclear factor κB signaling through its interaction with β-arrestin2. Carcinogenesis. 2009;30(8):1281–7.
141. Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-κB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene. 2007;26(5):711–24.
142. Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, et al. NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest. 2004;114(4):569–81.
143. Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. J Cell Biochem. 2003;88(4):660–72.
144. Payne SL, Hendrix MJ, Kirschmann DA. Paradoxical roles for lysyl oxidases in cancer–a prospect. J Cell Biochem. 2007;101 (6):1338–54.
145. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. Cancer Cell. 2005;8(3):241–54.
146. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891–906.
147. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell. 2009;15(1):35–44.
148. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature. 2006;440(7088):1222–6.
149. Payne SL, Hendrix MJ, Kirschmann DA. Lysyl oxidase regulates actin filament formation through the p130(Cas)/Crk/DOCK180 signaling complex. J Cell Biochem. 2006;98(4):827–37.
150. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+ CD11b+ myeloid cells that promote metastasis. Cancer Cell. 2008;13(1):23–35.
151. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci. 2007;98(10):1512–20.
152. Tomaskovic-Crook E, Thompson EW, Thiery JP. Epithelial to mesenchymal transition and breast cancer. Breast Cancer Res. 2009;11(6):213.
153. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420–8.
154. Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, et al. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell. 2008;133(1):66–77.
155. Furuse M. Molecular basis of the core structure of tight junctions. Cold Spring Harb Perspect Biol. 2010;2(1):a002907.
156. Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochim Biophys Acta. 2009;1788 (4):872–91.
157. Ebnet K, Suzuki A, Ohno S, Vestweber D. Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci. 2004;117(Pt 1):19–29.
158. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol. 2004;286(6):C1213–28.
159. Itoh M, Bissell MJ. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. J Mammary Gland Biol Neoplasia. 2003;8 (4):449–62.
160. Lacher MD, Tiirikainen MI, Saunier EF, Christian C, Anders M, Oft M, et al. Transforming growth factor-β receptor inhibition enhances adenoviral infectability of carcinoma cells via up-regulation of Coxsackie and Adenovirus Receptor in conjunction with reversal of epithelial-mesenchymal transition. Cancer Res. 2006;66(3):1648–57.
161. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition. Nat Cell Biol. 2009;11(8):943–50.
162. Grande M, Franzen A, Karlsson JO, Ericson LE, Heldin NE, Nilsson M. Transforming growth factor-β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. J Cell Sci. 2002;115(Pt 22):4227–36.
163. Shiou SR, Singh AB, Moorthy K, Datta PK, Washington MK, Beauchamp RD, et al. Smad4 regulates claudin-1 expression in a transforming growth factor-β-independent manner in colon cancer cells. Cancer Res. 2007;67(4):1571–9.
164. Barrios-Rodiles M, Brown KR, Ozdamar B, Bose R, Liu Z, Donovan RS, et al. High-throughput mapping of a dynamic signaling network in mammalian cells. Science. 2005;307 (5715):1621–5.
165. Murphy SJ, Dore JJ, Edens M, Coffey RJ, Barnard JA, Mitchell H, et al. Differential trafficking of transforming growth factor-β receptors and ligand in polarized epithelial cells. Mol Biol Cell. 2004;15(6):2853–62.
166. Yakovich AJ, Huang Q, Du J, Jiang B, Barnard JA. Vectorial TGFβ signaling in polarized intestinal epithelial cells. J Cell Physiol. 2010;224(2):398–404.
167. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer. 2004;4 (2):118–32.
168. Agiostratidou G, Hulit J, Phillips GR, Hazan RB. Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. J Mammary Gland Biol Neoplasia. 2007;12(2–3):127–33.
169. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin Cancer Res. 2007;13(23):7003–11.
170. Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, et al. Cadherin switching in human prostate cancer progression. Cancer Res. 2000;60(13):3650–4.
171. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol. 2000;148(4):779–90.
172. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia. 2010;15 (2):117–34.
173. Christofori G. Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J. 2003;22(10):2318–23.
174. Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, et al. Epithelial cell α3β1 integrin links β-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. J Clin Invest. 2009;119(1):213–24.
175. Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, et al. Integrin α3β1-dependent β-catenin phosphorylation links epithelial Smad signaling to cell contacts. J Cell Biol. 2009;184 (2):309–22.
176. Tian YC, Phillips AO. Interaction between the transforming growth factor-βtype II receptor/Smad pathway and β-catenin during transforming growth factor-β1-mediated adherens junction disassembly. Am J Pathol. 2002;160(5):1619–28.

177. Vogelmann R, Nguyen-Tat MD, Giehl K, Adler G, Wedlich D, Menke A. TGFβ-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN. J Cell Sci. 2005;118(Pt 20):4901–12.
178. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, et al. TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science. 2004;303(5659):848–51.
179. Kim BG, Li C, Qiao W, Mamura M, Kasprzak B, Anver M, et al. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature. 2006;441(7096):1015–9.
180. Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, et al. Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks. Oncogene. 2005;24(32):5053–68.
181. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature. 2004;432(7015):332–7.
182. Ignotz RA, Massague J. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem. 1986;261(9):4337–45.
183. Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, et al. Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res. 2008;68(15):6241–50.
184. Maschler S, Wirl G, Spring H, Bredow DV, Sordat I, Beug H, et al. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene. 2005;24(12):2032–41.
185. Wels J, Kaplan RN, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev. 2008;22(5):559–74.
186. Chandler EM, Saunders MP, Yoon CJ, Gourdon D, Fischbach C. Adipose progenitor cells increase fibronectin matrix strain and unfolding in breast tumors. Phys Biol. 2011;8(1):015008.
187. Barkan D, El Touny LH, Michalowski AM, Smith JA, Chu I, Davis AS, et al. Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. Cancer Res. 2010;70(14):5706–16.
188. Bianchini G, Qi Y, Alvarez RH, Iwamoto T, Coutant C, Ibrahim NK, et al. Molecular anatomy of breast cancer stroma and its prognostic value in estrogen receptor-positive and -negative cancers. J Clin Oncol. 2010;28(28):4316–23.
189. Wienke D, Davies GC, Johnson DA, Sturge J, Lambros MB, Savage K, et al. The collagen receptor Endo180 (CD280) Is expressed on basal-like breast tumor cells and promotes tumor growth in vivo. Cancer Res. 2007;67(21):10230–40.
190. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33.
191. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA. 2004;101(9):2999–3004.
192. Smeets A, Daemen A, Vanden Bempt I, Gevaert O, Claes B, Wildiers H, et al. Prediction of lymph node involvement in breast cancer from primary tumor tissue using gene expression profiling and miRNAs. Breast Cancer Res Treat. 2010: PMID: 21116709.
193. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65(16):7065–70.
194. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA. 2006;103(7):2257–61.
195. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, et al. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol. 2008;10(2):202–10.
196. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature. 2007;449(7163):682–8.
197. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 2008;18(3):350–9.
198. Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature. 2008;451(7175):147–52.
199. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
200. Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ. Transforming growth factor-β and microRNA:mRNA regulatory networks in epithelial plasticity. Cells Tissues Organs. 2007;185(1–3):157–61.
201. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem. 2007;282(19):14328–36.
202. Qian B, Katsaros D, Lu L, Preti M, Durando A, Arisio R, et al. High miR-21 expression in breast cancer associated with poor disease-free survival in early stage disease and high TGFβ1. Breast Cancer Res Treat. 2009;117(1):131–40.
203. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454(7200):56–61.
204. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010;29(34):4741–51.
205. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer. 2003;3(12):895–902.
206. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? Nat Med. 2009;15(9):1010–2.
207. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov. 2009;8(10):806–23.
208. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer. 2009;9(4):265–73.
209. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA. 2003;100(7):3983–8.
210. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730–7.
211. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663–76.
212. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318(5858):1917–20.
213. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.
214. Pera MF, Tam PP. Extrinsic regulation of pluripotent stem cells. Nature. 2010;465(7299):713–20.
215. Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. Genes Dev. 2010;24(20):2239–63.
216. Greber B, Lehrach H, Adjaye J. Fibroblast growth factor 2 modulates transforming growth factor βsignaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. Stem Cells. 2007;25(2):455–64.
217. James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFβ/activin/nodal signaling is necessary for the maintenance of

pluripotency in human embryonic stem cells. Development. 2005;132(6):1273–82.
218. Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol. 2009;11(12):1487–95.
219. Li HX, Han M, Bernier M, Zheng B, Sun SG, Su M, et al. Kruppel-like factor 4 promotes differentiation by transforming growth factor-βreceptor-mediated Smad and p38 MAPK signaling in vascular smooth muscle cells. J Biol Chem. 2010;285 (23):17846–56.
220. Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol. 1999;154(2):515–23.
221. Bennett CN, Green JE. Unlocking the power of cross-species genomic analyses: identification of evolutionarily conserved breast cancer networks and validation of preclinical models. Breast Cancer Res. 2008;10(5):213.
222. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. 1992;52(6):1399–405.
223. Santner SJ, Dawson PJ, Tait L, Soule HD, Eliason J, Mohamed AN, et al. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat. 2001;65(2):101–10.
224. Rak JW, McEachern D, Miller FR. Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression of murine mammary neoplasia. Br J Cancer. 1992;65(5):641–8.
225. Tsuji T, Ibaragi S, Shima K, Hu MG, Katsurano M, Sasaki A, et al. Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. Cancer Res. 2008;68(24):10377–86.
226. McAllister SS, Gifford AM, Greiner AL, Kelleher SP, Saelzler MP, Ince TA, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. Cell. 2008;133(6):994–1005.
227. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. Cell. 2009;139(7):1315–26.
228. Nomura M, Li E. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. Nature. 1998;393 (6687):786–90.
229. Waldrip WR, Bikoff EK, Hoodless PA, Wrana JL, Robertson EJ. Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. Cell. 1998;92 (6):797–808.
230. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 mutant mice develop metastatic colorectal cancer. Cell. 1998;94(6):703–14.
231. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor β-mediated signal transduction. Mol Cell Biol. 1999;19(4):2495–504.
232. Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J. 1999;18(5):1280–91.
233. Kretschmer A, Moepert K, Dames S, Sternberger M, Kaufmann J, Klippel A. Differential regulation of TGF-βsignaling through Smad2, Smad3 and Smad4. Oncogene. 2003;22(43):6748–63.
234. Dzwonek J, Preobrazhenska O, Cazzola S, Conidi A, Schellens A, van Dinther M, et al. Smad3 is a key nonredundant mediator of transforming growth factor βsignaling in Nme mouse mammary epithelial cells. Mol Cancer Res. 2009;7(8):1342–53.
235. Piek E, Ju WJ, Heyer J, Escalante-Alcalde D, Stewart CL, Weinstein M, et al. Functional characterization of transforming growth factor β signaling in Smad2- and Smad3-deficient fibroblasts. J Biol Chem. 2001;276(23):19945–53.
236. Brown KA, Pietenpol JA, Moses HL. A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-β signaling. J Cell Biochem. 2007;101(1):9–33.
237. Petersen M, Pardali E, van der Horst G, Cheung H, van den Hoogen C, van der Pluijm G, et al. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene. 2010;29(9):1351–61.
238. Tian F, DaCosta Byfield S, Parks WT, Yoo S, Felici A, Tang B, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res. 2003;63(23):8284–92.
239. Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, et al. TGF-βswitches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest. 2003;112 (7):1116–24.
240. Tian F, Byfield SD, Parks WT, Stuelten CH, Nemani D, Zhang YE, et al. Smad-binding defective mutant of transforming growth factor β type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res. 2004;64(13):4523–30.
241. Korpal M, Yan J, Lu X, Xu S, Lerit DA, Kang Y. Imaging transforming growth factor-β signaling dynamics and therapeutic response in breast cancer bone metastasis. Nat Med. 2009;15 (8):960–6.
242. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFβ signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol. 2009;11 (11):1287–96.
243. Giampieri S, Pinner S, Sahai E. Intravital imaging illuminates transforming growth factor β signaling switches during metastasis. Cancer Res. 2010;70(9):3435–9.
244. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. Nat Rev Cancer. 2009;9(2):108–22.
245. Paszek MJ, Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. J Mammary Gland Biol Neoplasia. 2004;9(4):325–42.
246. Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metastasis Rev. 2009;28(1–2):113–27.
247. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Girard OM, et al. Tissue-penetrating delivery of compounds and nanoparticles into tumors. Cancer Cell. 2009;16 (6):510–20.
248. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137(6):1005–17.
249. Tsuda N, Ishiyama S, Li Y, Ioannides CG, Abbruzzese JL, Chang DZ. Synthetic microRNA designed to target glioma-associated antigen 1 transcription factor inhibits division and induces late apoptosis in pancreatic tumor cells. Clin Cancer Res. 2006;12(21):6557–64.
250. Bhardwaj A, Singh S, Singh AP. MicroRNA-based cancer therapeutics: big hope from small RNAs. Mol Cell Pharmacol. 2010;2(5):213–9.
251. Weiler J, Hunziker J, Hall J. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? Gene Ther. 2006;13(6):496–502.
252. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438(7068):685–9.
253. Gumireddy K, Young DD, Xiong X, Hogenesch JB, Huang Q, Deiters A. Small-molecule inhibitors of microrna miR-21 function. Angew Chem Int Ed Engl. 2008;47(39):7482–4.
254. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods. 2007;4(9):721–6.

Video Article

In vivo Dual Substrate Bioluminescent Imaging

Michael K. Wendt, Joseph Molter, Christopher A. Flask, William P. Schiemann
Case Comprehensive Cancer Center, Case Western Reserve University

Correspondence to: William P. Schiemann at william.schiemann@case.edu

URL: http://www.jove.com/details.php?id=3245
DOI: 10.3791/3245

Keywords: Medicine, Issue 56, firefly luciferase, Renilla Luciferase, breast cancer, metastasis, Smad,

Date Published: 11/10/2011

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Citation: Wendt, M.K., Molter, J., Flask, C.A., Schiemann, W.P. In vivo Dual Substrate Bioluminescent Imaging. J. Vis. Exp. (56), e3245, DOI : 10.3791/3245 (2011).

Abstract

Our understanding of how and when breast cancer cells transit from established primary tumors to metastatic sites has increased at an exceptional rate since the advent of *in vivo* bioluminescent imaging technologies [1-3]. Indeed, the ability to locate and quantify tumor growth longitudinally in a single cohort of animals to completion of the study as opposed to sacrificing individual groups of animals at specific assay times has revolutionized how researchers investigate breast cancer metastasis. Unfortunately, current methodologies preclude the real-time assessment of critical changes that transpire in cell signaling systems as breast cancer cells *(i)* evolve within primary tumors, *(ii)* disseminate throughout the body, and *(iii)* reinitiate proliferative programs at sites of a metastatic lesion. However, recent advancements in bioluminescent imaging now make it possible to simultaneously quantify specific spatiotemporal changes in gene expression as a function of tumor development and metastatic progression *via* the use of dual substrate luminescence reactions. To do so, researchers take advantage for two light-producing luciferase enzymes isolated from the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*), both of which react to mutually exclusive substrates that previously facilitated their wide-spread use in *in vitro* cell-based reporter gene assays [4]. Here we demonstrate the *in vivo* utility of these two enzymes such that one luminescence reaction specifically marks the size and location of a developing tumor, while the second luminescent reaction serves as a means to visualize the activation status of specific signaling systems during distinct stages of tumor and metastasis development. Thus, the objectives of this study are two-fold. First, we will describe the steps necessary to construct dual bioluminescent reporter cell lines, as well as those needed to facilitate their use in visualizing the spatiotemporal regulation of gene expression during specific steps of the metastatic cascade. Using the 4T1 model of breast cancer metastasis, we show that the *in vivo* activity of a synthetic Smad Binding Element (SBE) promoter was decreased dramatically in pulmonary metastasis as compared to that measured in the primary tumor [4-6]. Recently, breast cancer metastasis was shown to be regulated by changes within the primary tumor microenvironment and reactive stroma, including those occurring in fibroblasts and infiltrating immune cells [7-9]. Thus, our second objective will be to demonstrate the utility of dual bioluminescent techniques in monitoring the growth and localization of two unique cell populations harbored within a single animal during breast cancer growth and metastasis.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=3245

Protocol

1. Stable Expression of CMV-Driven Renilla Luciferase

1. Transfection and selection of a stable clonal population is the preferred method for expression of this renilla luciferase reporter. This approach yields a more consistent and uniform renilla luciferase expression following the subsequent introduction of additional secondary reporter constructs (*e.g.*, firefly luciferase or fluorescent proteins).
2. Transfect malignant cells of interest with the expression vector encoding renilla luciferase, such as pcDNA3.1-Hygro or another plasmid harboring a selectable marker.
3. Following transfection, place the transfectants under an optimized antibiotic concentration for several days-to-weeks to facilitate the isolation of individual colonies, which are subsequently isolated individually and subcultured.
4. Select ≥10 individual renilla luciferase-expressing colonies to monitor the extent of renilla expression.
5. Colonies expressing high quantities of renilla luciferase are subsequently subjected to functional analyzes to ensure that the *(i)* pathophysiological properties of their parental counterparts are retained, and *(ii)* renilla expression values do not deviate or change over time. These steps are absolutely essential to avoid isolating and studying clonal variants/deviants, and to validate proper integration of the renilla construct into the genome.
6. Once a stable transfectant is isolated and verified, one can remove the selective pressure and be assured that the expression of renilla luciferase will remain constant over extended lengths of time both *in vitro* and *in vivo* .

2. Expression, Selection, and Functional Verification of Inducible Promoter-Driven Firefly Luciferase

1. Subclone the promoter of interest into a pGL4-luciferase reporter plasmid harboring a selectable marker (*e.g.*, puromycin) that is distinct from that used to select for stable renilla expression (*e.g.*, hygromycin).
2. Transfect malignant cells as in Step I, and subsequently select for a stable polyclonal population of firefly luciferase-expressing cells. Because the location where a report gene integrates into the genome can elicit erroneous effects on its expression and regulation, it is strongly recommended to select for polyclonal populations of firefly luciferase-expressing cells as opposed to clonal populations to ensure that *(i)* reporter gene expression more accurately reflects that of the endogenous gene in parental cells; and *(ii)* integration effects on gene expression are averaged and diminished across the heterogeneous cell population.
3. Malignant cells engineered to stably express both renilla and firefly luciferases are collectively referred to as dual bioluminescent reporter cells, or **DBR** cells.
4. Using traditional *in vitro* cell-based luciferase reporter gene assays, verify that the DBR cells regulate firefly luciferase expression, either positively or negatively, in a manner concordant with that observed in parental cells transiently transfected with these vectors. Likewise, verify that CMV-driven expression of renilla is not regulated by various cell stimulations or treatment conditions.
5. To do so, culture DBR and parental cells in 24-well plates, and subsequently transiently co-transfect parental cells with the original CMV-renilla and promoter-firefly constructs used to generate DBR cells.
6. Afterward, treat DBR and transfected parental cells with factors or pharmacological agents known to regulate the promoter of interest, and subsequently quantitate firefly and renilla luminescence using the Promega Dual Luciferase Assay Kit.

3. Establishing 4T1 Primary Mammary Tumors

1. Metastatic 4T1 mammary carcinoma cells that were found to lack adventitial rodent pathogens were engineered to stably express a CMV-driven renilla luciferase (pcDNA3.1-CMV-renilla luciferase-hygro) and a SBE-driven firefly luciferase (pGL4.2-SBE-firefly luciferase-puro) as described above. Orthotopic engraftment of these breast cancer cells results in the formation of spontaneous metastases primarily in the lungs.
2. 4T1 cells should not be allowed to reach confluence during their propagation in traditional 2-dimensional tissue culture systems, and they should be passaged the day before their *in vivo* inoculation.
3. 4T1 cells should be dissociated by trypsinization, washed thoroughly in growth media, and diluted in PBS to a concentration of $2x10^5$ cells/ml and immediately stored on ice.
4. Female Balb/C mice (4-6 weeks old) should be anesthetized with an induction dose of 3% isoflurane and maintained under anesthesia at a dose of 1% isoflurane. Prepare the injection site by swabbing with 70% isopropyl alcohol. Using a forceps, gently grasp and lift the 4th inguinal mammary pad. Carefully, place a 27.5 gauge needle bevel side up and inject into the mammary fat pad directly under the nipple, taking special care not push the needle into the abdominal cavity. Release the gland and slowly inject 50 µl of the cell suspension ($1x10^4$ cells) into the mammary fat pad.

4. Initial Dual Luminescent Imaging

1. Immediately after engrafting 4T1 cells onto the mammary fat pad and while the mice are still anesthetized, inject 100 µl of RediJect Coelenterazine into the lateral tail vein. This is the optimal substrate concentration recommended by the vendor and the best tolerated by the animal.
2. Immediately place the mouse into the isoflurane nose cone within the IVIS-200 imaging system and acquire a 0.5-1 minute luminescent image. Efficiency between injection and image acquisition is very important, as the signal for renilla luciferase drops precipitously ~30 seconds post-injection. Place the mouse back into its cage and allow it to recover for ≥1 hour, which ensures that any residual renilla luciferase signal has dissipated and that the mouse has fully recovered from the anesthesia.
3. Administer 150 mg/kg of D-luciferin potassium salt *via* I.P. injection and wait 5 minutes. This is the optimal concentration of D-luciferin, which provides a stable luminescent signal for 5-15 minutes following its injection into test animals. Anesthetize the mouse using isoflourane and replace in the IVIS-200, taking care to position the animal in a very similar position relative to the original renilla acquisition. The overall intensity of this firefly signal will depend on the activity of the promoter of interest, and as such, visualizing firefly luciferase activity may require extended acquisition times.
4. Using the IVIS Living Image software set to "photons" mode, establish values for both the renilla and firefly acquisitions as a means to establish baseline relative luminescence ratios (RLR).

5. Longitudinal Luminescent Imaging

1. 4T1 cells are highly aggressive, such that inoculation of $1x10^4$ cells typically leads to palpable tumor formation within 1 week, and to the lethality of the animal within 4-5 weeks. 4T1 tumors have an inherent propensity ulcerate in week 4. The growth and maintenance of ulcerated tumors may require separate IACUC approval. The 4T1 tumor studies shown herein have been approved by the IACUC at Case Western Reserve University.
2. As described above, mice should be injected weekly with RediJect Coelenterazine to monitor general tumor growth and metastasis, as well as with D-luciferin to monitor pathway-specific signaling during various stages of tumor development. Take care each time to ensure the renilla luciferase signal has completely dissipated prior to acquisition of the firefly luciferase signal.
3. As 4T1 tumors develop and progress, renilla luciferase acquisitions should be normalized to the initial renilla values measured at the time of tumor cell inoculation as a means to normalize and track primary tumor growth. More importantly, calculating RLR values over time will establish the temporal regulation of individual signaling systems relative to tumor growth and progression.
4. Overt pulmonary metastasis becomes apparent within 3-4 weeks following 4T1 cell engraftment onto the mammary fat pad. Comparing pulmonary RLR values *versus* those calculated for the primary tumor establishes the spatial regulation of individual signaling systems relative to tumor growth and metastasis.

6. Dual Bioluminescent Imaging of Two Unique Cell Types in a Single Animal

1. Engineer one breast cancer cell line to stably expression CMV-driven renilla luciferase as described above. Repeat this engineering process using CMV-driven firefly luciferase on a second distinct breast cancer cell line.
2. Herein we mixed renilla luciferase-expressing 4T1 cells with their nonmetastatic and isogenic firefly luciferase-expressing 4T07 counterparts [10, 11]. Varying ratios of these mixed breast cancer cell populations are subsequently injected into the mammary fat pad as described above.
3. Immediately acquire both firefly and renilla luciferase images to establish initial RLRs representative of a given inoculated cell mixture.

Copyright © 2011 Creative Commons Attribution License

4. Longitudinal dual bioluminescent imaging will visualize and track changes in the cellular composition within the primary tumor, as well as the spatiotemporal metastasis of each breast cancer derivative relative to tumor growth and progression.
5. Alternatively, individual breast cancer cell populations can be inoculated into different locations in the mouse (*i.e.* the right and left abdominal mammary fat pads) to assess the systemic influences these two populations exhibit over one another during various stages of tumor growth and metastasis.

7. Representative Results:

A major strength of dual bioluminescent imaging lies in the fact that each image is internally consistent and controlled, such that the continued signals derived from the primary tumor function as a important metric to gauge the overall success or failure of each renilla and firefly acquisition. This aspect of the imaging procedure is particularly important during acquisitions of the activity of renilla luciferase, whose Coelenterazine substrate is highly sensitive to oxidation that results in its ability to auto-luminescence. Figure 1A shows an example of this side effect, which immediately manifests as nonspecific luminescent signals derived from the intestinal tract, not the established primary tumor. Typically, these nonspecific Coelenterazine signals transpire following failed intravenous injections of this renilla luciferase substrate. However, once robust primary tumor-derived renilla signals have been obtained (Figure 1B, *left image*), it is safe to proceed in capturing pathway-specific signaling measurements derived from imaging firefly luciferase (Figure 1B, *right panel*), whose D-luciferin substrate is highly stable upon I.P. injection and produces negligible levels of background auto-luminescence.

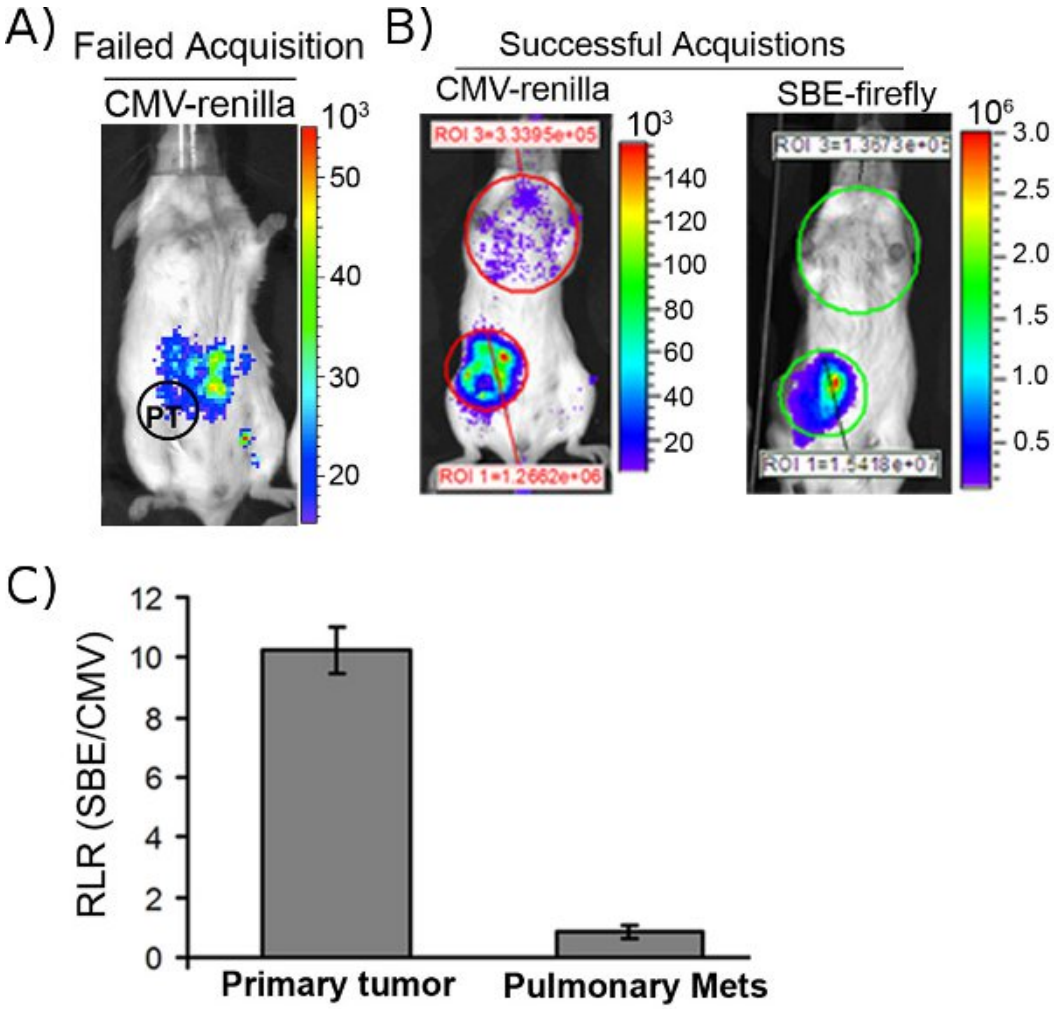


Figure 1. Acquiring renilla and firefly-derived bioluminescent images to calculate *in vivo* RLRs. **(A)** An example of a failed I.V. injection of Coelenterazine resulting in nonspecific signal from the intestinal tract. Circle and PT indicate the approximate location of the primary tumor. **(B)** A successful renilla acquisition of a mouse bearing a 4T1 primary tumor and pulmonary metastases (left panel; 4wk after fat pad engraftment). The corresponding firefly acquisition allows for calculation of RLRs from both the primary tumor and its pulmonary metastases. **(C)** Graphical representation of the primary tumor and pulmonary metastasis RLRs calculated from mice shown in panel B (n=4).

Discussion

The absolute power of bioluminescent imaging techniques lies in their ability to quantify tumor growth and metastasis in complex longitudinal studies, which herein involved the use of aggressive 4T1 breast cancer cells. Because these procedures rely on the stable integration of both luciferase reporter constructs, these techniques can be readily adapted and translated to other cancer cell lines of varying tumor latencies and metastatic capabilities. Similar to the results shown herein, calculating specific RLRs within slowly developing primary tumors and their eventual metastases allows for the real-time spatiotemporal identification of specific signaling events that transpire during the metastatic progression of tumors irrespective of their duration of latency. Upon identification of specific promoter regulatory events, it is important to excise both the primary tumor and its metastatic lesions for the performance of standard immunohistochemical and differential gene expression analyses to verify similar regulation of the endogenous gene and/or protein.

Copyright © 2011 Creative Commons Attribution License

Technically speaking, the primary challenge of dual bioluminescent analyses lies in the relatively short duration of the renilla luciferase signals. As such, this imaging technique requires significant optimization of the tail vein injection procedure, as well as the immediate imaging of individually injected animals, a process that is time consuming and somewhat inefficient relative to imaging firefly luciferase. Recently, Promega introduced "Viviren," which represents a second generation luciferase substrate whose sites of oxidation are blocked by esterification until this molecule gains entry into cells, at which point the molecule is rapidly de-esterified. Collectively, this novel substrate effectively lowers auto-luminescence and nonspecific modification and/or degradation coupled to renilla-induced auto-luminescence [12]. In doing so, this new renilla luciferase substrate produces brighter luminescent signals; however, the excessive costs associated with the acquisition and use of "Viviren" have provided relatively few studies necessary to evaluate the overall utility of this substrate in dual bioluminescent analyses. Finally, the sensitivity of any bioluminescent assay is critically dependent upon the CCD camera operant in acquiring individual light units. Indeed, as these technologies improve, we foresee a point in the future where our ability to visualize complex light-mediated bioluminescent reactions may transpire efficiently in free moving animals [13].

Disclosures

No conflicts of interest declared.

Acknowledgements

W.P.S. was supported in part by grants from the National Institutes of Health (CA129359), the Susan G. Komen for the Cure Foundation (BCTR0706967), the Department of Defense (BC084561), and the Seidman Cancer Center, while M.K.W. was supported by a fellowship from the American Cancer Society (PF-09-120-01). Additional support for this work was provided by the Case Center for Imaging Research, the Case Comprehensive Cancer Center (P30 CA43703), and the Cystic Fibrosis Center (P30 DK027651).

References

1. Wetterwald, A., *et al.* Optical imaging of cancer metastasis to bone marrow: a mouse model of minimal residual disease. *Am. J. Pathol.* **160**, 1143-1153 (2002).
2. Wendt, M.K., Drury, L.J., Vongsa, R.A., & Dwinell, M.B. Constitutive CXCL12 Expression Induces Anoikis in Colorectal Carcinoma Cells. *Gastroenterology.* **135**, 508 (2008).
3. Wendt, M. & Schiemann, W. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. *Breast Cancer Research.* **11**, R68 (2009).
4. Korpal, M., *et al.* Imaging transforming growth factor-beta signaling dynamics and therapeutic response in breast cancer bone metastasis. *Nat Med.* (2009).
5. Giampieri, S., *et al.* Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol.* **11**, 1287-1296 (2009).
6. Giampieri, S., Pinner, S., & Sahai, E. Intravital imaging illuminates transforming growth factor beta signaling switches during metastasis. *Cancer Res.* **70**, 3435-3439 (2010).
7. DeNardo, D.G., *et al.* CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell.* **16**, 91-102 (2009).
8. Wyckoff, J. *et al.* A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors. *Cancer Res.* **64**, 7022-7029 (2004).
9. Kim, M.Y., *et al.* Tumor self-seeding by circulating cancer cells. *Cell.* **139**, 1315-1326 (2009).
10. Aslakson, C.J. & Miller, F.R. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res.* **52**, 1399-1405 (1992).
11. Wendt, M.K., Smith, J.A., & Schiemann, W.P. Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. *Oncogene.* **29**, 6485-6498 (2010).
12. Hawkins, E., Unch, J., Murhpy, N., Vidugiriene, J., Scurria, M., Klaubert, D., & Wood, K. Bright Light, No Lysis. Measuring Renilla Luciferase Luminescence in Living Cells. http://www.promega.com/pnotes/90/12727_10/12727_10.pdf (2005).
13. Roncali, E., *et al.* New device for real-time bioluminescence imaging in moving rodents. *J. Biomed. Opt.* **13**, 054035 (2008).

Copyright © 2011 Creative Commons Attribution License

Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer

Michael K. Wendt, Molly A. Taylor, Barbara J. Schiemann, and William P. Schiemann
Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106

ABSTRACT **Reduced epithelial cadherin (E-cad) is a hallmark of invasive carcinomas that have acquired epithelial-mesenchymal transition (EMT) phenotypes. Here we show that down-regulated E-cad expression induced by transforming growth factor-β (TGF-β) and EMT preceded breast cancer outgrowth in three-dimensional (3D) organotypic assays and in the lungs of mice. Pharmacological inhibitors against focal adhesion kinase prevented metastatic outgrowth of newly seeded organoids, but not that of their fully established counterparts. Interrogating the D2-HAN (hyperplastic alveolar nodule) model of breast cancer dormancy and metastasis showed that dormant D2.OR cells produced branched organoid morphologies in 3D-cultures, and expressed robust quantities of E-cad that was uncoupled from regulation by TGF-β. In contrast, metastatic D2.A1 organoids were spherical and wholly lacked E-cad expression. Interestingly, D2.A1 cells engineered to re-express E-cad formed branched organoids, down-regulated β1 integrin expression, and failed to undergo metastatic outgrowth. The tumor-suppressing function of E-cad was inactivated by increased microenvironmental rigidity, and was not recapitulated by expression of an E-cad mutant lacking its extracellular domain. Twist expression, but not that of Snail, reinitiated metastatic outgrowth in dormant D2.OR cells. Our findings show that EMT and its down-regulated expression of E-cad circumvent breast cancer dormancy in part by facilitating β1 integrin expression necessary for metastatic outgrowth.**

Monitoring Editor
Kunxin Luo
University of California, Berkeley

Received: Apr 7, 2011
Revised: May 11, 2011
Accepted: May 17, 2011

INTRODUCTION

Dissemination of tumor cells from the primary lesion is the most common event in the metastatic process and leads to the shedding of millions of carcinoma cells into the circulation each day (Yoshida *et al.*, 2000; Cowin and Welch, 2007). Fortunately, metastasis is a highly inefficient and sequential cascade that requires carcinoma cells that have escaped the primary tumor to survive in the circulation, invade target organs, and reinitiate secondary tumor outgrowth. Indeed, initiation of metastatic outgrowth is the final critical event required to produce lethal metastatic disease. It therefore stands to reason that elucidating and targeting the molecular mechanisms that initiate the outgrowth of disseminated cancer cells could significantly improve the clinical course of patients with metastatic breast cancer. Expression of epithelial cadherin (E-cad) is a hallmark of a fully differentiated epithelium where it functions to maintain cell-cell junctions, thereby inhibiting aberrant cell proliferation and migration. Indeed, loss of E-cad function via genetic inactivation or epigenetic silencing is a common characteristic of systemically invasive carcinomas (Graff *et al.*, 1998, 2000; Nass *et al.*, 2000; Lombaerts *et al.*, 2006). Epithelial-mesenchymal transition (EMT) is a physiological process that is hijacked by breast cancer cells, which enables them to initiate systemic dissemination by 1) down-regulating E-cad expression or activity; 2) separating cell-cell junctions; 3) invading the surrounding tissues; and 4) intravasating the vasculature or lymphatic system (Thiery, 2002; Wendt *et al.*, 2009a). Recently, EMT and its

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-04-0306) on May 25, 2011.

Address correspondence to: William P. Schiemann (william.schiemann@case.edu).

Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; CMV, cytomegalovirus; E-cad, epithelial cadherin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion; GFP, green fluorescent protein; HAN, hyperplastic alveolar nodule; MEC, mammary epithelial cell; NM-E, NMuMG cells transformed by EGFR; RTK, receptor tyrosine kinase; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; VSVG, vesicular stomatitis virus-glycoprotein; WT, wild-type.

© 2011 Wendt *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

"ASCB®," "The American Society for Cell Biology®," and "Molecular Biology of the Cell®" are registered trademarks of The American Society of Cell Biology.

accompanying reduction in E-cad expression were shown to be essential for the extravasation of cancer cells into secondary organs (Drake *et al.*, 2009). Unfortunately, the specific impact of EMT and its inactivation of E-cad function in facilitating the metastatic outgrowth of disseminated breast cancer cells remains unknown.

The ability of breast cancer cells to initiate metastatic outgrowth has recently been linked to the expression and activity of β1 integrin and its downstream effector, focal adhesion kinase (FAK) (Barkan *et al.*, 2008; Shibue and Weinberg, 2009). Moreover, alterations within the cytoskeletal architecture also appear necessary to enable dormant breast cancer metastases to reinitiate proliferative programs coupled to metastatic outgrowth (Barkan *et al.*, 2008). EMT is classically associated with reorganization of the actin cytoskeleton (Miettinen *et al.*, 1994), and recent studies by our group and others have established integrins and FAK as essential mediators of EMT induced by transforming growth factor-β (TGF-β) in normal and malignant mammary epithelial cells (MECs) (Bhowmick *et al.*, 2001; Galliher and Schiemann, 2006; Wendt and Schiemann, 2009; Wendt *et al.*, 2010). In addition, TGF-β1 signaling has been associated with the selection and expansion of cancer stem cells, a phenomenon mimicked by the constitutive expression of EMT-associated transcription factors (e.g., Twist, Snail, and ZEB1) and by the targeted deletion of E-cad in MECs (Mani *et al.*, 2008; Battula *et al.*, 2010; Taube *et al.*, 2010). In light of these findings, we hypothesized that EMT endows disseminated cancer cells with the ability to overcome systemic dormancy and initiate metastatic outgrowth, leading to the subsequent formation of secondary macroscopic metastases.

Herein we engineered several breast cancer cell lines that possessed differing degrees of metastatic competency to stably express firefly luciferase, which was used to longitudinally track their growth in compliant and rigid three-dimensional (3D) cultures and in the lungs of mice. In doing so, we show that down-regulated E-cad expression induced by TGF-β and EMT was sufficient to prevent MEC differentiation and organoid branching, and instead produced dense, more spherical cultures that underwent metastatic outgrowth. We also characterized the EMT status of the D2-HAN (hyperplastic alveolar nodule) derivatives, D2.A1 and D2.OR, which are established models of the success and failure of pulmonary outgrowth, respectively. Indeed, recent studies have shown that these D2 cell derivatives differ not in their ability to extravasate into the lung, but in their ability to initiate metastatic outgrowth within the pulmonary microenvironment (Rak *et al.*, 1992; Morris *et al.*, 1994). Interestingly, the lung shares an elastic modulus reminiscent of that of the normal mammary gland (Butcher *et al.*, 2009), indicating that disseminated breast cancer cells endure dramatic changes in tissue compliance as a part of the metastatic cascade (i.e., compliant mammary gland → rigid primary tumor → compliant disseminated sites). Along these lines, the differential metastatic outgrowth activities exhibited by these D2 cell derivatives can be recapitulated in vitro using compliant 3D organotypic cultures (Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). We show that systemically dormant D2.OR cells express robust quantities of E-cad and readily differentiate into branching organoid structures in 3D cultures, whereas their outgrowth-proficient D2.A1 cell counterparts are devoid of E-cad expression and fail to undergo MEC differentiation programs. Importantly, heterologous E-cad expression in metastatic D2.A1 cells induced their formation of branched organoid structures, as well as ablated their outgrowth in 3D cultures. Interestingly, the ability of E-cad to prevent the outgrowth of D2.A1 cells in 3D cultrues could be circumvented by inclusion of collagen within recombinant basement membrane cushions, suggesting that microenvironmental rigidity negates the tumor-suppressing functions of E-cad. Mechanistically, the down-regulated expression of E-cad induced by TGF-β and Twist, but not by Snail, was both necessary and sufficient to stabilize β1 integrin expression needed for efficient outgrowth of metastatic breast cancer cells. Collectively our findings establish E-cad and its response to EMT induced by TGF-β as a critical determinant for whether disseminated breast cancer cells acquire dormant or proliferative metastatic programs.

RESULTS

Down-regulated E-cad expression is required for 3D outgrowth of breast cancer cells

We recently demonstrated that inducing EMT before the intravenous inoculation of epidermal growth factor receptor (EGFR)-transformed breast cancer cells dramatically increased their ability to colonize the lungs and initiate secondary tumor outgrowth (Wendt *et al.*, 2010). To address whether EMT induced by TGF-β could specifically increase the initiation of outgrowth, we treated these same EGFR-transformed NMuMG (NM-E) cells with TGF-β1 for 48 h to induce an EMT response that included decreased E-cad expression (Figure 1A, inset). Afterward, these pre- and post-EMT NM-E cell populations were sparsely seeded (5000 cells/cm^2) into compliant 3D cultures to mimic metastatic outgrowth in the pulmonary microenvironment (Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). Bioluminescence quantification showed that the ability of TGF-β to induce EMT readily enhanced the 3D outgrowth of NM-E cells in the absence or presence of exogenous EGF (Figure 1A). We also reexpressed E-cad in the mesenchymal-like and E-cad–deficient human MDA-MB-231 breast cancer cells, an experimental manipulation previously shown to decrease their metastatic potential and normalize their acinar morphology in 3D cultures (Mbalaviele *et al.*, 1996; Zantek *et al.*, 1999; Wang *et al.*, 2002). Accordingly, bioluminescence growth assays demonstrated that heterologous E-cad expression in MDA-MB-231 cells significantly inhibited their growth in 3D cultures (Figure 1B). We extended these analyses to murine 4T1 breast cancer cells, which are highly metastatic despite their robust expression of E-cad (Figure 1C; Galliher-Beckley and Schiemann, 2008; Wendt and Schiemann, 2009). Interestingly, 4T1 cells exhibited a biphasic growth pattern in 3D cultures that was characterized by an initial latency phase, followed by a dramatic proliferative phase (Figure 1C). Examination of 4T1 cells about to undertake the outgrowth process (i.e., 5 d postplating in 3D culture) revealed a dramatic down-regulation of E-cad protein as compared with cells grown for the same period of time on standard tissue culture plastic (Figure 1C).

To further explore the connections between E-cad expression and 4T1 cell proliferation, we engineered 4T1 cells that stably expressed 1) firefly luciferase under the control of the human E-cad promoter, and 2) renilla luciferase under the control of the constitutively active cytomegalovirus (CMV) promoter. Using this dual bioluminescence reporter system, we show that the initiation of 3D outgrowth (Day 5) was concomitant with dramatically diminished E-cad promoter activity, even in the absence of exogenous TGF-β (Figure 1D). Finally, we extended these analyses to 4T07 cells, which are isogenic to 4T1 cells and are 1) systemically invasive and unable to metastasize from orthotopic primary tumors (Aslakson and Miller, 1992; Wendt *et al.*, 2010); 2) competent to form lung tumors when injected into the lateral tail vein of syngeneic BALB/c mice (Figure 2, B and C); and 3) adept at down-regulating E-cad expression in response to TGF-β (Figure 2D). Similar to our findings in the 4T1 line, administration of TGF-β to 4T07

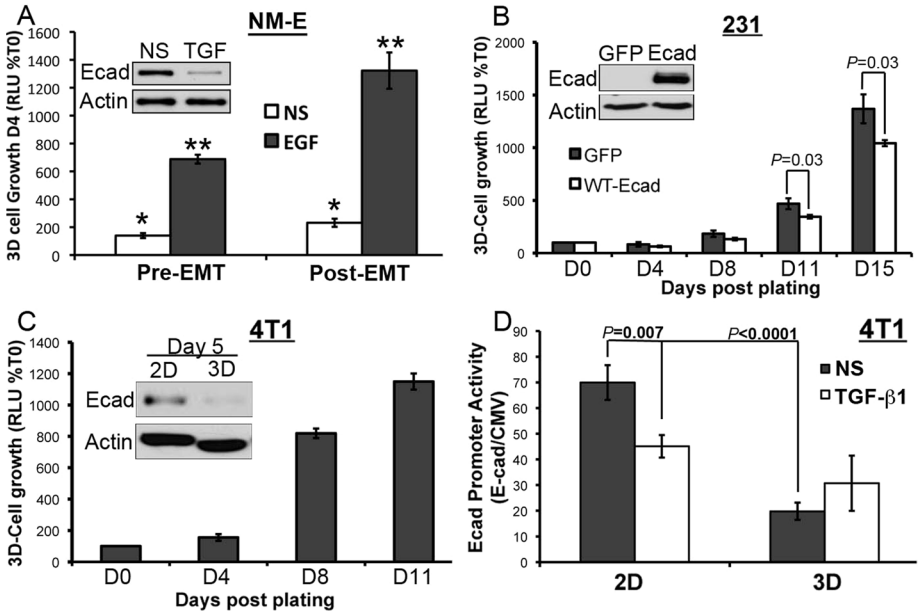


FIGURE 1: Down-regulated E-cad expression is required for 3D outgrowth of breast cancer cells. (A) NM-E cells were treated with TGF-β1 (5 ng/ml) for 48 h (Post-EMT) before their 3D culture for an additional 4 d in the absence (NS) or presence of EGF (50 ng/ml) as indicated; 3D outgrowth was monitored by a bioluminescence growth assay. Inset, Immunoblot verifying down-regulation of E-cad in NM-E cells upon TGF-β1–induced EMT. Data are the mean (±SE) of three independent experiments completed in triplicate. *, **, $p < 0.05$. (B) 3D outgrowth of human MDA-MB-231 breast cancer cells (231) expressing E-cad or GFP as a control was monitored longitudinally by bioluminescence. Data are the mean (±SE) of three independent experiments completed in triplicate. Inset, Immunoblot analysis verifying recombinant E-cad expression. (C) 3D outgrowth of murine 4T1 breast cancer cells was monitored by bioluminescence. Data are the mean (±SE) of three independent experiments completed in triplicate. Inset, E-cad protein levels were monitored by immunoblot analyses 5 d after propagating the cells in 2D or 3D cultures. (D) 4T1 cells engineered to stably express an E-cad–luciferase reporter construct were grown in the absence or presence of TGF-β1 (5 ng/ml) under 2D or 3D conditions as in (C). Data are the mean (±SE) E-cad–firefly luciferase/CMV–renilla luciferase ratios obtained from three independent experiments completed in triplicate.

cells dramatically down-regulated their expression of E-cad, as did their propagation in 3D cultures (Figure 2D). Interestingly, E-cad expression returned upon prolonged (11 d) culture under 3D conditions (Figure 2D). These data suggest that diminution of E-cad expression is required to initiate organoid outgrowth, whereas macroscopic metastasis formation requires a mesenchymal-epithelial transition (MET) that includes the reexpression of E-cad (Hugo *et al.*, 2007; Chao *et al.*, 2010). Moreover, the 3D outgrowth latency exhibited by 4T1 organoids was not observed in 4T07 organoids, presumably due to their lower levels of E-cad expression relative to their 4T1 counterparts (Figures 1C and 2, A and E). Importantly, treatment of 4T07 cells in 3D culture with TGF-β increased the initiation of 3D outgrowth, whereas inclusion of a small molecule antagonist to TβR-I (TGF-β receptor type I) dramatically inhibited the initiation of 3D outgrowth (Figure 2E). The diminished 3D outgrowth of 4T07 organoids brought about by the inhibition of autocrine TGF-β signaling resulted in MEC differentiation and the acquisition of branched organoid morphologies, which contrasted sharply with the increased appearance of dense and independent organoids elicited by administration of TGF-β (Figure 2F; Wendt *et al.*, 2010). Taken together, these findings are consistent with the notion that E-cad expression is down-regulated to allow breast cancer cells to abandon their inherent mammary branching phenotypes in favor of proliferative spheroids capable of initiating metastatic outgrowth.

TGF-β is sufficient to decrease mammary branching of D2.OR cells

We next sought to use the isogenic D2-HAN cell system, which represents an established model to recapitulate the success and failure of metastatic pulmonary outgrowth (Rak *et al.*, 1992; Morris *et al.*, 1994; Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). Morphologically, nonmetastatic D2.OR cells are less mesenchymal-like than their metastatic D2.A1 counterparts when grown on tissue-culture plastic (Figure 3A). More importantly, D2.OR cells displayed a branched morphology when propagated in 3D cultures, whereas D2.A1 cells grew as independent metastatic clusters (Figure 3A, Supplemental Figure S1, Supplemental Movie S1). Furthermore, propagating D2.OR cells at higher densities in 3D cultures resulted in a dramatic aggregation and formation of branched structures within 18 h of plating (Supplemental Figure S1). Similar manipulations to D2.A1 cells, however, showed these metastatic MECs to be immotile and immediately proficient to undergo proliferative programs (Supplemental Figure S1). Indeed, although traditional microscopy intuitively depicts branched D2.OR organoids as being outwardly invasive, our time-lapse microscopy clearly shows that these branched organoid structures formed in response to cellular aggregation and inward migration (Supplemental Movie S2). Thus the 3D culture morphology of D2.OR cells manifests independent of their ability to proliferate. We recently demonstrated the role of EMT induced by TGF-β to prevent organoid branching in 3D cultures, an event that correlated with enhanced pulmonary outgrowth of breast cancer cells in mice (Wendt *et al.*, 2010). Although identical experimental manipulations directed at D2.OR cells did reduce their ability to form branched organoid structures (Figure 3B), we were unable to rescue their 3D outgrowth in an EMT-dependent manner (Figure 3C). Moreover and irrespective of TGF-β signaling, we observed ~5–10% of D2.OR cell inoculated into the lateral tail veins of mice to remain dormant in the lungs for a span of up to 5 wk (Figure 3, D and E). Collectively these findings suggest that the inherent program of nonmetastatic breast cancer cells to communicate and migrate toward one another during the formation of branched, multicellular organoids may underlie their inability to initiate proliferative programs within compliant pulmonary microenvironments.

Outgrowth-proficient cells lack E-cad expression

Given the differential requirement of EMT to enhance the pulmonary outgrowth of NM-E cells (Figure 1A; Wendt *et al.*, 2010) and not that of D2.OR cells (Figure 3, C–E), we next sought to verify the response of D2-HAN derivatives to TGF-β by characterizing a repertoire of target genes known to be regulated by this multifunctional cytokine. Both D2.OR and D2.A1 cells readily up-regulated the expression of β3 integrin in response to TGF-β (Figure 4, A and B), a molecule we established as one of the most sensitive and robust markers of TGF-β signaling (Galliher and Schiemann,

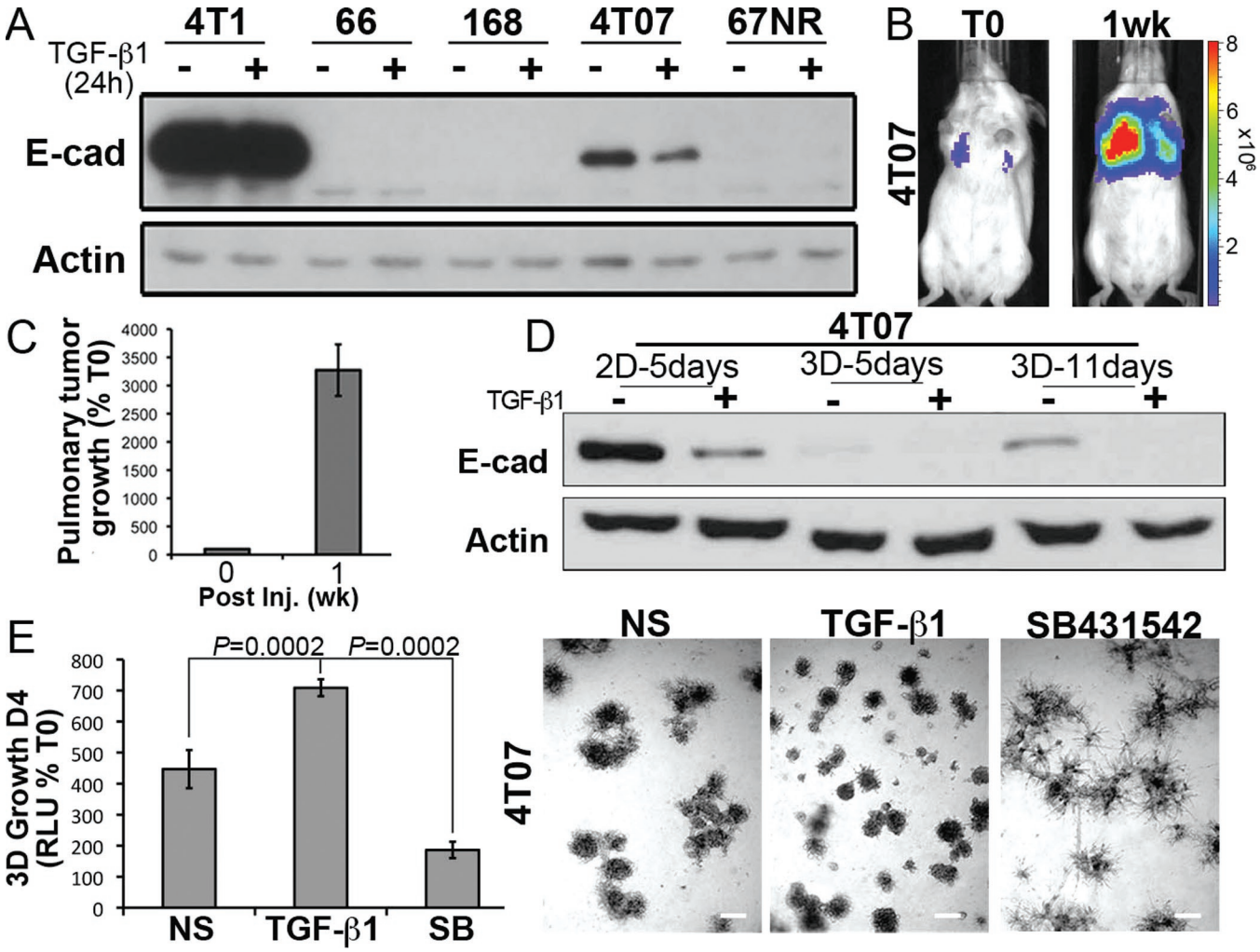


FIGURE 2: 4T07 cells down-regulate E-cad to initiate 3D outgrowth. (A) Members of the murine 4T1 progression series were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 24 h before monitoring their expression of E-cad and actin, which served as a loading control. (B) 4T07 cells (1×10^6) were injected into the lateral tail vein of 4-wk-old female BALB/c mice and imaged 30 min later (T0), and again 1 wk later, as indicated. Shown are bioluminescent images of representative mice from each time point. (C) Bioluminescence quantification of mice described in (B) (n = 4 mice). All mice succumbed to pulmonary tumor burden 12 d postinoculation. (D) 4T07 cells were grown in 2D or compliant 3D cultures for varying times in the absence or presence of TGF-β1 (5 ng/ml) as indicated. Afterward, E-cad expression was monitored by immunoblotting; actin immunoreactivity is provided as a loading control. Data are representative of three independent experiments. (E) Compliant 3D outgrowth of 4T07 cells propagated in the absence (NS) or presence of TGF-β1 (5 ng/ml) or the TβR-I inhibitor, SB431452 (SB, 10 μM), was quantified by bioluminescence. D4, Day 4 postplating. Afterward, the resulting organoids were imaged under phase-contrast microscopy (50×). Data are the mean (±SE) of two independent experiments completed in triplicate.

2006; Wendt and Schiemann, 2009; Wendt *et al.*, 2010). Moreover, both D2-HAN derivatives displayed enhanced actin stress fiber formation in response to TGF-β1 stimulation (Supplemental Figure S2). Examination of other EMT markers identified several absolute gene expression differences between these D2-HAN derivatives. For instance, systemically dormant D2.OR cells expressed abundant quantities of EGFR, Pyk2, and E-cad (Figure 4, A and B), all of which were conspicuously absent in their metastatic D2.A1 counterparts. Surprisingly, administration of TGF-β to D2.OR cells failed to down-regulate their expression of E-cad (Figure 4, A and B). Moreover, chronic and continued culture of D2.OR cells with TGF-β actually increased the levels of E-cad mRNA (unpublished data) and protein (Supplemental Figure S3A). Inclusion of a TβR-I antagonist, SB431542, to these cultures resulted in a dramatic diminution of E-cad expression (Supplemental Figure S3A). In stark contrast, chronic TGF-β treatment of the NM-E cells led to a robust EMT that included down-regulated E-cad expression that readily reversed upon removal of exogenous TGF-β (Supplemental Figure S3, B and C). These findings identify a clear defect in the ability of the D2.OR cells to down-regulate E-cad expression as part of their EMT program, a deficit that may underlie their preferential acquisition of dormant phenotypes during pulmonary metastasis.

We next examined the functional significance that results from the differential expression of EGFR observed between these D2-HAN derivatives. For instance, stimulating D2.OR cells with EGF resulted in a robust activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a response that was undetectable in D2.A1 cells (Figure 4C). D2.A1 cells, however, readily active ERK1/2 in response to either fibroblast growth factor and platelet-derived growth factor (Figure 4C), demonstrating the competency of the

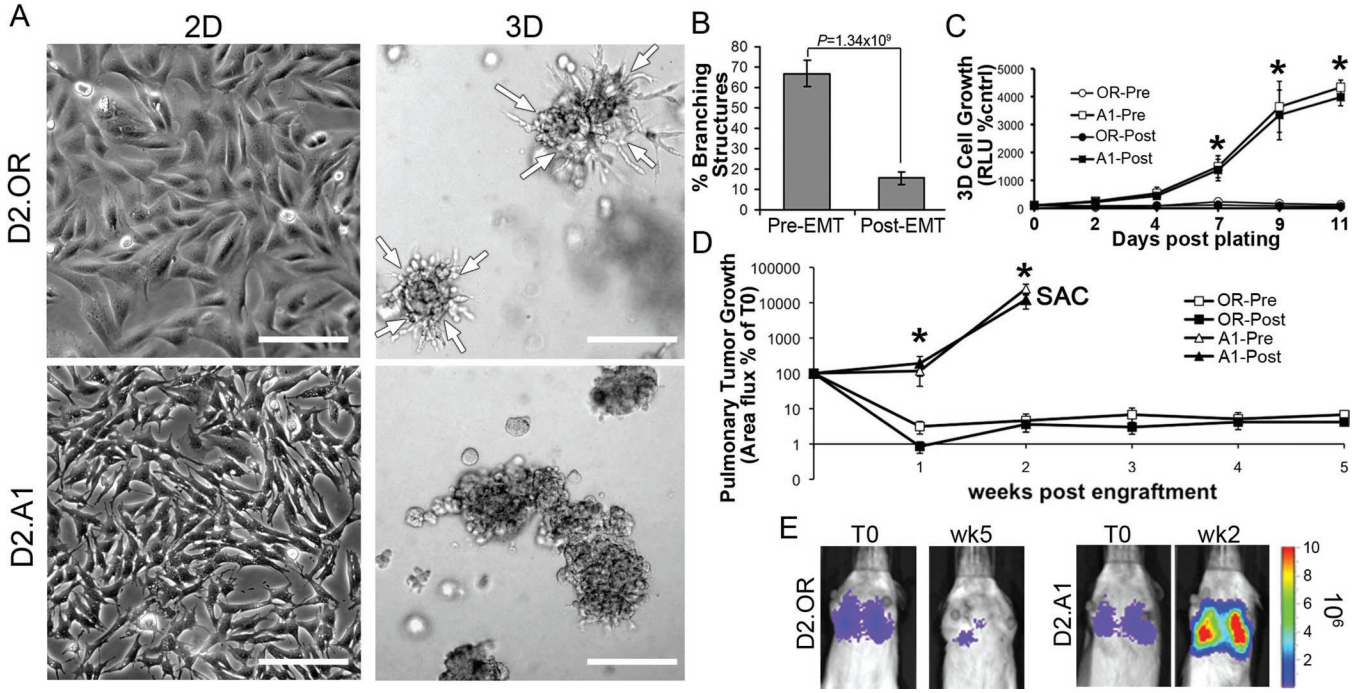


FIGURE 3: TGF-β–mediated EMT decreases mammary branching of D2.OR cells. (A) D2.OR or D2.A1 cells were plated on either tissue-culture plastic (2D) or reconstituted basement membrane (3D) for 5 d, and subsequently were imaged under phase-contrast microscopy (200×). Arrows indicate the inward directional migration of the D2.OR branching structures. (B) D2.OR cells were left untreated (Pre-EMT) or treated with TGF-β1 (Post-EMT) for 48 h on plastic. Afterward, the cells were subcultured and grown in compliant 3D cultures for 5 d, at which point the mean percentage of branching structures was quantified from nine random fields of view over three independent experiments. (C) 3D outgrowth of Pre- and Post-EMT D2.OR and D2.A1 cells was quantified by bioluminescence. Data are the mean (±SE) of three independent experiments completed in triplicate. (D) Pre- and Post-EMT D2.OR and D2.A1 cells were injected into the lateral tail vein of BALB/c mice (1×10^6 cells/mouse). Data are the mean area flux values (±SE, n = 5 per group) normalized to injected values. Mice that received injections of D2.A1 cells succumbed to pulmonary tumor burden 2 wk following tumor cell inoculation (SAC). (E) Bioluminescent images of representative mice described in (C), imaged at the time of injection (T0) and 2 (wk2) or 5 (wk5) wk later. In (C) and (D), * $p < 0.01$ between the D2.OR and D2.A1 cells.

Ras/mitogen-activated protein kinase pathway to be activated in these metastatic MECs. Along these lines, the 3D outgrowth of D2.OR organoids was strongly induced by EGF, which failed to enhance the growth of D2.A1 organoids presumably due to their deficiency in EGFR expression (Figure 4D). We also conducted 3D-outgrowth assays in the presence of either the dual FAK and Pyk2 inhibitor, PF-562,271 (PF271), or the FAK-specific inhibitor, PF-573,228 (PF228; Roberts *et al.*, 2008), both of which dramatically decreased the 3D outgrowth of D2.A1 organoids (Figure 4D). Because D2.A1 cells fail to express detectable levels of Pyk2 (Figure 4, A and B), these findings implicate FAK as a critical effector operant in mediating the 3D outgrowth of D2.A1 cells. This conclusion is supported by a recent study that observed genetic depletion of FAK to prevent pulmonary outgrowth of D2.A1 cells (Shibue and Weinberg, 2009). It remains unclear, however, as to whether FAK regulates the initiation, the maintenance, or both phases of metastatic outgrowth. To address this question, we performed a longitudinal D2.A1 outgrowth study in which the cells were immediately incubated with either diluent or PF228, or in which the cells were allowed to grow for 4 d before the addition of PF228. Figure 4E shows that FAK antagonism significantly impeded the initiation of D2.A1 outgrowth but provided no therapeutic benefit in preventing the outgrowth of established D2.A1 organoids. Thus FAK protein tyrosine kinase activity appears essential solely for the initiation of proliferative programs by metastatic cell clusters, not for their continued outgrowth. These findings are consistent with those of several recent reports showing that therapeutic targeting of FAK was effective in decreasing the establishment of pulmonary metastases, but not the later stages of their eventual outgrowth in the lung (van Nimwegen *et al.*, 2005; Wendt and Schiemann, 2009). Furthermore, given the established role of FAK in facilitating TGF-β–induced EMT (Cicchini *et al.*, 2008; Wendt and Schiemann, 2009), including the inactivation of E-cad function, our findings also demonstrate that the diminution of E-cad expression facilitates the initiation of metastatic outgrowth.

3D culture is required to manifest the TGF-β paradox

The switch in TGF-β function from a tumor suppressor to a tumor promoter is referred to as the "TGF-β Paradox" (Tian *et al.*, 2010). In breast cancer, this phenomenon is characterized by a decrease in Smad2/3 activity and acquired resistance to the cytostatic activities of TGF-β (Wendt *et al.*, 2009b), both of which are brought about by TGF-β stimulation of EMT (Supplemental Figure S4; Gal *et al.*, 2008; Neil *et al.*, 2008). Interestingly, the phosphorylation (Figure 5A), nuclear translocation (Figure 5B), and transcriptional activation (Figure 5C) mediated by Smad2/3 were similarly induced by TGF-β in both D2.OR and D2.A1 cells. Moreover, in traditional two-dimensional (2D) culture, both D2-HAN derivatives were similarly resistant to TGF-β–mediated growth arrest as compared with NM-E cells (Figure 5D). These findings, together with those in Figure 4, argue that the derivation of D2.A1 cells likely did not transpire via a TGF-β–driven EMT event. Along these lines, we observed dormant D2.OR cells to be more invasive than their D2.A1 counterparts in Matrigel-coated

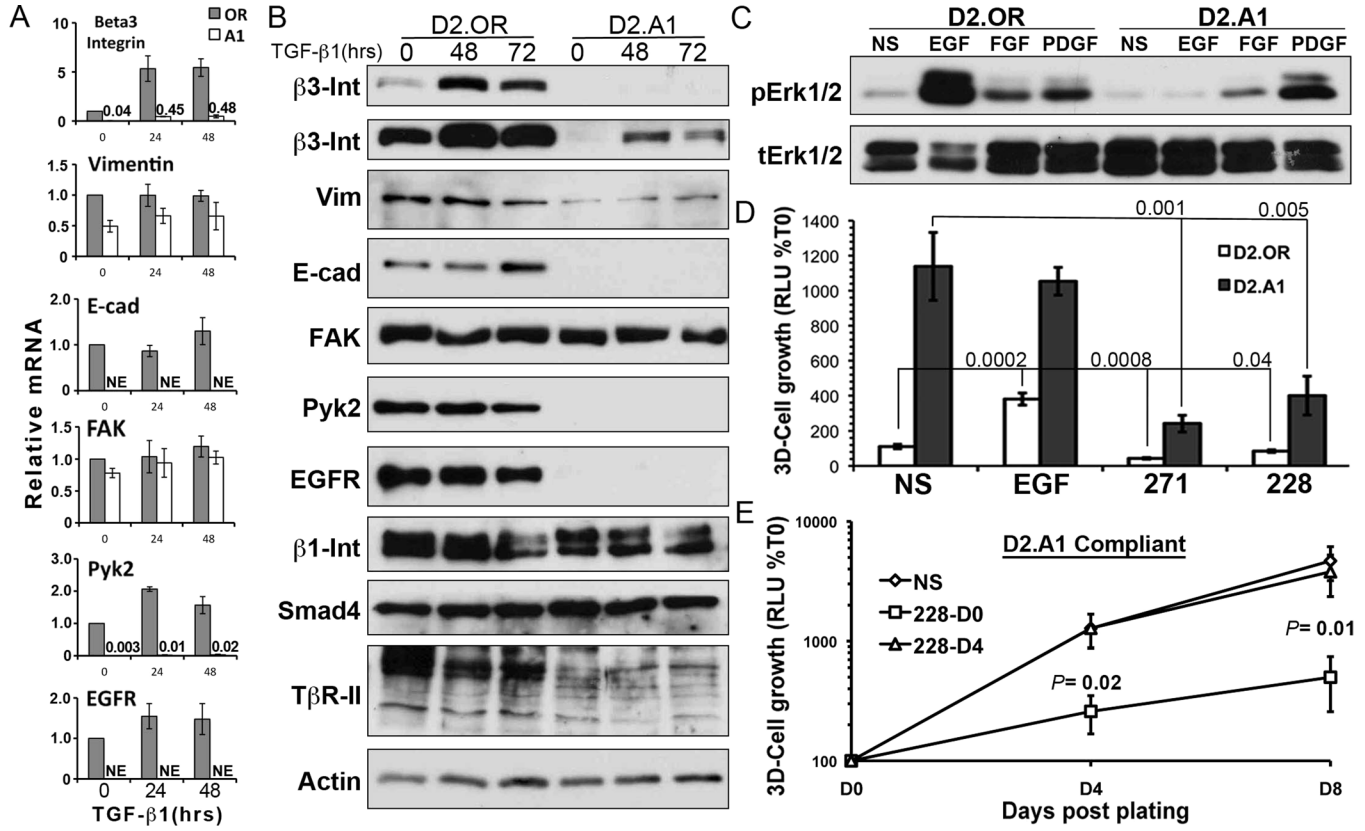


FIGURE 4: Outgrowth-proficient cells lack E-cad expression. (A) D2.OR and D2.A1 cells were stimulated with TGF-β1 (5 ng/ml) for 0–48 h and subsequently were analyzed for alterations in the indicated transcripts by RT-PCR. Data are the mean (±SE) of three independent experiments. *NE*, no expression. (B) D2.OR and D2.A1 cells were stimulated with TGF-β1 for the indicated times and analyzed by immunoblot for the indicated proteins. Data are representative of at least two independent analyses. Int, Integrin; Vim, Vimentin. (C) Quiescent D2.OR and D2.A1 cells were stimulated with the indicated growth factors for 30 min and analyzed for the presence of phospho-ERK1/2 (p-Erk1/2) and total Erk1/2 (t-Erk1/2) as a loading control. Data are representative of three independent experiments. (D) 3D-outgrowth of luminescent D2.OR and D2.A1 cells in the presence of EGF (50 ng/ml) or inhibitors. Data are the mean Day 5 values (±SE) of two independent experiments completed in triplicate. *NS*, no stimulation; *271*, FAK/Pyk2 inhibitor; *228*, FAK inhibitor. (E) 3D outgrowth of D2.A1 cells was monitored longitudinally in the absence or presence of the FAK inhibitor, PF228, that was added either at the initiation of the experiment (228-D0) or 4 d after cell plating (228-D4). Data are the mean (±SE) of three independent experiments completed in triplicate.

transwell assays (Figure 5E). Administration of TGF-β, however, significantly enhanced D2.A1 cell invasion, whereas identical TGF-β treatments of D2.OR cells failed to significantly enhance their invasion through reconstituted basement membranes (Figure 5E). Intriguingly, Figure 5F shows that propagating these D2.HAN derivatives in 3D cultures readily manifested and recapitulated the "TGF-β Paradox" in vitro. For instance, TGF-β dramatically inhibited the outgrowth of D2.OR organoids in compliant 3D cultures, but significantly stimulated the proliferation of D2.A1 organoids under identical culture conditions (Figure 5F). Additionally, D2.A1 cell outgrowth was critically dependent on autocrine TGF-β signaling as evidenced by the ability of TβR-I antagonism to significantly inhibit D2.A1 outgrowth (Figure 5F). We also incorporated type I collagen to these 3D cultures to make them mechanically rigid, which significantly enhanced the basal growth of D2-HAN derivatives (Figure 5F; Barkan *et al.*, 2010). Despite their enhanced growth in rigid 3D cultures, D2.OR cells remained sensitive to the cytostatic activities of TGF-β, whereas their D2.A1 counterparts remained insensitive to the antiproliferative activities of this cytokine (Figure 5F). Collectively these findings clearly demonstrate the necessity of 3D culture systems to manifest the "TGF-β Paradox." Moreover, our results also indicate that the function of TGF-β to either suppress or promote pulmonary outgrowth largely reflects its ability to govern the expression levels of E-cad in metastatic breast cancer cells.

Expression of E-cad is sufficient to block the initiation of pulmonary outgrowth

Given the differential expression of E-cad observed between D2.OR and D2.A1 cells, and the inability of D2.OR cells to down-regulate E-cad expression in response to TGF-β (Figure 4, A and B, and Supplemental Figure S3A), we next sought to investigate the mechanism by which loss of E-cad initiates metastatic outgrowth. In doing so, we engineered both D2-HAN derivatives to stably express either wild-type (WT) E-cad or a mutant E-cad molecule that lacked its extracellular domain (ΔE-Ecad) and functions as a dominant-negative protein through its ability to bind and sequester β-catenin in the cytoplasm (Dahl *et al.*, 1996; Onder *et al.*, 2008). Importantly, expression of WT E-cad, but not its ΔE-Ecad mutant, blocked the growth of D2.A1 organoids in 3D cultures (Figure 6B). Thus the homotypic binding properties of E-cad appear essential to its suppression of pulmonary outgrowth, whereas its ability to bind and sequester β-catenin appears dispensable for these events. Interestingly, the ability of E-cad to inhibit the 3D outgrowth of D2.A1 cells was circumvented by increased matrix rigidity

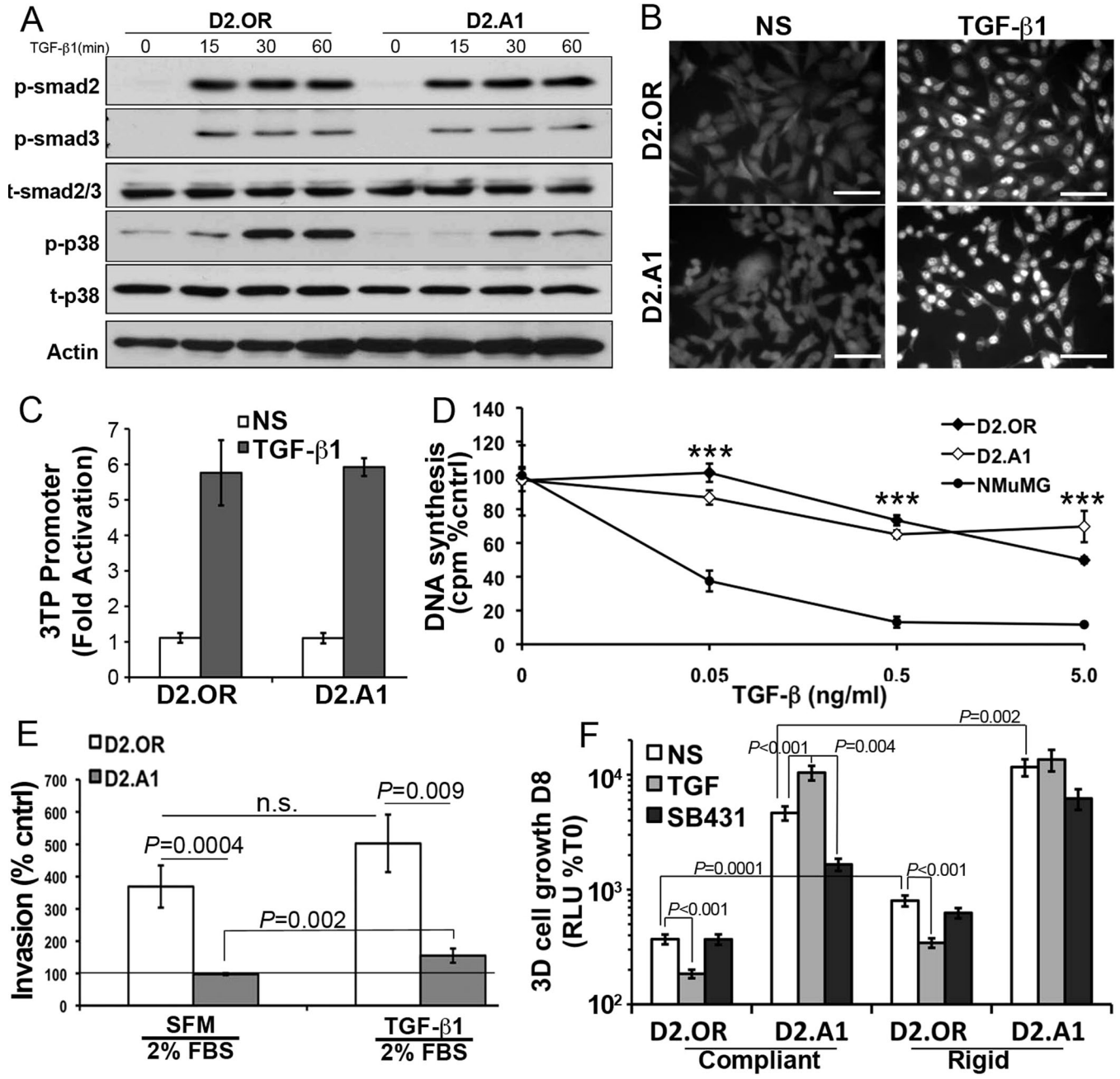


FIGURE 5: 3D culture is required to manifest the TGF-β paradox. (A) Quiescent D2.OR or D2.A1 cells were stimulated with TGF-β1 (5 ng/ml) for the indicated times and analyzed for the presence of phospho-Smad2 (p-smad2), phospho-Smad3 (p-smad3), and phospho-p38 mitogen-activated protein kinase (p-p38). Total Smad2/3 (t-smad2/3), total p38 (t-p38), and actin served as loading controls. Shown are representative immunoblots of three independent experiments. (B) Quiescent D2.OR and D2.A1 cells were stimulated with TGF-β1 as in (B) for 30 min before being processed for Smad2/3 immunofluorescence (400×). Data are representative of 10 random fields of view over two independent experiments. (C) D2.OR and D2.A1 cells were transfected with CMV-control and TGF-β–driven 3TP reporter constructs, and subsequently stimulated with TGF-β1 (5 ng/ml) for 18 h. Data are the mean (±SE) of three independent experiments completed in triplicate. (D) D2.OR, D2.A1, and NMuMG cells were incubated for 48 h in the absence or presence of increasing concentrations of TGF-β1 as indicated. Incorporation of [^{3}H]thymidine was determined as a measure of DNA synthesis. Data are normalized to untreated controls and are the mean (±SE) of three independent experiments completed in triplicate. ***, $p < 0.001$. (E) D2.OR or D2.A1 cells were inoculated into the top well of an invasion assay in the absence or presence of TGF-β1 (5 ng/ml). Data are normalized to serum-free medium (line) and are the mean (±SE) of two independent experiments completed in triplicate. n.s., not significant. (F) 3D outgrowth of D2.OR and D2.A1 cells under compliant and rigid conditions was quantified by bioluminescence. Where indicated, the cells were grown in the presence of TGF-β1 (5 ng/ml) or the TβR-I inhibitor, SB431542 (SB431, 10 μM). Data are the mean (±SE) of at least two independent experiments completed in triplicate.

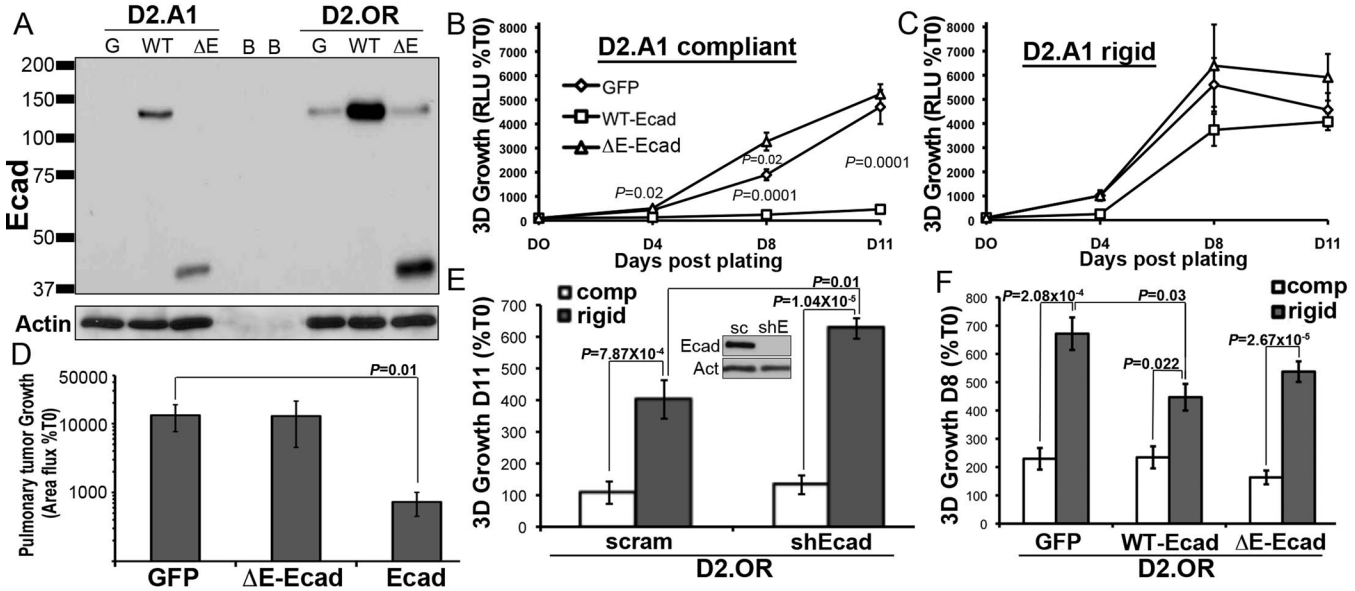


FIGURE 6: E-cad expression is sufficient to inhibit the initiation of pulmonary outgrowth. (A) Immunoblot showing recombinant expression of WT or ΔE-Ecad (ΔE) in the D2.OR and D2.A1 cells, and in GFP-expressing cells as a control (G). Blank wells are denoted as *B*. (B) The growth of control (GFP) or E-cad variant D2.A1 cells was quantified by bioluminescence in 3D outgrowth assays. (C) D2.A1 E-cad variant cells as in (B) were grown on 3D matrices that included type I collagen to increase its rigidity (Rigid). (D) Control (GFP) or E-cad variant D2.A1 cells were injected into the lateral tail vein of BALB/c mice, and pulmonary tumor growth was quantified using bioluminescence. Data are the mean (±SE; n = 10 mice per group) area flux values normalized to the injected values (%T0) 2 wk postinoculation. (E) E-cad deficiency significantly enhanced the growth of D2.OR cells in rigid 3D cultures. Inset, E-cad expression was stably depleted in D2.OR cells using shRNAs. sc, scrambled control; shE, Ecad-directed shRNA. Actin is shown as a loading control. (F) Overexpression of WT E-cad in D2.OR cells significantly inhibited their growth in rigid 3D cultures. Data for (B, C, E, and F) are the mean (±SE) of at least two independent experiments completed in triplicate.

(Figure 6C), suggesting that decreased tissue compliance may inactivate the tumor-suppressing activities of E-cad. Heterologous expression of E-cad in D2.A1 cells was resistant to administration of TGF-β or changes in matrix compliance and, more importantly, was able to elicit an epithelial morphology that prevented D2.A1 cells from undergoing EMT in response to TGF-β (Supplemental Figures S2 and S5). More importantly, Figure 6D shows that the expression of WT E-cad, but not its ΔE-Ecad mutant counterpart, effectively inhibited the initiation of metastatic outgrowth of D2.A1 cells in the lungs of BALB/c mice. Overexpression of WT or ΔE-Ecad had no effect on the dormancy of D2.OR cells under compliant pulmonary organotypic culture (Figure 6F); however, the enhanced growth of D2.OR cells on rigid matrices was 1) stimulated by depletion of endogenous E-cad (Figure 6E), 2) inhibited by the overexpression of WT E-cad (Figure 6F), and 3) unaffected by expression of ΔE-Ecad (Figure 6F). Taken together, these findings indicate that the extracellular domain of E-cad is sufficient to inhibit the initiation of pulmonary metastatic outgrowth by breast cancer cells.

E-cad regulates the expression of β1 integrin during 3D outgrowth

Our findings that increased matrix rigidity overcomes the ability of E-cad to suppress the metastatic outgrowth of breast cancer cells is supported by several recent studies that implicate β1 integrin as an essential mediator for the outgrowth of D2-HAN derivatives (Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). Unfortunately, D2.OR and D2.A1 cells express similar levels of β1 integrin (Figure 4B; Barkan *et al.*, 2010), and, as such, D2.OR cells would be expected to be similarly proficient in metastatic outgrowth as compared with their D2.A1 counterparts. Given these conflicting results, we instead hypothesized that E-cad may cross-talk with and/or regulate the expression and activity of β1 integrin in dormant breast cancer cells. To address this hypothesis, we first confirmed that the differential expression of E-cad was retained in the D2-HAN derivatives following their propagation in 3D cultures. Although TGF-β administration had no effect on E-cad expression in either D2-HAN derivative, this experimental condition did reduce β-catenin expression in D2.A1 cells, suggesting that dysregulated β-catenin signaling does not underlie the outgrowth of D2.A1 organoids (Figure 7A). Interestingly and consistent with a recent report by Green and colleagues (Barkan *et al.*, 2008), we found β1 integrin expression to be diminished in D2.OR cells propagated in 3D cultures (Figure 7A). More importantly, we observed heterologous expression of E-cad to suppress β1 integrin expression in D2.A1 cells (Figure 7B) and, conversely, depletion of E-cad expression in D2.OR cells to prevent their loss of β1 integrin expression in 3D cultures (Figure 7C). Equally intriguing, we found endogenous E-cad expression to be up-regulated significantly in dormant D2.OR cells upon their growth in 3D cultures (Figure 7C). These findings are in stark contrast to the down-regulated expression of E-cad observed in fully metastatic 4T1 cells (Figure 1, C and D), as well as in the outgrowth-proficient 4T07 cells (Figure 2D). Furthermore, the enhanced expression and/or stability of E-cad in D2.OR cells may explain our inability to reinitiate proliferation signals in D2.OR cells transduced with E-cad–directed shRNAs. Along these lines, reciprocal depletion of β1 integrin failed to alter E-cad expression in D2.A1 cells (Figure 7D); however, this cellular condition completely prevented these D2.A1 cells from forming any multicellular organoids, which contrasted sharply with the acquisition of branched cellular aggregates formed by E-cad–expressing D2.A1 cells (Figure 7E, Supplemental Figure S6). Therefore our findings have identified a novel mechanism

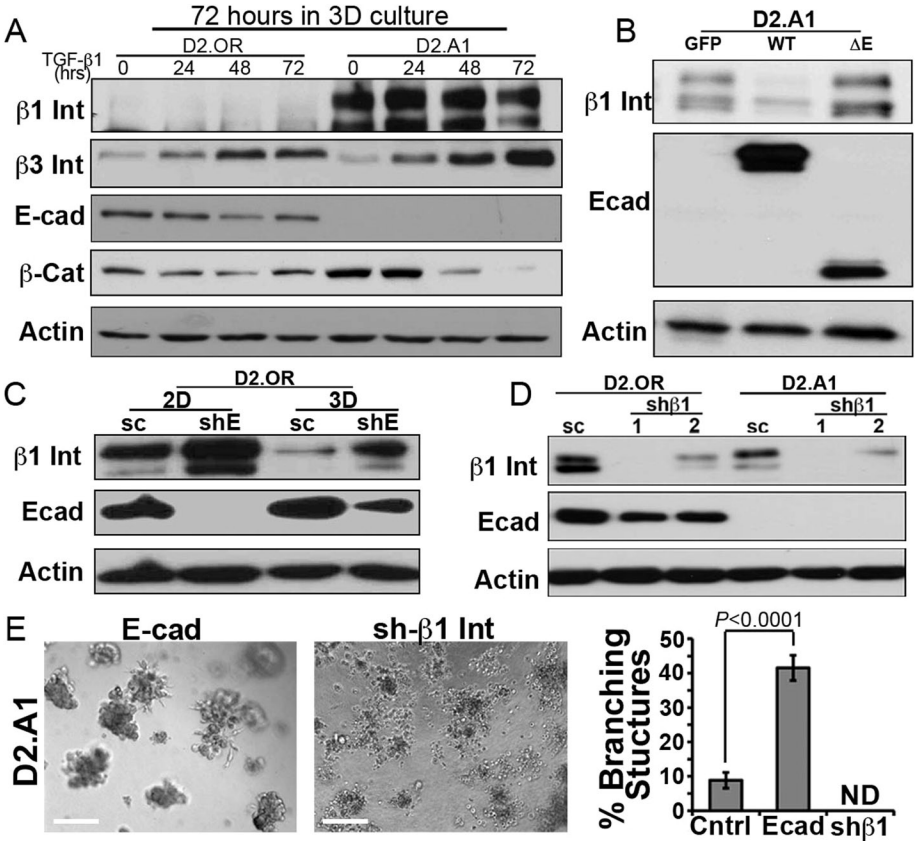


FIGURE 7: E-cad regulates β1 integrin expression during 3D outgrowth. (A) D2.OR and D2.A1 cells were propagated in 3D cultures for 72 h in the absence (0) or presence of TGF-β1 (5 ng/ml), and subsequently were analyzed for the expression of the indicated proteins by immunoblotting. Data are representative of at least three experiments. Int, integrin; Cat, catenin. (B) Control (GFP), WT E-cad (E-cad), or ΔE-Ecad (ΔE)-expressing D2.A1 cells were grown in 3D culture before visualizing the expression of β1 integrin (β1 Int), E-cad, and actin by immunoblotting. Data are representative of at least three experiments. (C) D2.OR cells expressing either a scrambled (sc) or an E-cad–specific (shE) shRNA were cultured in 2D or 3D conditions, and subsequently were analyzed for the expression of β1 integrin (β1 Int), E-cad, and actin as a loading control. (D) D2.OR and D2.A1 cells expressing either a scrambled (sc) or two distinct β1 integrin shRNAs (shβ1#1 and #2) were analyzed by immunoblotting to visualize the expression of β1 integrin (β1 Int), E-cad, and actin as a loading control. (E) D2.A1 cells expressing E-cad (E-cad) or depleted for β1 integrin (shβ1) were grown under compliant 3D cultures and imaged using phase-contrast microscopy (100×). Afterward, the percentage of branching structures was quantified. Data are the mean (±SE) of nine random fields of view over three independent experiments. ND, none detected.

a TGF-β–dependent or -independent manner. In doing so, we first monitored the expression of Twist, which is a master suppressor of E-cad expression (Yang *et al.*, 2004). Figure 8A shows that Twist expression was indeed significantly higher in metastatic D2.A1 cells as compared with their dormant D2.OR counterparts. Accordingly, transgenic expression of Twist in D2.OR cells was sufficient to initiate their outgrowth in 3D cultures (Figure 8B). The acquisition of 3D outgrowth by Twist-expressing D2.OR cells was accompanied by their abandonment of branched mammary structures in favor of dense metastatic cell spheroids (Figure 8C). Interestingly, although Twist expression failed to decrease that of E-cad in D2.OR cells propagated in traditional 2D cultures (Figure 8, A and D), this transcription factor readily inactivated E-cad expression in D2.OR cells propagated in 3D cultures (Figure 8D). Consistent with our findings in Figure 7, Twist-mediated down-regulation of E-cad expression in 3D cultures stabilized β1 integrin expression and created an E-cad$^{(low)}$/β1 integrin$^{(hi)}$ phenotype necessary for the initiation of 3D outgrowth (Figure 8D). Importantly, Figure 8E shows that Twist expression supported the initial metastatic outgrowth of D2.OR cells in the lungs of BALB/c mice. Finally, we also observed D2.A1 cells to express Snail (Figure 8F), which mediates EMT and down-regulates E-cad expression (Cano *et al.*, 2000). Overexpression of a green fluorescent protein (GFP)-Snail fusion protein, however, failed to down-regulate the expression of E-cad (Figure 8F) and, more importantly, was unable to initiate 3D outgrowth by D2.OR organoids (Figure 8G). Taken together, our findings suggest that elevated expression of the EMT transcription factor Twist, but not that of Snail, is sufficient to initiate pulmonary outgrowth.

whereby the extracellular domain of E-cad promotes the down-regulation of β1 integrin expression in breast cancer cells, an event coupled to MEC differentiation and metastatic dormancy. These data are supported by prior findings pointing to an extracellular interaction between E-cad and β1 integrin, an event that inhibits β1 integrin function and down-regulates its expression (Whittard *et al.*, 2002; Zhang *et al.*, 2006). Thus down-regulation of E-cad in metastatic cells permits their expression and activation of β1 integrin, which facilitates the initiation of metastatic outgrowth.

Twist is sufficient to elicit an outgrowth initiation competent phenotype

Having established E-cad as a molecular determinant of β1 integrin expression and metastatic outgrowth, we next sought to examine the role of known transcriptional regulators of E-cad expression in 1) governing the ability of TGF-β to regulate E-cad in systemically dormant breast cancer cells and 2) driving metastatic outgrowth in

DISCUSSION

EMT is a normal physiological process essential for proper development and wound healing (Taylor *et al.*, 2010); however, aberrant initiation of oncogenic EMT can promote the acquisition of invasive phenotypes in developing and progressing carcinomas, thereby driving their systemic dissemination (Wendt *et al.*, 2009a; Taylor *et al.*, 2010; Tian *et al.*, 2010). More recently, TGF-β stimulation of EMT was shown to establish a population of MECs that possess stem cell–like properties (Mani *et al.*, 2008). Thus the ability of individual breast cancer cells to undergo EMT in response to TGF-β may represent the molecular crux that endows TGF-β with oncogenic activity. Indeed, we recently found EMT induced by TGF-β to bestow EGF with oncogenic activity in breast cancers, as well as to enhance pulmonary tumor formation by breast cancer cells typically unable to undergo metastatic outgrowth (Wendt *et al.*, 2010). In the current study, we used a 3D organotypic culture system to investigate the molecular mechanisms of metastatic dormancy and its

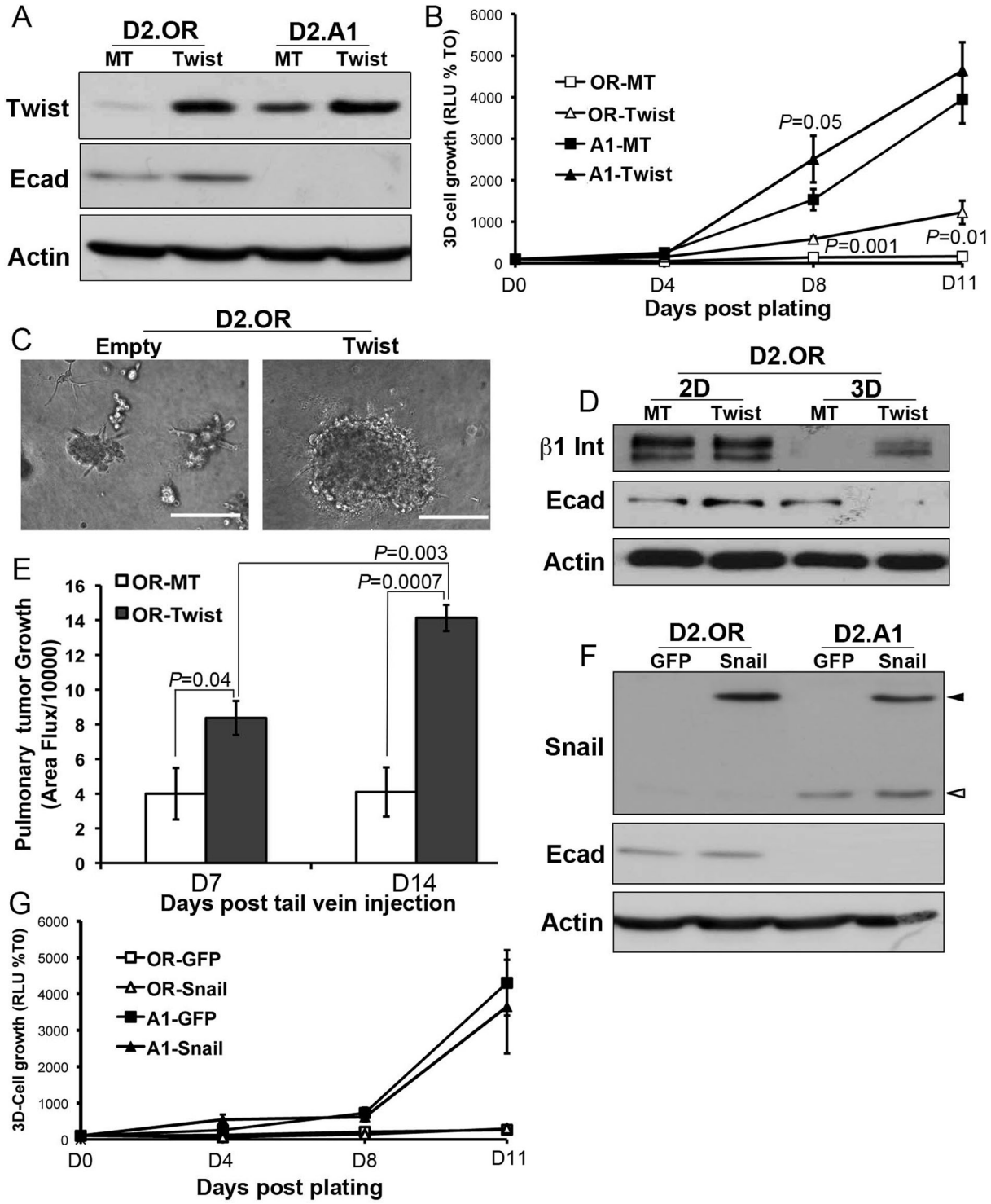


FIGURE 8: Twist expression is sufficient to initiate pulmonary outgrowth. (A) Immunoblot analysis of Twist and E-cad (Ecad) expression in D2.OR and D2.A1 cells transduced with Twist or empty vector (MT). (B) The 3D outgrowth values of the cells described in (A) were quantified using bioluminescence and are the mean (±SE) of three independent experiments completed in triplicate. (C) Representative photomicrographs (200×) of control (Empty) and Twist-expressing D2.OR cells propagated in 3D cultures. (D) Control (MT) and Twist-expressing D2.OR cells were propagated for 5 d under 2D or 3D culture conditions before analyzing their expression of β1 integrin (β1 Int), E-cad, or actin as a loading control. (E) Control (MT) and Twist-expressing D2.OR cells were injected into the lateral tail vein of BALB/c mice, and pulmonary tumor formation was quantified by bioluminescence. Data are the mean (±SE) area flux values of five mice per group at the indicated time points. (F) Immunoblot analysis showing Snail and E-cad (Ecad) expression in D2.OR and D2.A1 cells stably transfected with GFP-Snail or with GFP as a control. Open arrowhead indicates endogenous Snail; closed arrowhead indicates the GFP-Snail fusion protein. (G) The 3D outgrowth of the cells described in (E) was quantified using bioluminescence assays. Data are the mean (±SD) of triplicate wells from a representative experiment that was performed three times with similar results.

potential regulation by TGF-β and EMT. In doing so, we established down-regulated E-cad expression as a critical event in EMT-driven initiation of metastatic outgrowth. Moreover, characterization of the EMT status of the D2-HAN model of pulmonary outgrowth revealed that dormant breast cancer cells expressed abundant levels of E-cad, which was notably absent in their metastatic proficient counterparts, suggesting that an EMT event had transpired (Rak *et al.*, 1992; Morris *et al.*, 1994; Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). Accordingly, heterologous E-cad expression significantly inhibited the outgrowth of metastatic D2.A1 and MDA-MB-231 cells propagated in 3D cultures. Finally, unlike metastatic breast cancer cells that do express E-cad constitutively (e.g., 4T1 cells), systemically dormant breast cancer cells were incapable of down-regulating E-cad expression when propagated in 3D cultures or when treated with TGF-β. Importantly, we demonstrated that combining Twist expression with compliant 3D cultures did result in the down-regulation of E-cad expression and the initiation of 3D outgrowth.

Recent studies using the D2-HAN system to model metastatic dormancy versus proliferative outgrowth have demonstrated the necessity of β1 integrin in mediating 3D outgrowth of these cells (Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009). Indeed, propagation of dysmorphic, malignant MEC organoids in 3D cultures requires aberrant expression and activity of β1 integrin (Weaver *et al.*, 1997). Unfortunately, similar levels of β1 integrin between the D2-HAN derivatives suggest that its expression and activity cannot solely account for metastatic outgrowth failure (D2.OR) or success (D2.A1). Given the dramatic differences in E-cad expression between the D2 cell lines, we hypothesized that this adherens molecule is a key determinant in regulating β1 integrin action under compliant 3D growth conditions. Along these lines, the extracellular domain of E-cad can regulate β1 integrin function by acting as a heterotypic binding partner (Whittard *et al.*, 2002) and by down-regulating β1 integrin expression (Zhang *et al.*, 2006). Moreover, ectopic E-cad expression in the MDA-MB-231 cells, which significantly reduced their 3D outgrowth (Figure 1), effectively inhibited their expression of β1 integrin and activation of FAK (Wu *et al.*, 2006). These findings wholly support our conclusion that the heterotypic interaction of E-cad with β1 integrin results in a loss of β1 integrin expression coupled to metastatic dormancy. In contrast to β1 integrin, we observed D2.OR cells to express markedly higher levels of β3 integrin as compared with D2.A1 cells. Given the essential role of β3 integrin in regulating TGF-β stimulation of breast cancer EMT and metastasis (Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Wendt and Schiemann, 2009; Wendt *et al.*, 2010), we suspect that elevated β3 integrin expression underlies the 1) enhanced invasive and migratory capacity of D2.OR cells, and 2) elevated invasion of D2.A1 cells in response to TGF-β. Thus β3 integrin expression represents one of the most sensitive and robust markers of TGF-β signaling during the invasive progression of metastatic mammary tumors. Collectively these findings highlight the dynamic interactions that transpire between carcinoma cells and their microenvironments in dictating metastatic proficiency of breast cancers; they also suggest that the delivery of extracellular E-cad mimetics may prevent the initiation of metastatic outgrowth by disseminated breast cancers through interaction and suppression of β1 integrin.

We recently demonstrated the ability of EMT induced by TGF-β to stabilize EGFR expression, an event that conferred oncogenic and invasive capacity to EGF (Wendt *et al.*, 2010). Following chronic TGF-β treatment of NM-E cells, however, we observed their subsequent withdrawal and recovery from TGF-β to elicit a mesenchymal-epithelial transition (MET) that generated a population of MECs that lacked EGFR expression. This intriguing finding is consistent with the 1) malfunction of D2.A1 cells to express EGFR (Figure 4); 2) selection of highly mesenchymal E-cad$^{(neg)}$/EGFR$^{(neg)}$ subpopulation of cells in response to TGF-β (Wendt *et al.*, 2010); and 3) initiation of "RTK (receptor tyrosine kinase) switching" in post-EMT breast and lung cancer cells that acquire resistance to EGFR-directed therapies (Barr *et al.*, 2008; Thomson *et al.*, 2008). Future studies need to determine the molecular mechanisms whereby TGF-β, EMT, and E-cad dictate the repertoire of RTKs expressed in systemically disseminated breast cancer cells. This knowledge, together with enhanced understanding of how metastatic microenvironments and niches govern RTK switching, will enable the development of specialized therapies against dormant micrometastases harbored within vital tissues.

FAK is an essential signaling node targeted by E-cad, various RTKs, and β1 and β3 integrins. We (Wendt and Schiemann, 2009) and others (Cicchini *et al.*, 2008) established FAK as an essential mediator of EMT induced by TGF-β and of metastatic outgrowth by D2.A1 cells (Shibue and Weinberg, 2009). We now show that FAK activity is absolutely essential to the initiation of 3D organotypic outgrowth but not to the maintained growth of established 3D organoids (Figure 4). Collectively these findings coalesce to support the notion that 1) EMT is required for the initiation of micrometastatic outgrowth, which is clearly FAK-dependent, and 2) MET is required for the continued proliferation and expansion of macrometastastic growth, which may be FAK-independent (Figure 4; Hugo *et al.*, 2007; Chao *et al.*, 2010). These findings have important therapeutic ramifications because FAK inhibitors are currently being evaluated in clinical trials (Lim *et al.*, 2008); however, our present findings do not address the potential role of nuclear FAK and its FERM (Band 4.1/Ezrin/Radixin/Moesin homology) domain to promote carcinoma growth and survival, independent of its PTK (protein tyrosine kinase) activity (Lim *et al.*, 2008). Indeed, we have recently observed TGF-β to promote the proliferation of breast cancer cells in part by stimulating the nuclear accumulation of FAK and its FERM domain, findings that are currently under investigation (M. K. Wendt, B. J. Schiemann, and W. P. Schiemann, unpublished data). Finally, Twist expression, but not that of Snail, initiated the pulmonary outgrowth of D2.OR cells. Despite this initial proliferative event, Twist-expressing D2.OR cells ultimately failed to form lethal macroscopic pulmonary lesions in the lungs of mice (unpublished data). These findings, together with those detecting the reexpression of E-cad in fully formed 3D organoids (Figure 2), strongly suggest that MET is required for the maintenance and continued outgrowth of pulmonary metastases, a reaction that fails to occur in cells engineered to constitutively express master EMT drivers such as Twist (Yang *et al.*, 2004). Furthermore, these data suggest that Twist and Snail mediate distinct and nonredundant functions during EMT and metastasis. Indeed, recent findings delineate unique functions for these transcription factors in regulating carcinoma invasion, metastasis, and chemoresistance (Gupta *et al.*, 2009; Casas *et al.*, 2011). Future studies need to further delineate the downstream effectors of Twist and its control over EMT that are operant in overcoming senescence to generate metastatic initiating cells, and how these processes then convert to a MET to support the formation of lethal macroscopic pulmonary lesions (Ansieau *et al.*, 2008; Mani *et al.*, 2008). Experiments are currently under way to address these important questions.

MATERIALS AND METHODS

Cell lines and reagents

D2-HAN (D2.OR and D2.A1) and 4T1 derivatives (67NR, 168FARN, 66cl4, 4T07, and 4T1) were obtained from Fred Miller (Wayne State University, Detroit, MI) and cultured in DMEM supplemented

with 10% fetal bovine serum and 1% Pen/Strep as described previously (Wendt *et al.*, 2010). Bioluminescent D2-HAN and 4T1 derivatives were engineered to stably express luciferase by transfection with pNifty-CMV-luciferase as described (Wendt *et al.*, 2008; Wendt and Schiemann, 2009). Dual bioluminescent 4T1 cells were generated by transfection with pcDNA3.1-renilla luciferase, followed by hygromycin selection. Afterward, renilla luciferase-expressing 4T1 cells were transfected with firefly luciferase the expression of which was driven by the human E-cad promoter (pGL4.20, –108 to +125 base pairs), followed by selection with puromycin. Expression of WT or a dominant-negative E-cad mutant lacking its extracellular domain (ΔE-Ecad; Dahl *et al.*, 1996; Onder *et al.*, 2008) was achieved by vesicular stomatitis virus-glycoprotein (VSVG) retroviral transduction of pWZL and selection with blastocidin (2–10 μg/ml). Cellular depletion of E-cad and β1 integrin expression was achieved by VSVG lentiviral transduction of pLKO.1 shRNA vectors (Open Biosystems, Huntsville, AL; Supplemental Table S2) as described (Wendt and Schiemann, 2009). Expression of Twist was achieved by VSVG retroviral transduction of pBabe and selected with puromycin. A pEGFP-C2 construct encoding GFP-Snail was provided by Thomas T. Egelhoff (Cleveland Clinic Foundation, Cleveland, OH), and breast cancer cells stably expressing this fusion protein were selected with G418.

In vivo bioluminescence imaging

WT D2.A1 and D2.OR (1×10^6 cells), E-cad–expressing D2.A1 (1×10^5 cells), or Twist-expressing D2.OR (1×10^6 cells) were injected into the lateral tail vein of 4-wk-old BALB/c mice, and pulmonary tumor development was assessed by weekly bioluminescence imaging normalized to an initial reading conducted immediately after inoculation. Bioluminescence imaging was performed on an IVIS-200 (Caliper Life Sciences, Hopkinton, MA) as described (Wendt and Schiemann, 2009; Wendt *et al.*, 2009b, 2010), and in accordance with the Institutional Animal Care and Use Committees for the University of Colorado Denver and Case Western Reserve University.

3D organotypic growth assays

Malignant MECs were diluted in complete medium supplemented with 5% Cultrex (Trevigen, Gaithersburg, MD) and seeded onto solidified Cultrex cushions (50 μl/well) contained in 96-well plates (5000 cells/cm^2). Where indicated, the cells were grown in the presence of 1) EGF (50 ng/ml); 2) the dual FAK/Pyk2 inhibitor, PF562271 (10 μM; Pfizer, Groton, CT); and 3) the FAK-specific inhibitor, PF573228 (10 μM; Pfizer). The medium/Cultrex mixtures were replaced every 4 d, and cellular outgrowth was detected by the addition of D-luciferin potassium salt (Caliper Life Sciences) to induce bioluminescence, which was quantified using a GloMax-Multi detection system (Promega, Madison, WI). Longitudinal cell growth was normalized to an initial reading taken 18 h after cell plating. Where indicated, collagen type I (3 mg/ml; BD Biosciences, Bedford, MA) was incorporated into Cultrex cushions to increase their rigidity. For long-term (5–14 d) morphology experiments, the cells were propagated on 175 μl Cultrex cushions contained within 48-well plates (5×10^4 cells/cm^2), and short-term (24 h) cellular aggregation experiments transpired on 400 ml Cultrex cushions contained within 12-well dishes (7.5×10^4 cells/cm^2). Time-lapse microscopy was performed using a Leica DMI6000 over a span of 18 h with the resulting images being captured once every 10 min.

Immunoblot assays

Lysates generated from 3D cultures were prepared by removing the medium/Cultrex overlay and adding lysis buffer directly to the organoids grown on top of Cultrex cushions. The resulting mixtures were incubated and shaken continuously for 60 min before harvesting the solubilized extract away from the remaining intact Cultrex cushion. Afterward, the whole cell extracts were processed for immunoblotting as described (Wendt and Schiemann, 2009; Wendt *et al.*, 2009b, 2010). Antibodies used herein are described in Supplemental Table S1.

Cell biological assays

The ability of TGF-β1 (5 ng/ml) to alter serum-induced invasion of D2.OR and D2.A1 cells was analyzed using a Matrigel-coated transwell assay as described (Galliher and Schiemann, 2006). Incorporation of [^{3}H]thymidine into cellular DNA to monitor DNA synthesis was accomplished as described (Wendt *et al.*, 2009b), as was the detection luciferase reporter gene expression regulated by TGF-β and by 3D cultures (Wendt *et al.*, 2010). For real-time PCR analysis, D2.OR and D2.A1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 or 48 h, at which point total RNA was isolated using an RNeasy Plus Kit (Qiagen, Valencia, CA). Afterward, total RNA was reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad, Hercules, CA), and semiquantitative real-time PCR was conducted using iQ SYBR Green (Bio-Rad) as described previously (Wendt and Schiemann, 2009). The oligonucleotide primer pairs used are provided in Supplementary Table S2. Indirect immunofluorescence of Smad2/3 subcellular localization or direct phalloidin fluorescence to visualize the actin cytoskeleton was accomplished as described previously (Wendt and Schiemann, 2009; Wendt *et al.*, 2009b)

Statistical analyses

Statistical values were defined using an unpaired Student's *t* test, where a p value < 0.05 was considered significant. Values of p for all experiments analyzed are indicated.

ACKNOWLEDGMENTS

We thank Pfizer for generously providing the small molecule inhibitors against FAK and Pyk2. W.P.S. was supported in part by grants from the National Institutes of Health (CA129359), the Susan G. Komen for the Cure Foundation (BCTR0706967), and the Department of Defense (BC084561). M.K.W. was supported by a fellowship from the American Cancer Society (PF-09–120-01).

REFERENCES

Ansieau S *et al.* (2008). Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell 14, 79–89.

Aslakson CJ, Miller FR (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 52, 1399–1405.

Barkan D *et al.* (2008). Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68, 6241–6250.

Barkan D *et al.* (2010). Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. Cancer Res 70, 5706–5716.

Barr S *et al.* (2008). Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. Clin Exp Metastasis 25, 685–693.

Battula VL *et al.* (2010). Epithelial-mesenchymal transition-derived cells exhibit multilineage differentiation potential similar to mesenchymal stem cells. Stem Cells 28, 1435–1445.

Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL (2001). Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. J Biol Chem 276, 46707–46713.

Butcher DT, Alliston T, Weaver VM (2009). A tense situation: forcing tumour progression. Nat Rev Cancer 9, 108–122.

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000). The transcription factor Snail controls

epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2, 76–83.
Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J (2011). Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res 71, 245–254.
Chao YL, Shepard CR, Wells A (2010). Breast carcinoma cells reexpress E-cadherin during mesenchymal to epithelial reverting transition. Mol Cancer 9, 179.
Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A, Fantoni A, Amicone L, Tripodi M (2008). TGF-beta-induced EMT requires focal adhesion kinase (FAK) signaling. Exp Cell Res 314, 143–152.
Cowin P, Welch DR (2007). Breast cancer progression: controversies and consensus in the molecular mechanisms of metastasis and EMT. J Mammary Gland Biol Neoplasia 12, 99–102.
Dahl U, Sjodin A, Semb H (1996). Cadherins regulate aggregation of pancreatic beta-cells in vivo. Development 122, 2895–2902.
Drake JM, Strohbehn G, Bair TB, Moreland JG, Henry MD (2009). ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. Mol Biol Cell 20, 2207–2217.
Gal A, Sjoblom T, Fedorova L, Imreh S, Beug H, Moustakas A (2008). Sustained TGF beta exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. Oncogene 27, 1218–1230.
Galliher AJ, Schiemann WP (2006). Beta3 integrin and Src facilitate transforming growth factor-beta mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 8, R42.
Galliher AJ, Schiemann WP (2007). Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res 67, 3752–3758.
Galliher-Beckley AJ, Schiemann WP (2008). Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-beta. Carcinogenesis 29, 244–251.
Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG (2000). Methylation patterns of the E-cadherin 5′ CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem 275, 2727–2732.
Graff JR, Greenberg VE, Herman JG, Westra WH, Boghaert ER, Ain KB, Saji M, Zeiger MA, Zimmer SG, Baylin SB (1998). Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. Cancer Res 58, 2063–2066.
Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 138, 645–659.
Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW (2007). Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. J Cell Physiol 213, 374–383.
Lim ST, Mikolon D, Stupack DG, Schlaepfer DD (2008). FERM control of FAK function: implications for cancer therapy. Cell Cycle 7, 2306–2314.
Lombaerts M *et al*. (2006). E-cadherin transcriptional down-regulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br J Cancer 94, 661.
Mani SA *et al*. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133, 704–715.
Mbalaviele G, Dunstan CR, Sasaki A, Williams PJ, Mundy GR, Yoneda T (1996). E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. Cancer Res 56, 4063–4070.
Miettinen PJ, Ebner R, Lopez AR, Derynck R (1994). TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol 127, 2021–2036.
Morris VL, Koop S, MacDonald IC, Schmidt EE, Grattan M, Percy D, Chambers AF, Groom AC (1994). Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metastasis 12, 357–367.
Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE, Graff JR (2000). Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. Cancer Res 60, 4346–4348.
Neil JR, Johnson KM, Nemenoff RA, Schiemann WP (2008). Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-beta through a PGE2-dependent mechanisms. Carcinogenesis 29, 2227–2235.
Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA (2008). Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 68, 3645–3654.
Rak JW, McEachern D, Miller FR (1992). Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression of murine mammary neoplasia. Br J Cancer 65, 641–648.
Roberts WG *et al*. (2008). Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. Cancer Res 68, 1935–1944.
Shibue T, Weinberg RA (2009). Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. Proc Natl Acad Sci USA 106, 10290–10295.
Taube JH *et al*. (2010). Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA 107, 15449–15454.
Taylor MA, Parvani JG, Schiemann WP (2010). The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia 15, 169–190.
Thiery JP (2002). Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2, 442–454.
Thomson S, Petti F, Sujka-Kwok I, Epstein D, Haley JD (2008). Kinase switching in mesenchymal-like non-small cell lung cancer lines contributes to EGFR inhibitor resistance through pathway redundancy. Clin Exp Metastasis 25, 843–854.
Tian M, Neil JR, Schiemann WP (2010). Transforming growth factor-beta and the hallmarks of cancer. Cell Signal 23, 951–962.
van Nimwegen MJ, Verkoeijen S, van Buren L, Burg D, van de Water B (2005). Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. Cancer Res 65, 4698–4706.
Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ (2002). Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst 94, 1494–1503.
Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 137, 231–245.
Wendt M, Schiemann W (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. Breast Cancer Research 11, R68.
Wendt MK, Allington TM, Schiemann WP (2009a). Mechanisms of the epithelial-mesenchymal transition by TGF-beta. Future Oncol 5, 1145–1168.
Wendt MK, Cooper AN, Dwinell MB (2008). Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. Oncogene 27, 1461–1471.
Wendt MK, Smith JA, Schiemann WP (2009b). p130Cas is required for mammary tumor growth and transforming growth factor-beta-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem 284, 34145–34156.
Wendt MK, Smith JA, Schiemann WP (2010). Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene 29, 6485–6498.
Whittard JD, Craig SE, Mould AP, Koch A, Pertz O, Engel JR, Humphries MJ (2002). E-cadherin is a ligand for integrin alpha2-beta1. Matrix Biol 21, 525–532.
Wu H, Liang YL, Li Z, Jin J, Zhang W, Duan L, Zha X (2006). Positive expression of E-cadherin suppresses cell adhesion to fibronectin via reduction of alpha5-beta1 integrin in human breast carcinoma cells. J Cancer Res Clin Oncol 132, 795–803.
Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117, 927–939.
Yoshida BA, Sokoloff MM, Welch DR, Rinker-Schaeffer CW (2000). Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl Cancer Inst 92, 1717–1730.
Zantek ND, Azimi M, Fedor-Chaiken M, Wang B, Brackenbury R, Kinch MS (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. Cell Growth Differ 10, 629–638.
Zhang W, Alt-Holland A, Margulis A, Shamis Y, Fusenig NE, Rodeck U, Garlick JA (2006). E-cadherin loss promotes the initiation of squamous cell carcinoma invasion through modulation of integrin-mediated adhesion. J Cell Sci 119, 283–291.

Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression

Michael K. Wendt, Maozhen Tian & William P. Schiemann

Cell and Tissue Research

ISSN 0302-766X
Volume 347
Number 1

Cell Tissue Res (2012) 347:85-101
DOI 10.1007/s00441-011-1199-1

Springer

Your article is protected by copyright and all rights are held exclusively by Springer-Verlag. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your work, please use the accepted author's version for posting to your own website or your institution's repository. You may further deposit the accepted author's version on a funder's repository at a funder's request, provided it is not made publicly available until 12 months after publication.

Author's personal copy

Cell Tissue Res (2012) 347:85–101
DOI 10.1007/s00441-011-1199-1

REVIEW

Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression

Michael K. Wendt · Maozhen Tian · William P. Schiemann

Received: 25 April 2011 / Accepted: 1 June 2011 / Published online: 21 June 2011
© Springer-Verlag 2011

Abstract Transforming growth factor-β (TGF-β) is a potent pleiotropic cytokine that regulates mammalian development, differentiation, and homeostasis in essentially all cell types and tissues. TGF-β normally exerts anticancer activities by prohibiting cell proliferation and by creating cell microenvironments that inhibit cell motility, invasion, and metastasis. However, accumulating evidence indicates that the process of tumorigenesis, particularly that associated with metastatic progression, confers TGF-β with oncogenic activities, a functional switch known as the "TGF-β paradox." The molecular determinants governing the TGF-β paradox are complex and represent an intense area of investigation by researchers in academic and industrial settings. Recent findings link genetic and epigenetic events in mediating the acquisition of oncogenic activity by TGF-β, as do aberrant alterations within tumor microenvironments. These events coalesce to enable TGF-β to direct metastatic progression via the stimulation of epithelial-mesenchymal transition (EMT), which permits carcinoma cells to abandon polarized epithelial phenotypes in favor of apolar mesenchymal-like phenotypes. Attempts to deconstruct the EMT process induced by TGF-β have identified numerous signaling molecules, transcription factors, and microRNAs operant in mediating the initiation and resolution of this complex transdifferentiation event. In addition to its ability to enhance carcinoma cell invasion and metastasis, EMT also endows transitioned cells with stem-like properties, including the acquisition of self-renewal and tumor-initiating capabilities coupled to chemoresistance. Here, we review recent findings that delineate the pathophysiological mechanisms whereby EMT stimulated by TGF-β promotes metastatic progression and disease recurrence in human carcinomas.

Keywords Cancer stem cells · Chemoresistance · Epithelial-mesenchymal transition · Integrins · Metastasis · Transforming growth factor-β · Tumor microenvironment

Introduction

Transforming growth factor-β (TGF-β) is a ubiquitously expressed cytokine that regulates an assortment of biological activities in essentially all cell types and tissues. In addition to its role in regulating cell development, differentiation, and survival, TGF-β inhibits the proliferation of epithelial, endothelial, and hematopoietic cell lineages (Blobe et al. 2000; Massague 2008; Tian et al. 2011). Interestingly, resistance to TGF-β-mediated cytostasis is a hallmark of neoplastic transformation, which ultimately transforms the signals produced by this cytokine into oncogenic activities, particularly enhanced cancer cell invasion and metastasis. This conversion in TGF-β function is known as the "TGF-β paradox" (Rahimi and Leof 2007; Schiemann 2007; Tian and Schiemann 2009) and underlies the adverse prognosis associated with elevated TGF-β levels in developing carcinomas, including their acquisition of epithelial-mesenchymal transition (EMT),

M.K.W and M.T. have equally contributed to this review.

W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), the Department of Defense (BC084651), the Case Comprehensive Cancer Center, and the University Hospitals Seidman Cancer Center. M.K.W. was supported by the American Cancer Society (PF-09-120-01-CS).

M. K. Wendt · M. Tian · W. P. Schiemann (✉)
Case Comprehensive Cancer Center, Division of General Medical Sciences—Oncology, Case Western Reserve University, Wolstein Research Building, 2103 Cornell Road, Cleveland OH 44106, USA
e-mail: william.schiemann@case.edu

Author's personal copy

metastatic, and chemoresistant phenotypes (Taylor et al. 2010; Tian and Schiemann 2009; Wendt et al. 2009a). The molecular mechanisms whereby TGF-β suppresses tumor formation remain to be fully elucidated, as does the manner in which this cytokine promotes the progression of late-stage carcinomas. Historically, investigations into TGF-β action have applied "cell-centric" approaches to interrogate the TGF-β paradox by means of monitoring the differential expression of gene transcripts, proteins, and microRNAs that exist between normal and malignant cells as a potential means to explain the dichotomous functions of TGF-β. Although enormous strides have been made through these analyses (Kang et al. 2003; Minn et al. 2005; Zavadil et al. 2001), the results have collectively failed both to explain the contradictory nature of TGF-β and to recapitulate accurately the TGF-β paradox in experimental settings. Because of these limitations, recent studies have begun to investigate the impact that dynamic alterations in carcinoma microenvironments play in dictating the behaviors of TGF-β. Indeed, numerous findings point to a prominent role of EMT in manifesting the TGF-β paradox and its ability to couple TGF-β to metastatic progression in human carcinomas. Here, we review recent results that directly impact our understanding of the role of TGF-β in mediating the initiation and resolution of EMT, and the way that these events underlie the pathophysiology of TGF-β in human malignancies.

TGF-β signaling

Mammals express three genetically distinct TGF-β ligands (e.g., TGF-βs 1–3) whose mature and biologically active forms are ~97% identical and exhibit virtually indistinguishable actions in vitro (Blobe et al. 2000, Massague 1998). Individual TGF-β ligands are expressed spatiotemporally during embryogenesis and tissue morphogenesis, events contributing to the vast array of diverse and nonredundant phenotypes displayed by mice lacking distinct TGF-β isoforms (Chang et al. 2002). TGF-β signaling commences by its binding to three high-affinity receptors, TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan) receptors. When expressed, TβR-III is the most abundant TGF-β receptor present on the cell surface where it (1) functions as an accessory receptor that binds and modulates TGF-β function in responsive cells and (2) mediates the tumor-suppressing activities of TGF-β in tissues of the breast, ovary, prostate, lung, pancreas, kidney, and endometrium (Gatza et al. 2010). Although TβR-III lacks intrinsic enzymatic activity, TβR-I and TβR-II both harbor Ser/Thr protein kinases in their cytoplasmic domains that initiate intracellular signaling (Feng and Derynck 2005; Massague and Gomis 2006; Shi and Massague 2003). The binding of TGF-β to TβR-II enables this polypeptide to transphosphorylate and activate TβR-I, which subsequently binds, phosphorylates, and stimulates the latent transcription factors, Smad2 and Smad3 (Feng and Derynck 2005; Massague and Gomis 2006; Shi and Massague 2003). Phosphorylated Smad2/3 rapidly form high order complexes with the common Smad, Smad4, which enables the resulting heterotrimeric complexes to take up residence in the nucleus where they regulate transcription in a gene- and cell-specific manner (Feng and Derynck 2005; Massague and Gomis 2006; Shi and Massague 2003). Changes in cell behavior regulated by the activation of Smad2/3 are referred to as "canonical TGF-β signaling", and these events are modulated in all subcellular compartments by numerous effector molecules.

In addition to its activation of canonical Smad2/3 signaling, TGF-β also couples to a variety of noncanonical effector molecules (i.e., Smad2/3-independent), including (1) the mitogen-activated protein (MAP) kinases, ERK1/2, p38MAPK, and JNK; (2) the cell survival mediators, PI3K, AKT1/2, and mTOR; (3) the inflammation mediators, NF-κB, cyclooxygenase-2 (Cox-2), and prostaglandins; (4) the small GTP-binding proteins, Ras, RhoA (Ras homolog gene family, member A), Rac1, and Cdc42; and (5) the nonreceptor protein tyrosine kinases, Src, FAK, and Abl (Kang et al. 2009; Moustakas and Heldin 2005; Parvani et al. 2011). Interestingly, recent findings indicate that the coupling of TGF-β to its noncanonical effectors appears inappropriately amplified in metastatic cancer cells, thereby generating a signaling imbalance that overrides and/or dampens the tumor-suppressing messages transduced by Smad2/3 in human tumors (Wendt et al. 2009b). Figure 1 depicts the interactome of noncanonical effector molecules targeted by TGF-β during EMT and metastatic progression. Unfortunately, the way that these noncanonical effectors are precisely activated by TGF-β in normal and malignant cells remains to be determined definitively, as does the manner in which these pathways become dysregulated and magnified during the acquisition of metastatic phenotypes by cancer cells. These relationships and their regulation by tumor microenvironments during the initiation of EMT and metastatic progression driven by TGF-β are discussed below.

Epithelial-mesenchymal transitions

Definition and classical EMT phenotypes

EMT is a normal physiological process that is essential for embryogenesis and tissue morphogenesis and for tissue remodeling and repair during wound healing (Wendt et al. 2009a). However, pathological EMT is increasingly recog-

Author's personal copy

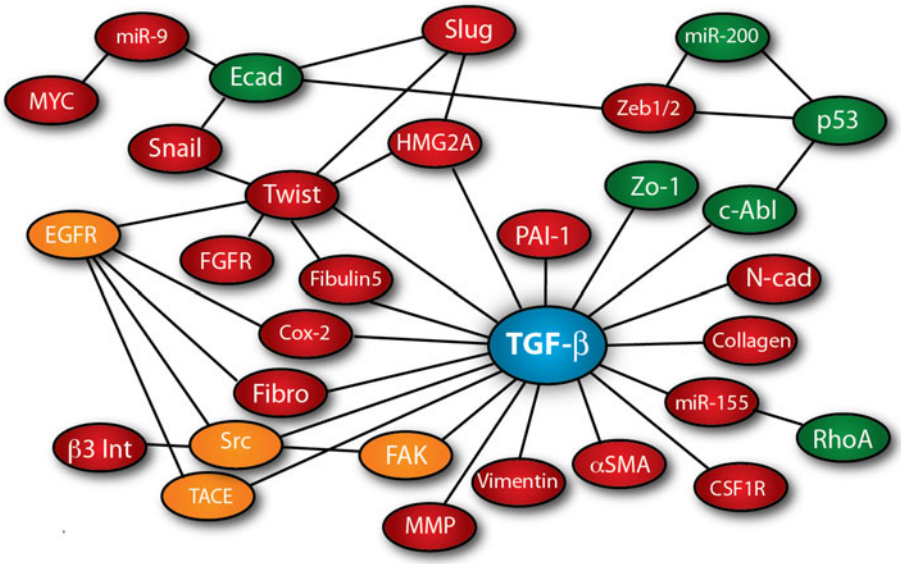


Fig. 1 Epithelial-mesenchymal transition (EMT) signaling network and transcriptome regulated by TGF-β (*red ellipses* genes whose expression is increased by TGF-β during EMT, *green ellipses* genes whose expression is decreased by TGF-β during EMT, *orange ellipses* increased enzymatic activity stimulated by TGF-β during EMT, *lines between nodes* describe the interaction or transactivation between these molecules during EMT)

nized to play an important role during the development of human diseases, including chronic inflammation, fibrosis, rheumatoid arthritis, and cancer invasion and metastasis (Micalizzi et al. 2010a; Taylor et al. 2010; Thiery et al. 2009). EMT was first described almost a century ago with the observation that epithelial cells in chick embryos acquire mesenchymal-like features (Thiery 2002), an event officially recognized as being a discrete physiological process by Greenberg and Hay in 1982 (Greenburg and Hay 1982). The ability of TGF-β to induce EMT was initially described by Derynck and colleagues in 1994 (Miettinen et al. 1994). During the intervening years since this important discovery, the findings of numerous studies have coalesced in establishing TGF-β as a master regulator of the initiation and resolution of EMT under a variety of pathophysiological contexts (Taylor et al. 2010; Wendt et al. 2009a). Typically, differentiated epithelium is composed of a single layer of polarized epithelial cells that possess an apical and basolateral orientation and of an actin cytoskeleton organized into defined "cobblestones" attributable to the concentration of polymerized actin fibers at cell-cell junctions. The EMT process occurs when fully differentiated epithelial cells undergo a transdifferentiation process that gives rise to their bearing fibroblastoid-like phenotypes (Micalizzi et al. 2010a; Taylor et al. 2010; Thiery et al. 2009). Additionally, transdifferentiating epithelial cells exhibit a variety of common molecular features, including (1) the downregulated expression of polarized epithelial cell markers (e.g., E-cadherin [E-cad], zonula occludens-1 [ZO-1], β4 integrin, and cytokeratin 19); (2) rearrangements of the actin, intermediate filaments, and myosin cytoskeletal systems; (3) redistribution of intracellular organelles; and (4) the upregulated expression of mesenchymal cell markers (e.g., N-cadherin, vimentin, and α-smooth muscle actin) and invasive factors (e.g., matrix metalloproteinases [MMPs] and extracellular matrix [ECM] molecules; Micalizzi et al. 2010a; Taylor et al. 2010; Thiery et al. 2009). Figure 2 depicts these events and highlights many of the most well-established and best characterized markers of EMT, including (1) E- and N-cadherins (Bhowmick et al. 2001a; Hazan et al. 2000; Miettinen et al. 1994); (2) vimentin (Grunert et al. 2003; Mendez et al. 2010); (3) fibronectin (Ignotz and Massague 1986); (4) α-smooth muscle actin (Masszi et al. 2003; Yazhou et al. 2004); (5) MMPs (Duivenvoorden et al. 1999; E.S. Kim et al. 2007; Lee et al. 2008); and (6) β3 integrin (Galliher and Schiemann 2006; Wendt and Schiemann 2009).

An important feature of the EMT process manifests through the disintegration and disassembly of epithelial cell-cell adhesion complexes, including desmosomes and gap, tight, and adherens junctions that function in maintaining epithelial cell polarity and in restricting cell motility (Cavallaro and Christofori 2004; Christofori 2003). Inactivating the expression and activity of these junctional complexes enables transitioning epithelial cells to abandon their immotile and polarized architectures and to assume highly motile and apolar architectures characteristic of mesenchymal-like cells (Fig. 2). For example, tight junctions arise from the collective actions of claudins, occludins, and junctional adhesion molecules, which are connected to the actin cytoskeleton via the scaffold proteins ZO-1, -2, and −3 (Ebnet et al. 2004; Schneeberger and Lynch 2004). Par6 (partitioning-defective 6) plays an essential role in overseeing (1) the formation of tight junctions; (2) the generation of apical-basolateral polarity; and (3) the initiation of polarized cell migration (Bose and Wrana 2006). Par6 also interacts physically with TGF-β receptors and can be phosphorylated by TβR-II, leading to

Author's personal copy

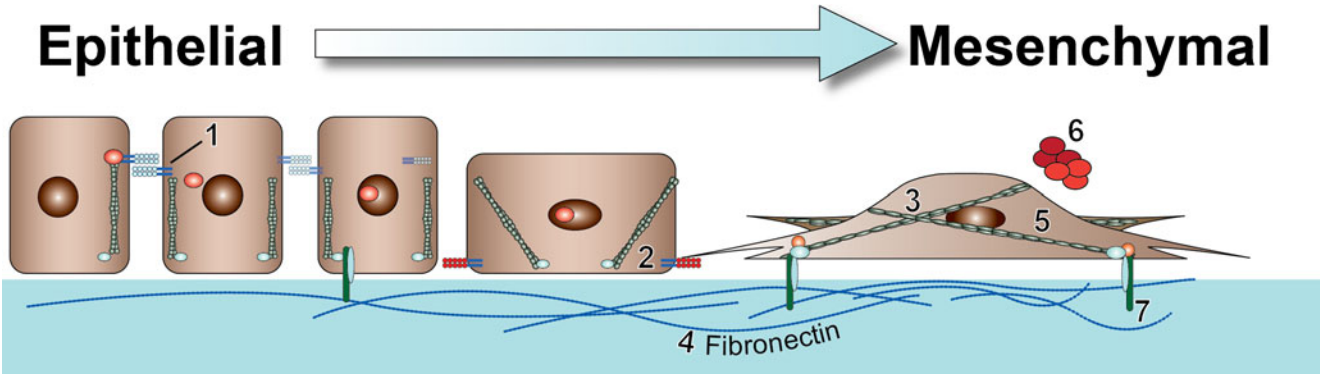


Fig. 2 Transdifferentiation from polarized epithelial-like to mesenchymal-like morphologies (*numbers* classical biomarkers of initiated EMT programs. (1) E-cadherin, the primary component of adherens junctions. During EMT, TGF-β represses E-cadherin expression and induces its internalization from the plasma membrane (Miettinen et al. 1994). (2) N-cadherin, an adhesion molecule that promotes cellular migration (Hazan et al. 2000). EMT stimulated by TGF-β is associated with increased expression of N-cadherin (Bhowmick et al. 2001a). (3) Vimentin, an intermediate filament protein that is expressed in all primitive cell types, but not in differentiated epithelial cells. Vimentin might function to drive EMT programs (Mendez et al. 2010) and also serves as a canonical marker for detecting transdifferentiated mesenchymal cell types (Grunert et al. 2003). (4) Fibronectin, a critical extracellular matrix (ECM) component whose elevated production by cancer cells is classically associated with EMT programs. TGF-β is a potent inducer of fibronectin production and its deposition into the ECM (Ignotz and Massague 1986). (5) α-Smooth muscle actin (α-SMA), a major component of contractile microfilaments and a canonical marker for detecting myofibroblasts. TGF-β stimulation of EMT elicits α-SMA expression (Masszi et al. 2003), which strongly associates with increased tumor invasion and with decreased patient survival (Yazhou et al. 2004). (6) Matrix metalloproteinases (MMPs). These enzymes proteolytically degrade basement membranes to allow primary tumor cells to invade the surrounding tissue and intrasvasate into tumor-associated vasculature. Whereas several MMPs are induced during EMT, MMP-9 is a well-established target of TGF-β signaling (Duivenvoorden et al. 1999; E.S. Kim et al. 2007; Lee et al. 2008). (7) β3 Integrin, a transmembrane protein that physically links the ECM to intracellular signaling systems and the cytoskeleton via focal adhesion complexes. β3 Integrin is rapidly and robustly upregulated by TGF-β and interacts physically with TGF-β type II receptor (TβR-II) via a FAK-dependent mechanism (Galliher and Schiemann 2006; Wendt and Schiemann 2009)

the formation of Par6:Smurf1 complexes that promote the ubiquitination and degradation of RhoA (Ozdamar et al. 2005). These events culminate in the dissolution of tight junctions during EMT stimulated by TGF-β. Moreover, uncoupling the ability of TGF-β to regulate Par6 activity is sufficient to prevent EMT induced by TGF-β (Ozdamar et al. 2005) and its stimulation of breast cancer metastasis in mice (Viloria-Petit et al. 2009).

In contrast to tight junctions, adherens-based junctional complexes are comprised primarily of the transmembrane protein, epithelial cadherin (E-cad), which connects to the actin cytoskeleton via α- and β-catenins (Niessen 2007). TGF-β stimulation of EMT inactivates E-cad function by repressing the synthesis of E-cad transcripts or by delocalizing and internalizing E-cad proteins from the cell membrane, an event coupled to a loss of Rac1 activity (Takaishi et al. 1997). The dissolution of cell-cell contacts accompanies the formation of prominent actin filaments and the appearance of fibroblastoid-like phenotypes in transitioning epithelial cells. The ability of TGF-β to remodel the actin cytoskeleton arises through the activation of RhoA (Bhowmick et al. 2001a; Ridley and Hall 1992), which together with diminished cell adhesion elicits cell migration and invasion. TGF-β also modulates the adhesion of transitioning epithelial cells by stimulating their expression of MMPs, while simultaneously inhibiting that of their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs); this collectively leads to the degradation of various basement membrane constituents such as collagen IV and laminin (Duivenvoorden et al. 1999). Collectively, these phenotypic and morphologic changes conspire to transform immobile multicellular epithelial sheets into highly motile, independent units that are poised for invasion and metastasis.

Classifying EMT subtypes

The process of EMT has recently been categorized into three distinct subtypes based the biological settings and contexts in which the EMT event is activated. Importantly, the generic features attributed to EMT are readily observed in all of the three major EMT subtypes. For instance, type 1 EMT is activated during embryogenesis and tissue morphogenesis, whereas type 2 EMT is initiated during tissue regeneration, wound healing, and inflammation-driven fibrosis. Finally, type 3 EMT is employed by carcinoma cells during their acquisition of invasive and metastatic phenotypes (Kalluri and Weinberg 2009). In the following sections, we highlight evidence linking TGF-β to the regulation of individual EMT subtypes, particularly that of type 3 EMT during metastatic progression stimulated by TGF-β.

Author's personal copy

Type 1 EMT

Type 1 EMT is synonymous with developmental EMT, and as such, this EMT subtype occurs during embryogenesis to facilitate the faithful synthesis of organs and tissues. Initiation of type 1 EMT promotes gastrulation and the subsequent formation of ectodermal, mesodermal, and endodermal tissues from the invaginating primitive streak (Micalizzi and Ford 2009; Yang and Weinberg 2008). In conjunction with the Wnt signaling pathway, primitive streak cells readily undergo EMT when stimulated by the TGF-β superfamily members, Nodal, Vg1 (a *Xenopus laevis* homolog of human TGF-beta), and bone morphogenetic protein-2 (Chea et al. 2005; Conlon et al. 1994; Raible 2006; Skromne and Stern 2001; Zhou et al. 1993). Type 1 EMT is also initiated during neurulation to facilitate neural tube formation during spinal and cerebral development, and again by neural crest cells to generate the adrenal medulla and the skeletal and peripheral nervous systems (Sauka-Spengler and Bronner-Fraser 2008). Additional insights associating TGF-β signaling to developmental EMT programs have been gleaned by genetically engineering mice to lack the expression of individual TGF-β isoforms and their receptors. For example, homozygous deletion of TGF-β2 elicits perinatal lethality attributable to multiple defects in the initiation of type 1 EMT during organogenesis and tissue morphogenesis, particularly that linked to atrioventricular valve formation by transitioned endocardial cells (Sanford et al. 1997). Likewise, the disruption of the TGF-β3 locus also produces perinatal lethality resulting from defective type 1 EMT, which occurs during lung and palate development (Kaartinen et al. 1995). Finally, the expression and activity of TβR-III are essential for TGF-β to stimulate type 1 EMT necessary for normal heart and secondary palate formation (Brown et al. 1999; Nakajima et al. 2007).

Type 2 EMT

Type 2 EMT plays an essential role in governing tissue homeostasis via its ability to induce the healing and remodeling of wounded tissues. Unlike type I EMT, transdifferentiation initiated by type 2 EMT is driven by inflammatory reactions, whose termination is sufficient to resolve the EMT event following wound repair (Kalluri and Weinberg 2009). The series of events that underlie type 2 EMT during wound healing and tissue remodeling are highly complex and require coordinated interactions between multiple cell types, such as fibroblasts and endothelial and immune cells, to facilitate the re-epithelialization of damaged epithelium. TGF-β plays a key role in mediating these events, doing so in part by promoting myofibroblast activation and differentiation coupled to the ECM remodeling necessary for tissue repair (Taylor et al. 2010). Interestingly, chronic inflammatory reactions can trigger a dysregulated type 2 EMT response that engenders the formation of a variety of fibroproliferative disorders, particularly in the lung, liver, kidney, and retina (Wynn 2007; Wynn 2008). Similarly, the inhibition of collagen cross-linking during mammary fibrosis significantly reduces breast cancer metastasis in mice (Levental et al. 2009), findings consistent with the notion that desmoplastic and fibrotic reactions enhance the incidence of mammary tumorigenesis (Boyd et al. 2007). Thus, the chemotherapeutic targeting of the TGF-β signaling system might abrogate neoplastic progression by simultaneously inactivating the deleterious activities associated with pathologic type 2 and type 3 EMTs (see below).

Type 3 EMT

Type 3 EMT is synonymous with oncogenic EMT and is essential in facilitating the acquisition of invasive and metastatic phenotypes by developing neoplasms (Micalizzi et al. 2010a; Taylor et al. 2010; Thiery et al. 2009). Oncogenic EMTs are readily distinguished from their normal counterparts, because these events arise in malignant cells that harbor a host of genetic and epigenetic abnormalities that coalesce in hijacking the EMT process during metastatic dissemination. Importantly, EMT stimulated by TGF-β has been linked to the selection and expansion of cancer stem cells (CSCs; Ben-Porath et al. 2008; Mani et al. 2008; Morel et al. 2008; Shipitsin et al. 2007) and, by extension, to the development of chemoresistance and disease recurrence (Frank et al. 2010; Singh and Settleman 2010). Even more remarkably, recent findings have implicated EMT as the molecular determinant that manifests the TGF-β paradox and the conversion of TGF-β function from that of a tumor suppressor to an inducer of metastatic progression. In the succeeding sections, we present evidence linking EMT to the acquisition of oncogenic TGF-β signaling and provide an overview of the way that TGF-β stimulates type 3 EMT in developing neoplasms.

TGF-β paradox

The initial framework underlying the TGF-β paradox began nearly 30 years ago when TGF-β was originally defined by its ability to promote the morphological transformation and anchorage-independent growth of normal rat kidney fibroblasts (NRK-49 cells; Moses et al. 1981; Roberts et al. 1981). Shortly thereafter, opposing findings obtained in epithelial cells established TGF-β as a potent and powerful inhibitor of cell proliferation (Carr et al. 1986). Thus, TGF-

Author's personal copy

β stimulates the growth of mesenchymal cell lineages but inhibits that of epithelial cell lineages. Interestingly, studies from this same era demonstrated that transformed cells secrete 40-fold more TGF-β as compared with their nontransformed counterparts (Anzano et al. 1985). This important observation strongly suggested that cellular transformation accompanies a loss in the tumor-suppressing functions of TGF-β. The merits of this supposition were quickly translated and verified in mouse models of tumorigenesis. For instance, implanting slow-release TGF-β pellets in female mice reversibly inhibits mammary gland growth and morphogenesis (Silberstein and Daniel 1987). In contrast, inoculation with neutralizing TGF-β antibodies enhances the ability of natural killer cells to eradicate breast cancer development and progression in mice (Arteaga et al. 1993). A specific role for TGF-β in promoting metastasis was established by the conditional expression of constitutively active TGF-β1, which selectively enhances the pulmonary metastasis of mammary tumors without altering their growth and proliferation (Muraoka-Cooket al. 2004). Likewise, transgenic expression of constitutively active TGF-β1 in mouse keratinocytes promotes their proliferation and eventual progression to spindle cell carcinoma in a chemical carcinogenesis model of skin cancer (Cui et al. 1996; Fowlis et al. 1996). More recently, conditional deletion of TβR-I expression in the oral cavity of mice has been observed to facilitate carcinogen-induced tumor formation in part via constitutive PI3K/AKT activity (Bian et al. 2009), whereas the combination of TβR-I haploinsufficiency with an Apc($^{Min/+}$) background produces twice as many intestinal tumors as compared with wild-type mice (Zeng et al. 2009). Collectively, these and other findings establish a paradigm whereby TGF-β potently inhibits tumor initiation and the development of early-stage carcinomas but enthusiastically drives the metastatic progression of late-stage carcinomas.

In addition to its ability to induce cytostasis in epithelial cells, TGF-β also governs the behaviors of adjacent fibroblasts and their synthesis and secretion of paracrine factors and ECM molecules that collectively suppress carcinoma development. For instance, the targeted deletion of TβR-II in mammary carcinoma cells facilitates the inappropriate activation of two distinct paracrine signaling axes, i.e., SDF-1:CXCR4 and CXCL5:CXCR2, which leads to the recruitment of immature GR1^{+}CD11b^{+} myeloid cells that drive breast cancer metastasis by inhibiting host tumor immunosurveillance and by inducing MMP expression (Yang et al. 2008). Likewise, rendering fibroblasts deficient in TβR-II expression elicits prostate intraepithelial neoplasia and invasive carcinoma of the forestomach resulting from disruptions in tumor-suppressing paracrine signaling networks (Bhowmick et al. 2004). Moreover, similar inactivation of TβR-II in mammary fibroblasts greatly exaggerates the growth and invasion of breast cancer cells simultaneously engrafted under the renal capsule. The proliferation-promoting activities of TβR-II-deficient fibroblasts derive from their upregulated expression of TGF-α, MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor; Cheng et al. 2005). Finally, the genetic inactivation of Smad4 in T cells elicits carcinoma formation within the gastrointestinal tract (e.g., colon, rectum, intestine, and stomach) attributable to aberrant stromal expansion and signaling (Kim et al. 2006). Collectively, these findings highlight the paradoxical activities of TGF-β in normal and malignant cells and, in doing so, point to the potential difficulties in developing effective therapies capable of predictably circumventing the context- and stage-specific behaviors of this cytokine in neoplastic tissues.

Mechanisms underlying type 3 EMT driven by TGF-β

TGF-β and transcriptional regulators of EMT

Attempts to deconstruct the EMT process have identified a variety of transcription factors that mediate essential functions in activating the "EMT transcriptome" necessary to bring about the epithelial transdifferentiation process (Table 1). Indeed, heterologous expression of the transcription factor Twist, which fulfils a critical role during mesoderm development (Leptin 1991), results in the initiation of the EMT program. Consistent with a role for EMT in driving carcinoma metastasis, rendering metastatic breast cancer cells deficient in Twist expression abrogates their ability to metastasize (Yang et al. 2004). Snail-1 is similarly required during mesoderm development and its expression readily represses that of E-cad (Cano et al. 2000). More recently, Snail-2 (also know as Slug) has been identified as a downstream effector of Twist, one that plays an essential role in facilitating Twist-mediated downregulation of E-cad (Casas et al. 2011; Mani et al. 2008). In addition to inducing the expression of Twist (Allington et al. 2009; Lee et al. 2008; Mani et al. 2008), TGF-β is also coupled to the expression of high mobility group A2 (HMGA2), leading to the robust downregulation of E-cad expression concomitant with the induction of Snail-1, Snail-2, and Twist (Thuault et al. 2006). Similarly, the findings of several studies support the notion that E-cad plays an active role in maintaining differentiated and polarized epithelial phenotypes, and indeed, E-cad deficiency is sufficient to elicit a robust EMT in mammary epithelial cells (Battula et al. 2010; Mani et al. 2008; Onder et al. 2008; Taube et al. 2010). Interestingly, recent evidence suggests that the expression and activity of these EMT transcription factors elicits a "feed forward" response

Author's personal copy

coupled to increased autocrine TGF-β production and signaling (Taube et al. 2010). Elucidation of the molecular mechanisms operant in mediating autocrine TGF-β signaling and its stabilization of the EMT process might shed new light on and provide a better understanding of the differences between physiological (i.e., types 1 and 2) and pathological (type 3) EMT. Indeed, prolonged induction of EMT results in the recruitment of DNA methyltransferases and chromatin remodeling enzymes to the promoters of EMT-regulated genes targeted by TGF-β, including estrogen receptor-α and E-cad (Dumont et al. 2008). Moreover, the inhibition of canonical Smad2 signaling, either by the inactivation of its expression or by the overexpression of the inhibitory Smad, Smad7, is sufficient to induce a mesenchymal-epithelial transition (MET) initiated by the loss of epigenetic silencing of epithelial-associated genes (Papageorgis et al. 2010). Collectively, these findings provide an initial framework to begin delineating the sequential nature of events associated with EMT induced by TGF-β and its ability to dictate epigenetic silencing as a means to stabilize and maintain oncogenic EMT programs.

TGF-β and microRNAs

Recent evidence has associated the expression of microRNAs with the initiation of the TGF-β paradox and its promotion of oncogenic TGF-β signaling (Table 1). For instance, EMT induced by TGF-β downregulates the expression of the microRNA-200 (miR-200) family, which facilitates the expression of ZEB1/2 (zinc finger E-box-binding homeobox 1/2) and their repression of E-cad expression in transitioning cells (Gregory et al. 2008; Korpal et al. 2008). Moreover, the absence of miR-200 expression has predictive value in identifying highly invasive and metastatic carcinomas (Burk et al. 2008; Singh et al. 2008). Interestingly, loss of miR-220c expression has been linked to inactivation of p53 function, an event coupled to the acquisition of EMT and stem-cell-like phenotypes (Chang et al. 2011). Similarly, we have recently identified c-Abl as a potent suppressor of EMT stimulated by TGF-β (Allington et al. 2009), an event that occurs via c-Abl-mediated reactivation of p53 expression and its induction of p21 (T.M.A. and W.P.S., unpublished). Likewise, elevated ZEB1/2 expression and the accompanying EMT are sufficient to overcome senescence induced by either p53 activation or epidermal growth factor receptor (EGFR) overexpression (Ohashi et al. 2010). In addition to its regulation of the miR-220 family of microRNAs, TGF-β also stimulates the processing of primary miR-21 transcripts into their pre-miR-21 counterparts, an event requiring the formation of Smad2/3:DROSHA complexes. Interestingly, these miR maturation events occur normally

Table 1 Master regulators of transforming growth factor-β (TGF-β)-induced epithelial-mesenchymal transition (EMT)

Regulator	Gene target	Transcript target
Transcriptional regulators		
Twist	Snail-2, TGF-β (Taube et al. 2010)	–
Snail	E-cadherin (E-cad; Cano et al. 2000), estrogen receptor-α (ER-α; Dhasarathy et al. 2007)	–
HMG2a (high mobility group A2)	Snail-1, Snail-2, Twist (Thuault et al. 2006)	–
Snail-2	E-cad (Hajra et al. 2002)	–
Zeb1/2 (Zinc finger E-box-binding homeobox 1/2	E-cad (Comijn et al. 2001)	–
Dnmt1 (DNA [cytosine-5]-methyltransferase 1)	E-cad (Papageorgis et al. 2010)	–
Six1 (Sine oculis homeobox homolog 1)	TGF-β type I receptor (TβR-I; Micalizzi et al. 2010b)	–
FOXC2 (Forkhead 1)	Vimentin, fibronectin, α-smooth muscle actin, N-cadherin (Mani et al. 2007)	–
MicroRNAs		
miR-200c	–	ZEB1, ZEB2 (Gregory et al. 2008)
miR-9	–	E-cad (Ma et al. 2010)
miR-155	–	RhoA (Ras homolog gene family, member A; Kong et al. 2008)
miR-21	–	Tiam1 (T-cell lymphoma invasion and metastasis 1; Cottonham et al. 2010; Davis et al. 2008; Zavadil et al. 2007)

Author's personal copy

in Smad4-deficient cells (Davis et al. 2008), thereby identifying a novel bifurcation in the canonical TGF-β signaling system that couples specifically to miR-processing events. TGF-β stimulation of miR-21 and miR-31 expression serve in promoting EMT programs coupled to cell migration and invasion, presumably via the downregulated expression of the guanine exchange factor, Tiam1 (T-cell lymphoma invasion and metastasis 1; Cottonham et al. 2010; Zavadil et al. 2007). The induction of miR-155 expression by TGF-β proceeds through a Smad4-dependent pathway and is necessary in mediating downregulated RhoA expression during EMT reactions (Kong et al. 2008). Finally, Myc amplification has recently been shown to induce miR-9 expression, which enhances breast cancer metastasis by downregulating E-cad expression (Ma et al. 2010). Although a direct role for TGF-β in regulating these events has not been examined, these findings nonetheless might provide new insights into the way that aberrant Myc expression overrides the cytostatic actions of TGF-β (Alexandrow et al. 1995; Chen et al. 2001). Figure 1 depicts the relationship between various microRNAs and TGF-β effector molecules operant in eliciting EMT programs.

Impact of integrins during EMT induced by TGF-β

As discussed above, variations in the composition of the ECM can elicit profound alterations in the way that normal and malignant cells respond to TGF-β. Mechanistically, these disparate functions of TGF-β have been linked to the expression and activity originating from integrins. For example, we have observed TβR-II to interact physically with β3 integrin following its activation by vitronectin or following its elevated expression, which arises during EMT induced by TGF-β (Galliher and Schiemann 2006; Galliher and Schiemann 2007; Galliher-Beckley and Schiemann 2008). Additionally, the formation of β3 integrin:TβR-II complexes is critically dependent upon the expression and activity of FAK, such that depleting FAK expression disrupts the interaction between these receptors and markedly reduces the ability of TGF-β to stimulate breast cancer invasion and metastasis (Wendt and Schiemann 2009). Interestingly, the FAK effector, p130Cas, has been found to bind and sequester Smad3, thereby preventing its phosphorylation by TβR-I and its subsequent translocation into the nucleus (Kim et al. 2008). Similarly, we have observed that elevated p130Cas expression marks metastatic progression and distorts the delicate balance between canonical and noncanonical TGF-β signaling systems in metastatic breast cancers (Wendt et al. 2009b). Indeed, rendering metastatic breast cancer cells deficient in p130Cas expression drastically reduces breast cancer development and progression stimulated by TGF-β (Wendt et al. 2009b), presumably by enhancing the sensitivity of p130Cas-deficient cells to apoptotic stimuli (Cabodi et al. 2006). Interestingly, TβR-II also interacts physically with β1 integrin (Galliher and Schiemann 2006), which is required for TGF-β to induce EMT and activate p38 MAPK (Bhowmick et al. 2001b). Taken together, these findings highlight the direct influence that integrins and focal adhesion complexes have in promoting EMT and metastatic progression stimulated by TGF-β.

Additional insights into the role of integrins in regulating normal and malignant cell responses to TGF-β have been gleaned through the use of three-dimensional (3D)-organotypic culture systems. For instance, the inactivation of β4 integrin function in normal mammary epithelial cells elicits their formation of dysmorphic acinar structures reminiscent of those produced by malignant mammary epithelial cells (Weaver et al. 2002). Conversely, the inactivation of β1 integrin function in breast cancer cells elicits their formation of spherical acinar structures reminiscent of normal mammary epithelial cells (Weaver et al. 1997). More recently, collagen has been incorporated within these 3D-organotypic cultures as a means to better recapitulate the microenvironmental compliance experienced by palpable primary tumors and to initiate mechanotransduction pathways coupled to integrin activation (Paszek et al. 2005). In undertaking similar analyses, we have observed compliant 3D-organotypic cultures to be sufficient in restoring the cytostatic activities of TGF-β in metastatic breast cancer cells that are normally completely resistant to the anti-proliferative activities of TGF-β in traditional two-dimensional culture systems (Allington et al. 2009; Taylor et al. 2011). Importantly, the inclusion of type I collagen in these 3D-organotypic cultures to increase their rigidity (i.e., decreased compliance) and initiate mechanotransduction has been observed to dose-dependently uncouple TGF-β from the regulation of cell cycle progression (Allington et al. 2009). Conversely, the treatment of developing organoids with the small molecule TβR-I inhibitor (TβR-I Inhibitor II) stimulates the growth of 4T1 cells in compliant 3D-organotypic cultures, wherein TGF-β functions as a tumor suppressor. However, administration of this same pharmacologic regimen to rigid 3D-organotypic cultures, wherein TGF-β functions as a tumor promoter, greatly inhibits the growth of the resultant 4T1 organoids (Taylor et al. 2011), thereby validating the first in vitro system that recapitulates the TGF-β paradox solely by modulating the tension sensed by malignant mammary epithelial cells. Importantly, the expression and activity of lysyl oxidase stimulated by TGF-β contributes to its acquisition of oncogenic behavior and the induction of EMT in normal and malignant mammary epithelial cells (Taylor et al. 2011). Oncogenic TGF-β signaling brought about by mechanotransduction is partially neutralized by rendering

Author's personal copy

breast cancer cells deficient in lysyl oxidase expression, which enhances E-cad expression and activity in developing organoids (Taylor et al. 2011). Similarly, an increase in ECM rigidity not only induces dramatic differences in cell proliferation, but also suppresses E-cad expression by promoting EMT programs (Tilghman et al. 2010), presumably via rigidity-induced TGF-β synthesis and autocrine signaling coupled to the integrin-mediated activation of latent TGF-β complexes (Taylor et al. 2011; Wipff et al. 2007). Recently, the initiation of MET programs and the elevated expression of E-cad have been observed to promote the metastatic outgrowth of disseminated breast cancer cells in the lungs of mice (Chao et al. 2010; Dykxhoorn et al. 2009). Somewhat paradoxically, E-cad expression has also been shown to normalize acinar morphology and growth (Wang et al. 2002), presumably via heterotypic binding to and the regulation of β1 integrin expression (Whittard et al. 2002; Wu et al. 2006; Zhang et al. 2006). As a means to explain these contrasting findings, we have recently found that the heterotypic binding of E-cad to β1 integrin suppresses its expression and inhibits the initiation of metastatic outgrowth by breast micrometastases. Moreover, the conversion from dormancy to proliferative programs capable of supporting metastatic outgrowth requires a transient EMT event, which downregulates E-cad expression and upregulates that of β1 integrin necessary for micrometastatic outgrowth (M.K.W. and W.P.S., unpublished). Collectively, these studies provide novel insights into the role of mechanotransduction in mediating oncogenic TGF-β signaling and its coupling to type 3 EMT during distinct stages of the metastatic cascade (Drake et al. 2009; Wendt et al. 2010).

Impact of growth factor receptors during EMT induced by TGF-β

As mentioned above, recent advancements in the use of 3D-organotypic cultures have shed new light onto the importance of cell and tumor microenvironments in regulating the TGF-β stimulation of EMT. In a similar avenue of research, studies are now investigating the specific impact played by other tumor-derived growth factors and chemokines in coupling TGF-β to EMT programs. For instance, the expression and activity of Cox-2 are essential in facilitating TGF-β stimulation of EMT in normal and malignant mammary epithelial cells (Neil et al. 2008; Tian and Schiemann 2010). Importantly, the coupling of TGF-β to Cox-2 expression is increased synergistically by co-stimulation with EGF and its activation of MAP kinases (Saha et al. 1999). Interestingly, TGF-β can transactivate EGFR via a Src-dependent pathway coupled to the activation of TACE/ADAM17 (TNF-α-converting enzyme) and its cleavage of EGF-like ligands (Murillo et al. 2005; S. E. Wang et al. 2009). In agreement, two-photon intravital imaging analyses have assisted in identifying a paracrine signaling axis comprised of carcinoma-cell-derived colony-stimulating factor-1 (CSF-1) and macrophage-derived EGF, which facilitates tumor cell migration and invasion (Patsialou et al. 2009; Wang et al. 2007; Wyckoff et al. 2004). Importantly, TGF-β plays an essential role in establishing this paracrine signaling network through its ability to upregulate CSF-1 receptor expression (Patsialou et al. 2009). EGFR also activates the transcription factor STAT3 (signal transducer and activator of transcription-3), which induces the Twist expression necessary to induce autocrine TGF-β signaling (Lo et al. 2007). We have recently demonstrated that TGF-β stimulation of EMT confers invasive activity to EGFR in part by (1) stabilizing its expression at the cell surface, and (2) facilitating its coupling to Src and p38 MAPK (Wendt et al. 2010). Interestingly, the chronic activation of EMT programs by TGF-β results in the acquisition of resistance to EGFR inhibitors (Barr et al. 2008). Thus, these findings bolster the notion that transient EMT programs support oncogenic EGFR signaling, whereas those encompassing prolonged EMT programs support "kinase switching" away from EGFR to depend instead upon the actions of fibroblast growth factor receptor or platelet-derived growth factor receptor (Thomson et al. 2008). Finally, insulin-like growth factor-1 (IGF-1) and its receptor (IGF1R) also induce cell transformation, invasion, and metastasis (Zha and Lackner 2010), presumably via an EMT event involving the actions of Snail-2, ZEB1/2, and Akt2 (Graham et al. 2008; Irie et al. 2005; H.J. Kim et al. 2007; Sivakumar et al. 2009). Similarly, we find IGF1R expression to render transitioning mammary epithelial cells resistant to apoptotic stimuli when undergoing EMT induced by TGF-β and to play an essential role in mediating cell migration and invasion stimulated by TGF-β (M.T. and W.P.S., unpublished). Future studies need to determine the molecular mechanisms that elicit kinase switching and the spatiotemporal events that influence their initiation and resolution.

EMT, CSCs, and chemoresistance

EMT and CSCs

Solid tumors are notoriously heterogeneous with respect to their collective morphologies, proliferative indices, genetic lesions, and responsiveness to chemotherapeutic regimens (Visvader 2011). Housed within this heterogeneous environment lies a subpopulation of CSCs that possess self-renewal and tumor-initiating properties that give rise to metastatic progression and disease recurrence (Pardal et al. 2003). Although considerable debate remains regarding the

Author's personal copy

"true" identity of CSCs, several recent studies have identified a variety of proteins that serve to mark tumor-initiating cells (Table 2). For example, $CD44^{+}/CD24^{-/low}$, $CD29^{hi}/CD24^{med}$, or aldehyde dehydrogenase 1 (ALDH1) can differentiate breast CSCs from those lacking tumor-initiating properties (Al-Hajj et al. 2003; Ginestier et al. 2007), whereas detection of the expression of CD133 has been proposed as a marker for CSCs derived from cancers of the brain, colon, and pancreas (Hermann et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Moreover, cellular efflux of Hoechst stains can visualize a heterogeneous "side-population" of cells enriched in stem cell-like properties (Challen and Little 2006), as can detection of the expression of EpCAM (epithelial cell adhesion molecule; Munz et al. 2009). Importantly, the initiation of EMT programs elicits the selection and expansion of CSCs (Ben-Porath et al. 2008; Morel et al. 2008; Shipitsin et al. 2007), leading to the development of chemoresistant phenotypes and disease recurrence (Creighton et al. 2010; Singh and Settleman 2010; Turley et al. 2008). Consistent with its function as a master regulator of EMT, TGF-β stimulation of carcinoma cells facilitates the appearance of CSCs in an EMT-dependent manner, suggesting that targeting the TGF-β signaling system can suppress tumor development and progression by inhibiting CSC function. Accordingly, the inactivation of TGF-β signaling in metastatic breast cancers results in a MET that re-establishes a more epithelial-like phenotype in these aggressive cells, whereas elevated TGF-β signatures are associated with metastatic progression and poorer clinical outcomes (Shipitsin et al. 2007). Future studies need to determine the impact that might be achieved on tumor development, progression, and recurrence by combining targeted TGF-β chemotherapies with agents capable of selectively targeting CSCs, including the delivery of (1) chemotherapeutic agents coupled to monoclonal antibodies directed at CSC cell-surface markers (Deonarain et al. 2009) or (2) pharmaceuticals capable of inactivating CSC drug transporters (Baguley 2010).

EMT and chemoresistance

The most common failure in successfully treating cancers is their development of chemoresistance, which typically falls into two major categories: (1) de novo drug resistance, which manifests in the absence of drug selection attributable to a pre-existing phenotype, and (2) acquired drug resistance, which manifests as an adaptive response to drug selection. Mechanistically, acquired drug resistance typically reflects the ability of cells to upregulate their expression of drug efflux transporters or of metabolizing enzymes or to downregulate their drug-induced apoptotic machinery (Gottesman 2002). As mentioned above, the initiation of EMT programs underlies disease progression and the emergence of chemoresistance; this has led to the notion that the inactivation of EMT or induction of MET might serve to resensitize carcinoma cells to previously effective treatment regimens (Table 2). For instance, anti-EGFR inhibitors such as Erlotinib, Gefitinib, or Cetuximab are widely used clinically to treat a variety of human cancers; however, the effectiveness of these drugs is inversely

Table 2 Cancer stem cell (CSC) markers and their relationship to chemoresistant phenotypes (*Stro* antigen used to identify bone marrow stromal cell precursors and bone marrow stem cells, *uPAR* urokinase plasminogen activator surface receptor, *5-FU* 5-fluorouracil, *AML* acute myeloid leukemia, *CML* chronic myelogenous leukemia, *SCLC* small cell lung cancer, *hi* high, *med* medium)

CSC markers	Cancer type	Conferred drug resistance
$CD133^{+}/CD117^{+}$	Ovarian	Carboplatin; Paclitaxel; Cisplatin (Hu et al. 2010; Shi et al. 2010)
$Stro\text{-}1^{+}$	Osteosarcoma	Doxorubicin (Adhikari et al. 2010)
$CD117^{+}/ABCG2^{+}$	Prostate	Arsenite; Cisplatin; Paclitaxel; Adriamycin; Methotrexate (Liu et al. 2010; Tokar et al. 2010)
$CD133^{+}/ABCG2^{+}$	SCLC	5-FU, Cisplatin; Etoposide (Gutova et al. 2007; Wang et al. 2010)
$CD34^{+}CD38^{-}$	AML	Ara-C (Ishikawa et al. 2007; Saito et al. 2010)
$CD34^{-}$	CML	Imatinib (Lemoli et al. 2009)
$uPAR^{+}$	SCLC	5-FU, Cisplatin; Etoposide (Gutova et al. 2007; Wang et al 2010)
$Hoechst^{+}$	Liver	Doxorubicin; Methotrexate (Zhang et al. 2010)
$CD29^{hi}/CD24^{med}$	Breast	Paclitaxel; Cisplatin (Shafee et al. 2008; To et al. 2010)
$CD44^{+}/CD24^{-/low}$	Breast and pancreatic	Paclitaxel; Cisplatin; Gemcitabine (Hermann et al. 2007; Hong et al. 2009; Shafee et al. 2008; To et al. 2010)
$CD44^{+}$	Pancreatic	Gemcitabine (Hermann et al. 2007; Hong et al. 2009)
$CD133^{+}$	Brain and colon	Carboplatin; Paclitaxel; Irinotecan; Temozolomide; Etoposide; Oxaliplatin; 5-FU; Cyclophosphamide (Dylla et al. 2008; Fang et al. 2010; Liu et al. 2006; Ong et al. 2010; Todaro et al. 2007)

Author's personal copy

correlated to the completeness of the EMT program and acquisition of mesenchymal-like phenotypes attained in cancers of the lung, head, and neck (Chin et al. 2008; Frederick et al. 2007; Thomson et al. 2005, 2008; Yauch et al. 2005). Likewise, ZEB1-driven EMT in pancreatic cancers elicits their resistance to Gemcitabine, 5-fluorouricil (5-FU), and Cisplatin (Arumugam et al. 2009). Importantly, rendering pancreatic cancer cells deficient in the expression of either ZEB1 or Notch is sufficient to elicit a MET and restore sensitivity to these cytotoxic agents (Arumugam et al. 2009; Z. Wang et al. 2009). Similarly, the appearance of acquired resistance reflects the completion of EMT programs in (1) ovarian cancer cells that develop resistance to Paclitaxel (Kajiyama et al. 2007); (2) bladder cancer cells that develop resistance to radiotherapy and Cisplatin, an event mediated by ZEB2 (Sayan et al. 2009); and (3) luminal breast cancer cells that develop resistance to Paclitaxel, Docetaxel, or Doxorubicin, events mediated by downregulated expression of estrogen receptor-α and upregulated expression of TβR-II (Iseri et al. 2011). Interestingly, Doxorubicin treatment of breast cancer cells elicits an EMT program sufficient to enhance their metastasis to the lungs and bone in mice. Importantly, administration of the TβR-I antagonist neutralizes the metastasis promoting activities of Doxorubicin (Bandyopadhyay et al. 2010), suggesting that the ability of cytotoxic agents to induce EMT depends upon the initiation of autocrine TGF-β signaling. Future studies need to determine the overall reliance of EMT induced by TGF-β in mediating acquired resistance to chemotherapeutics and to develop reliable biomarkers capable of typing the function of TGF-β within these developing and progressing neoplasms.

Concluding remarks

The current EMT paradigm states that oncogenic EMT is a metastable and transient process that affords carcinoma cells the ability to escape the confines of the primary tumor and to disseminate throughout the body via the systemic circulation. Similarly, EMT is proficient in generating unique cell populations endowed with self-renewal and stem-like qualities, thereby rendering transitioned cells insensitive to apoptosis, hypoxia, and traditional chemotherapies. At present, the origin of "EMT-competent" carcinoma cells, the location in which they reside within heterogeneous tumor microenvironments, and the extent to which these events can be driven by TGF-β at distinct stages of tumor development remain uncertain. Likewise, whether the EMT phenotype is stable or malleable throughout the evolution of a given tumor and its spatiotemporal susceptibility to the EMT process (Fig. 3) are still ambiguous. Equally unclear is the precise role played by "EMT-incompetent" cells in (1) supporting the selection, expansion, and dissemination of transitioned carcinoma cells during metastatic progression; (2) enabling transitioned cells to subvert the effects of neoadjuvant chemotherapies; and (3) promoting transitioned cells to escape metastatic dormancy and reinitiate proliferative programs coupled to lethal bouts of disease recurrence. Future studies need to address and fill these gaps in our knowledge, and in doing so, science and medicine might develop the tools necessary to circumvent the TGF-β paradox and its ability to promote EMT, metastatic progression, and disease recurrence in patients harboring metastatic disease.

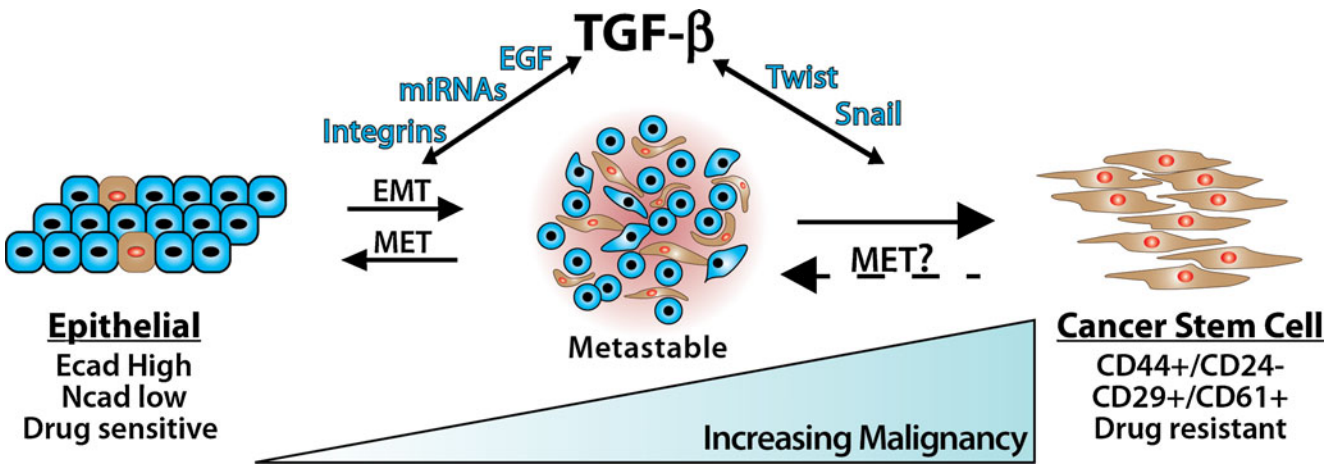


Fig. 3 Consequences of EMT programs induced by TGF-β. Administration of TGF-β readily elicits the formation of a metastable EMT state in normal and malignant cells. The restoration of epithelial phenotypes occurs through mesenchymal-epithelial transitions (METs), which occur normally during developmental EMTs and perhaps during metastatic outgrowth associated with oncogenic EMTs. Prolonged exposure of cells to TGF-β or other EMT-initiating factors supports the continued development and expansion of cancer stem cells, which collectively are chemoresistant and underlie disease recurrence. How, when, and where cancer stems cells undergo MET during secondary tumor outgrowth remains to be determined definitively

Author's personal copy

Acknowledgements We thank members of the Schiemann Laboratory for critical comments and for reading the manuscript.

References

Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR, Iwakuma T (2010) CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 70:4602–4612

Alexandrow MG, Kawabata M, Aakre M, Moses HL (1995) Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor β1. Proc Natl Acad Sci USA 92:3239–3243

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:3983–3988

Allington TM, Galliher-Beckley AJ, Schiemann WP (2009) Activated Abl kinase inhibits oncogenic transforming growth factor-β signaling and tumorigenesis in mammary tumors. FASEB J 23:4231–4243

Anzano MA, Roberts AB, De Larco JE, Wakefield LM, Assoian RK, Roche NS, Smith JM, Lazarus JE, Sporn MB (1985) Increased secretion of type β transforming growth factor accompanies viral transformation of cells. Mol Cell Biol 5:242–247

Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT (1993) Anti-transforming growth factor (TGF)-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. J Clin Invest 92:2569–2576

Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W (2009) Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. Cancer Res 69:5820–5828

Baguley BC (2010) Multiple drug resistance mechanisms in cancer. Mol Biotechnol 46:308–316

Bandyopadhyay A, Wang L, Agyin J, Tang Y, Lin S, Yeh IT, De K, Sun LZ (2010) Doxorubicin in combination with a small TGFβ inhibitor: a potential novel therapy for metastatic breast cancer in mouse models. PLoS One 5:e10365

Barr S, Thomson S, Buck E, Russo S, Petti F, Sujka-Kwok I, Eyzaguirre A, Rosenfeld-Franklin M, Gibson NW, Miglarese M, Epstein D, Iwata KK, Haley JD (2008) Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. Clin Exp Metastasis 25:685–693

Battula VL, Evans KW, Hollier BG, Shi Y, Marini FC, Ayyanan A, Wang RY, Brisken C, Guerra R, Andreeff M, Mani SA (2010) Epithelial-mesenchymal transition-derived cells exhibit multilineage differentiation potential similar to mesenchymal stem cells. Stem Cells 28:1435–1445

Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40:499–507

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL (2001a) Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 12:27–36

Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL (2001b) Integrin β1 signaling is necessary for transforming growth factor-β activation of p38MAPK and epithelial plasticity. J Biol Chem 276:46707–46713

Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL (2004) TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303:848–851

Bian Y, Terse A, Du J, Hall B, Molinolo A, Zhang P, Chen W, Flanders KC, Gutkind JS, Wakefield LM, Kulkarni AB (2009) Progressive tumor formation in mice with conditional deletion of TGF-β signaling in head and neck epithelia is associated with activation of the PI3K/Akt pathway. Cancer Res 69:5918–5926

Blobe GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor-β in human disease. N Engl J Med 342:1350–1358

Bose R, Wrana JL (2006) Regulation of Par6 by extracellular signals. Curr Opin Cell Biol 18:206–212

Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E, Jong RA, Hislop G, Chiarelli A, Minkin S, Yaffe MJ (2007) Mammographic density and the risk and detection of breast cancer. N Engl J Med 356:227–236

Brown CB, Boyer AS, Runyan RB, Barnett JV (1999) Requirement of type III TGF-β receptor for endocardial cell transformation in the heart. Science 283:2080–2082

Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9:582–589

Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, Sapino A, Arisio R, Cavallo F, Forni G, Glukhova M, Silengo L, Altruda F, Turco E, Tarone G, Defilippi P (2006) p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-Neu oncogene-dependent breast tumorigenesis. Cancer Res 66:4672–4680

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2:76–83

Carr BI, Hayashi I, Branum EL, Moses HL (1986) Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type β transforming growth factor. Cancer Res 46:2330–2334

Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J (2011) Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res 71:245–254

Cavallaro U, Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer 4:118–132

Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. Stem Cells 24:3–12

Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, Yu WH, Rehman SK, Hsu JL, Lee HH, Liu M, Chen CT, Yu D, Hung MC (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 13:317–323

Chang H, Brown CW, Matzuk MM (2002) Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocr Rev 23:787–823

Chao YL, Shepard CR, Wells A (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. Mol Cancer 9:179

Chea HK, Wright CV, Swalla BJ (2005) Nodal signaling and the evolution of deuterostome gastrulation. Dev Dyn 234:269–278

Chen CR, Kang Y, Massague J (2001) Defective repression of c-Myc in breast cancer cells: a loss at the core of the transforming growth factor β growth arrest program. Proc Natl Acad Sci USA 98:992–999

Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, Arteaga CL, Neilson EG, Hayward SW, Moses HL (2005) Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-,

Author's personal copy

MSP- and HGF-mediated signaling networks. Oncogene 24:5053–5068

Chin TM, Quinlan MP, Singh A, Sequist LV, Lynch TJ, Haber DA, Sharma SV, Settleman J (2008) Reduced Erlotinib sensitivity of epidermal growth factor receptor-mutant non-small cell lung cancer following Cisplatin exposure: a cell culture model of second-line Erlotinib treatment. Clin Cancer Res 14:6867–6876

Christofori G (2003) Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J 22:2318–2323

Comijn J, Berx G, Vermassen P, Verschueren K, Grunsven L van, Bruyneel E, Mareel M, Huylebroeck D, Roy F van (2001) The two-handed E box binding zinc finger protein SIP1 down-regulates E-cadherin and induces invasion. Mol Cell 7:1267–1278

Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ (1994) A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120:1919–1928

Cottonham CL, Kaneko S, Xu L (2010) miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. J Biol Chem 285:35293–35302

Creighton CJ, Chang JC, Rosen JM (2010) Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. J Mammary Gland Biol Neoplasia 15:253–260

Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ (1996) TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell 86:531–542

Davis BN, Hilyard AC, Lagna G, Hata A (2008) SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454:56–61

Deonarain MP, Kousparou CA, Epenetos AA (2009) Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? MAbs 1:12–25

Dhasarathy A, Kajita M, Wade PA (2007) The transcription factor Snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor α. Mol Endocrinol 21:2907–2918

Drake JM, Strohbehn G, Bair TB, Moreland JG, Henry MD (2009) ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. Mol Biol Cell 20:2207–2217

Duivenvoorden WC, Hirte HW, Singh G (1999) Transforming growth factor β1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. Clin Exp Metastasis 17:27–34

Dumont N, Wilson MB, Crawford YG, Reynolds PA, Sigaroudinia M, Tlsty TD (2008) Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. Proc Natl Acad Sci 105:14867–14872

Dykxhoorn DM, Wu Y, Xie H, Yu F, Lal A, Petrocca F, Martinvalet D, Song E, Lim B, Lieberman J (2009) miR-200 enhances mouse breast cancer cell colonization to form distant metastases. PLoS One 4:e7181

Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL (2008) Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. PLoS One 3:e2428

Ebnet K, Suzuki A, Ohno S, Vestweber D (2004) Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci 117:19–29

Fang DD, Kim YJ, Lee CN, Aggarwal S, McKinnon K, Mesmer D, Norton J, Birse CE, He T, Ruben SM, Moore PA (2010) Expansion of CD133(+) colon cancer cultures retaining stem cell properties to enable cancer stem cell target discovery. Br J Cancer 102:1265–1275

Feng XH, Derynck R (2005) Specificity and versatility in TGF-β signaling through Smads. Annu Rev Cell Dev Biol 21:659–693

Fowlis DJ, Cui W, Johnson SA, Balmain A, Akhurst RJ (1996) Altered epidermal cell growth control in vivo by inducible expression of transforming growth factor β1 in the skin of transgenic mice. Cell Growth Differ 7:679–687

Frank NY, Schatton T, Frank MH (2010) The therapeutic promise of the cancer stem cell concept. J Clin Invest 120:41–50

Frederick BA, Helfrich BA, Coldren CD, Zheng D, Chan D, Bunn PA Jr, Raben D (2007) Epithelial to mesenchymal transition predicts Gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma. Mol Cancer Ther 6:1683–1691

Galliher AJ, Schiemann WP (2006) Beta3 integrin and Src facilitate transforming growth factor-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 8:R42

Galliher AJ, Schiemann WP (2007) Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res 67:3752–3758

Galliher-Beckley AJ, Schiemann WP (2008) Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis 29:244–251

Gatza CE, Oh SY, Blobe GC (2010) Roles for the type III TGF-β receptor in human cancer. Cell Signal 22:1163–1174

Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1:555–567

Gottesman MM (2002) Mechanisms of cancer drug resistance. Annu Rev Med 53:615–627

Graham TR, Zhau HE, Odero-Marah VA, Osunkoya AO, Kimbro KS, Tighiouart M, Liu T, Simons JW, O'Regan RM (2008) Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 68:2479–2488

Greenburg G, Hay ED (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol 95:333–339

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10:593–601

Grunert S, Jechlinger M, Beug H (2003) Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 4:657–665

Gutova M, Najbauer J, Gevorgyan A, Metz MZ, Weng Y, Shih CC, Aboody KS (2007) Identification of uPAR-positive chemoresistant cells in small cell lung cancer. PLoS One 2:e243

Hajra KM, Chen DY, Fearon ER (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res 62:1613–1618

Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 148:779–790

Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1:313–323

Hong SP, Wen J, Bang S, Park S, Song SY (2009) CD44-positive cells are responsible for Gemcitabine resistance in pancreatic cancer cells. Int J Cancer 125:2323–2331

Author's personal copy

Hu Z, Zhang Z, Guise T, Seth P (2010) Systemic delivery of an oncolytic adenovirus expressing soluble transforming growth factor-β receptor II-Fc fusion protein can inhibit breast cancer bone metastasis in a mouse model. Hum Gene Ther 21:1623–1629

Ignotz RA, Massague J (1986) Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345

Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, Natesan S, Brugge JS (2005) Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol 171:1023–1034

Iseri OD, Kars MD, Arpaci F, Atalay C, Pak I, Gunduz U (2011) Drug resistant MCF-7 cells exhibit epithelial-mesenchymal transition gene expression pattern. Biomed Pharmacother 65:40–45

Ishikawa F, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, Nakamura R, Tanaka T, Tomiyama H, Saito N, Fukata M, Miyamoto T, Lyons B, Ohshima K, Uchida N, Taniguchi S, Ohara O, Akashi K, Harada M, Shultz LD (2007) Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol 25:1315–1321

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J (1995) Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 11:415–421

Kajiyama H, Shibata K, Terauchi M, Yamashita M, Ino K, Nawa A, Kikkawa F (2007) Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. Int J Oncol 31:277–283

Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–1428

Kang JS, Liu C, Derynck R (2009) New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol 19:385–394

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549

Kim BG, Li C, Qiao W, Mamura M, Kasprzak B, Anver M, Wolfraim L, Hong S, Mushinski E, Potter M, Kim SJ, Fu XY, Deng C, Letterio JJ (2006) Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature 441:1015–1019

Kim ES, Sohn YW, Moon A (2007) TGF-β-induced transcriptional activation of MMP-2 is mediated by activating transcription factor (ATF)2 in human breast epithelial cells. Cancer Lett 252:147–156

Kim HJ, Litzenburger BC, Cui X, Delgado DA, Grabiner BC, Lin X, Lewis MT, Gottardis MM, Wong TW, Attar RM, Carboni JM, Lee AV (2007) Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-κB and snail. Mol Cell Biol 27:3165–3175

Kim W, Seok Kang Y, Soo Kim J, Shin NY, Hanks SK, Song WK (2008) The integrin-coupled signaling adaptor p130Cas suppresses Smad3 function in transforming growth factor-β signaling. Mol Biol Cell 19:2135–2146

Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, Cheng JQ (2008) MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol 28:6773–6784

Korpal M, Lee ES, Hu G, Kang Y (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 283:14910–14914

Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP (2008) Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-β in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis 29:2243–2251

Lemoli RM, Salvestrini V, Bianchi E, Bertolini F, Fogli M, Amabile M, Tafuri A, Salati S, Zini R, Testoni N, Rabascio C, Rossi L, Martin-Padura I, Castagnetti F, Marighetti P, Martinelli G, Baccarani M, Ferrari S, Manfredini R (2009) Molecular and functional analysis of the stem cell compartment of chronic myelogenous leukemia reveals the presence of a CD34- cell population with intrinsic resistance to Imatinib. Blood 114:5191–5200

Leptin M (1991) Twist and Snail as positive and negative regulators during *Drosophila* mesoderm development. Genes Dev 5:1568–1576

Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM (2009) Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139:891–906

Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL, Yu JS (2006) Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 5:67

Liu T, Xu F, Du X, Lai D, Zhao Y, Huang Q, Jiang L, Huang W, Cheng W, Liu Z (2010) Establishment and characterization of multi-drug resistant, prostate carcinoma-initiating stem-like cells from human prostate cancer cell lines 22RV1. Mol Cell Biochem 340:265–273

Lo HW, Hsu SC, Xia W, Cao X, Shih JY, Wei Y, Abbruzzese JL, Hortobagyi GN, Hung MC (2007) Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. Cancer Res 67:9066–9076

Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S, Westermann F, Speleman F, Vandesompele J, Weinberg RA (2010) miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nat Cell Biol 12:247–256

Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, Weinberg RA (2007) Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci USA 104:10069–10074

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704–715

Massague J (1998) TGF-β signal transduction. Annu Rev Biochem 67:753–791

Massague J (2008) TGF β in cancer. Cell 134:215–230

Massague J, Gomis RR (2006) The logic of TGFβ signaling. FEBS Lett 580:2811–2820

Masszi A, Di Ciano C, Sirokmany G, Arthur WT, Rotstein OD, Wang J, McCulloch CA, Rosivall L, Mucsi I, Kapus A (2003) Central role for Rho in TGF-β1-induced α-smooth muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol 284:F911–F924

Mendez MG, Kojima S, Goldman RD (2010) Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J 24:1838–1851

Micalizzi DS, Ford HL (2009) Epithelial-mesenchymal transition in development and cancer. Future Oncol 5:1129–1143

Micalizzi DS, Farabaugh SM, Ford HL (2010a) Epithelial-mesenchymal transition in cancer: parallels between normal

Author's personal copy

development and tumor progression. J Mammary Gland Biol Neoplasia 15:117–134
Micalizzi DS, Wang CA, Farabaugh SM, Schiemann WP, Ford H (2010b) Homeoprotein Six1 increases TGF-β type I receptor and converts TGF-β signaling from suppressive to supportive for tumor growth. Cancer Res 70:10371–10380
Miettinen PJ, Ebner R, Lopez AR, Derynck R (1994) TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol 127:2021–2036
Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005) Genes that mediate breast cancer metastasis to lung. Nature 436:518–524
Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 3:e2888
Moses HL, Branum EL, Proper JA, Robinson RA (1981) Transforming growth factor production by chemically transformed cells. Cancer Res 41:2842–2848
Moustakas A, Heldin CH (2005) Non-Smad TGF-β signals. J Cell Sci 118:3573–3584
Munz M, Baeuerle PA, Gires O (2009) The emerging role of EpCAM in cancer and stem cell signaling. Cancer Res 69:5627–5629
Muraoka-Cook RS, Kurokawa H, Koh Y, Forbes JT, Roebuck LR, Barcellos-Hoff MH, Moody SE, Chodosh LA, Arteaga CL (2004) Conditional overexpression of active transforming growth factor β1 in vivo accelerates metastases of transgenic mammary tumors. Cancer Res 64:9002–9011
Murillo MM, Castillo G del, Sanchez A, Fernandez M, Fabregat I (2005) Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. Oncogene 24:4580–4587
Nakajima A, Ito Y, Asano M, Maeno M, Iwata K, Mitsui N, Shimizu N, Cui XM, Shuler CF (2007) Functional role of transforming growth factor-β type III receptor during palatal fusion. Dev Dyn 236:791–801
Neil JR, Johnson KM, Nemenoff RA, Schiemann WP (2008) Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-beta through a PGE2-dependent mechanisms. Carcinogenesis 29:2227–2235
Niessen CM (2007) Tight junctions/adherens junctions: basic structure and function. J Invest Dermatol 127:2525–2532
O'Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
Ohashi S, Natsuizaka M, Wong GS, Michaylira CZ, Grugan KD, Stairs DB, Kalabis J, Vega ME, Kalman RA, Nakagawa M, Klein-Szanto AJ, Herlyn M, Diehl JA, Rustgi AK, Nakagawa H (2010) Epidermal growth factor receptor and mutant p53 expand an esophageal cellular subpopulation capable of epithelial-to-mesenchymal transition through ZEB transcription factors. Cancer Res 70:4174–4184
Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 68:3645–3654
Ong CW, Kim LG, Kong HH, Low LY, Iacopetta B, Soong R, Salto-Tellez M (2010) CD133 expression predicts for non-response to chemotherapy in colorectal cancer. Mod Pathol 23:450–457
Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL (2005) Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science 307:1603–1609
Papageorgis P, Lambert AW, Ozturk S, Gao F, Pan H, Manne U, Alekseyev YO, Thiagalingam A, Abdolmaleky HM, Lenburg M, Thiagalingam S (2010) Smad signaling is required to maintain epigenetic silencing during breast cancer progression. Cancer Res 70:968–978
Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902
Parvani JG, Taylor MA, Schiemann WP (2011) Noncanonical TGF-β signaling during mammary tumorigenesis. J Mammary Gland Biol Neoplasia 16:127–146
Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM (2005) Tensional homeostasis and the malignant phenotype. Cancer Cell 8:241–254
Patsialou A, Wyckoff JB, Wang Y, Goswami S, Stanley ER, Condeelis JS (2009) Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. Cancer Res 69:9498–9506
Rahimi RA, Leof EB (2007) TGF-β signaling: a tale of two responses. J Cell Biochem 102:593–608
Raible DW (2006) Development of the neural crest: achieving specificity in regulatory pathways. Curr Opin Cell Biol 18:698–703
Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399
Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB (1981) New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci USA 78:5339–5343
Saha D, Datta PK, Sheng H, Morrow JD, Wada M, Moses HL, Beauchamp RD (1999) Synergistic induction of cyclooxygenase-2 by transforming growth factor-β1 and epidermal growth factor inhibits apoptosis in epithelial cells. Neoplasia 1:508–517
Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, Najima Y, Takagi S, Aoki Y, Wake A, Taniguchi S, Shultz LD, Ishikawa F (2010) Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat Biotechnol 28:275–280
Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. Development 124:2659–2670
Sauka-Spengler T, Bronner-Fraser M (2008) A gene regulatory network orchestrates neural crest formation. Nat Rev Mol Cell Biol 9:557–568
Sayan AE, Griffiths TR, Pal R, Browne GJ, Ruddick A, Yagci T, Edwards R, Mayer NJ, Qazi H, Goyal S, Fernandez S, Straatman K, Jones GD, Bowman KJ, Colquhoun A, Mellon JK, Kriajevska M, Tulchinsky E (2009) SIP1 protein protects cells from DNA damage-induced apoptosis and has independent prognostic value in bladder cancer. Proc Natl Acad Sci USA 106:14884–14889
Schiemann WP (2007) Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? Expert Rev Anticancer Ther 7:609–611
Schneeberger EE, Lynch RD (2004) The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286:C1213–C1228
Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, Stanbridge EJ, Lee EY (2008) Cancer stem cells contribute to Cisplatin resistance in BRCA1/p53-mediated mouse mammary tumors. Cancer Res 68:3243–3250
Shi MF, Jiao J, Lu WG, Ye F, Ma D, Dong QG, Xie X (2010) Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line. Cell Mol Life Sci 67:3915–3925
Shi Y, Massague J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113:685–700

Author's personal copy

Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K (2007) Molecular definition of breast tumor heterogeneity. Cancer Cell 11:259–273

Silberstein GB, Daniel CW (1987) Reversible inhibition of mammary gland growth by transforming growth factor-β. Science 237:291–293

Singh A, Settleman J (2010) EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 29:4741–4751

Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR, Darling DS, Shroyer KR, Horwitz KB, Broaddus RR, Richer JK (2008) ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease. Mod Pathol 21:912–923

Sivakumar R, Koga H, Selvendiran K, Maeyama M, Ueno T, Sata M (2009) Autocrine loop for IGF-I receptor signaling in SLUG-mediated epithelial-mesenchymal transition. Int J Oncol 34:329–338

Skromne I, Stern CD (2001) Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. Development 128:2915–2927

Takaishi K, Sasaki T, Kotani H, Nishioka H, Takai Y (1997) Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK cells. J Cell Biol 139:1047–1059

Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW, Hollier BG, Ram PT, Lander ES, Rosen JM, Weinberg RA, Mani SA (2010) Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA 107:15449–15454

Taylor MA, Parvani JG, Schiemann WP (2010) The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia 15:169–190

Taylor MA, Amin J, Kirschmann DA, Schiemann WP (2011) Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor-β signaling in breast cancer cells. Neoplasia 13:406–418

Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454

Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–890

Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N, Iwata KK, Gibson N, Haley JD (2005) Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer Res 65:9455–9462

Thomson S, Petti F, Sujka-Kwok I, Epstein D, Haley JD (2008) Kinase switching in mesenchymal-like non-small cell lung cancer lines contributes to EGFR inhibitor resistance through pathway redundancy. Clin Exp Metastasis 25:843–854

Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A (2006) Transforming growth factor-β employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol 174:175–183

Tian M, Schiemann WP (2009) The TGF-β paradox in human cancer: an update. Future Oncol 5:259–271

Tian M, Schiemann WP (2010) PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J 24:1105–1116

Tian M, Neil JR, Schiemann WP (2011) Transforming growth factor-β and the hallmarks of cancer. Cell Signal 23:951–962

Tilghman RW, Cowan CR, Mih JD, Koryakina Y, Gioeli D, Slack-Davis JK, Blackman BR, Tschumperlin DJ, Parsons JT (2010) Matrix rigidity regulates cancer cell growth and cellular phenotype. PLoS One 5:e12905

To K, Fotovati A, Reipas KM, Law JH, Hu K, Wang J, Astanehe A, Davies AH, Lee L, Stratford AL, Raouf A, Johnson P, Berquin IM, Royer HD, Eaves CJ, Dunn SE (2010) Y-box binding protein-1 induces the expression of CD44 and CD49f leading to enhanced self-renewal, mammosphere growth, and drug resistance. Cancer Res 70:2840–2851

Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G (2007) Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 1:389–402

Tokar EJ, Qu W, Liu J, Liu W, Webber MM, Phang JM, Waalkes MP (2010) Arsenic-specific stem cell selection during malignant transformation. J Natl Cancer Inst 102:638–649

Turley EA, Veiseh M, Radisky DC, Bissell MJ (2008) Mechanisms of disease: epithelial-mesenchymal transition—does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol 5:280–290

Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, Roncari L, Narimatsu M, Bose R, Moffat J, Wong JW, Kerbel RS, O'Malley FP, Andrulis IL, Wrana JL (2009) A role for the TGFβ-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci USA 106:14028–14033

Visvader JE (2011) Cells of origin in cancer. Nature 469:314–322

Wang B, Yang H, Huang YZ, Yan RH, Liu FJ, Zhang JN (2010) Biologic characteristics of the side population of human small cell lung cancer cell line H446. Chin J Cancer 29:254–260

Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ (2002) Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst 94:1494–1503

Wang SE, Xiang B, Zent R, Quaranta V, Pozzi A, Arteaga CL (2009) Transforming growth factor β induces clustering of HER2 and integrins by activating Src-focal adhesion kinase and receptor association to the cytoskeleton. Cancer Res 69:475–482

Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, Condeelis JS (2007) Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. Cancer Res 67:3505–3511

Wang Z, Li Y, Kong D, Banerjee S, Ahmad A, Azmi AS, Ali S, Abbruzzese JL, Gallick GE, Sarkar FH (2009) Acquisition of epithelial-mesenchymal transition phenotype of Gemcitabine-resistant pancreatic cancer cells is linked with activation of the Notch signaling pathway. Cancer Res 69:2400–2407

Weaver VM, Peterson OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ (1997) Reversion of the malignant phenotype of human breast cancer cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 137:231–245

Weaver VM, Lelievre S, Lakins JN, Chrenek MA, Jones JC, Giancotti F, Werb Z, Bissell MJ (2002) Beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2:205–216

Wendt MK, Schiemann WP (2009) Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. Breast Cancer Res 11:R68

Wendt MK, Allington TM, Schiemann WP (2009a) Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol 5:1145–1168

Wendt MK, Smith JA, Schiemann WP (2009b) p130Cas is required for mammary tumor growth and transforming growth factor-β-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem 284:34145–34156

Author's personal copy

Wendt MK, Smith JA, Schiemann WP (2010) Transforming growth factor-β-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene 29:6485–6498

Whittard JD, Craig SE, Mould AP, Koch A, Pertz O, Engel J, Humphries MJ (2002) E-cadherin is a ligand for integrin α2β1. Matrix Biol 21:525–532

Wipff PJ, Rifkin DB, Meister JJ, Hinz B (2007) Myofibroblast contraction activates latent TGF-β1 from the extracellular matrix. J Cell Biol 179:1311–1323

Wu H, Liang YL, Li Z, Jin J, Zhang W, Duan L, Zha X (2006) Positive expression of E-cadherin suppresses cell adhesion to fibronectin via reduction of alpha5beta1 integrin in human breast carcinoma cells. J Cancer Res Clin Oncol 132:795–803

Wyckoff JB, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall JE, Condeelis JS (2004) A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64:7022–7029

Wynn TA (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest 117:524–529

Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 214:199–210

Yang J, Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 14:818–829

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927–939

Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, Moses HL (2008) Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+myeloid cells that promote metastasis. Cancer Cell 13:23–35

Yauch RL, Januario T, Eberhard DA, Cavet G, Zhu W, Fu L, Pham TQ, Soriano R, Stinson J, Seshagiri S, Modrusan Z, Lin CY, O'Neill V, Amler LC (2005) Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of Erlotinib in lung cancer patients. Clin Cancer Res 11:8686–8698

Yazhou C, Wenlv S, Weidong Z, Licun W (2004) Clinicopathological significance of stromal myofibroblasts in invasive ductal carcinoma of the breast. Tumour Biol 25:290–295

Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, Piek E, Bottinger EP (2001) Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proc Natl Acad Sci USA 98:6686–6691

Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ (2007) Transforming growth factor-β and microRNA:mRNA regulatory networks in epithelial plasticity. Cells Tissues Organs 185:157–161

Zeng Q, Phukan S, Xu Y, Sadim M, Rosman DS, Pennison M, Liao J, Yang GY, Huang CC, Valle L, Di Cristofano A, Chapelle A de la, Pasche B (2009) Tgfbr1 haploinsufficiency is a potent modifier of colorectal cancer development. Cancer Res 69:678–686

Zha J, Lackner MR (2010) Targeting the insulin-like growth factor receptor-1R pathway for cancer therapy. Clin Cancer Res 16:2512–2517

Zhang N, Li R, Tao KS, Cao DY, Ti ZY, Ding R, Cai L, Zhang FQ, Dou KF (2010) Characterization of a stem-like population in hepatocellular carcinoma MHCC97 cells. Oncol Rep 23:827–831

Zhang W, Alt-Holland A, Margulis A, Shamis Y, Fusenig NE, Rodeck U, Garlick JA (2006) E-cadherin loss promotes the initiation of squamous cell carcinoma invasion through modulation of integrin-mediated adhesion. J Cell Sci 119:283–291

Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR (1993) Nodal is a novel TGF-β-like gene expressed in the mouse node during gastrulation. Nature 361:543–547

Gene Expression, Vol. 15, pp. 117–132
Printed in the USA. All rights reserved.
Copyright © 2011 Cognizant Comm. Corp.

1052-2166/11 $90.00 + .00
DOI: http://dx.doi.org/10.3727/105221611X13176664479322
E-ISSN 1555-3884
www.cognizantcommunication.com

Role of TGF-β and the Tumor Microenvironment During Mammary Tumorigenesis

MOLLY A. TAYLOR,[1] YONG-HUN LEE,[1] AND WILLIAM P. SCHIEMANN

Case Comprehensive Cancer Center, Division of General Medical Sciences-Oncology, Case Western Reserve University, Cleveland, OH, USA

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that functions to inhibit mammary tumorigenesis by directly inducing mammary epithelial cells (MECs) to undergo cell cycle arrest or apoptosis, and to secrete a variety of cytokines, growth factors, and extracellular matrix proteins that maintain cell and tissue homeostasis. Genetic and epigenetic events that transpire during mammary tumorigenesis typically inactivate the tumor suppressing activities of TGF-β and ultimately confer this cytokine with tumor promoting activities, including the ability to stimulate breast cancer invasion, metastasis, angiogenesis, and evasion from the immune system. This dramatic conversion in TGF-β function is known as the "TGF-β paradox" and reflects a variety of dynamic alterations that occur not only within the developing mammary carcinoma, but also within the cellular and structural composition of its accompanying tumor microenvironment. Recent studies have begun to elucidate the critical importance of mammary tumor microenvironments in manifesting the TGF-β paradox and influencing the response of developing mammary carcinomas to TGF-β. Here we highlight recent findings demonstrating the essential function of tumor microenvironments in regulating the oncogenic activities of TGF-β and its stimulation of metastatic progression during mammary tumorigenesis.

Key words: Mammary tumorigenesis; Metastasis; Microenvironment; Transforming growth factor-β (TGF-β)

INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that suppresses tumorigenesis within the mammary epithelium by inhibiting cell cycle progression, by inducing apoptosis, and by maintaining cellular and tissue homeostasis. Although TGF-β possesses powerful cytostatic activity in normal mammary epithelial cells (MECs), its ability to do so in malignant MECs is frequently inactivated, an event that often gives rise to the acquisition of oncogenic activity by TGF-β in developing and progressing mammary tumors (72,130). This malicious switch in TGF-β function is referred to as the "TGF-β paradox" and is supported by a variety of genetic and epigenetic events that ultimately underlie the adverse prognosis associated with elevated TGF-β production in developing mammary carcinomas (132). At present, the precise sequence of events that manifest the TGF-β paradox remain to be fully elucidated, as does the manner in which these events dictate the extent to which TGF-β mediates its oncogenic activities across genetically distinct breast cancer subtypes (97,121,125). Genomic and proteomic technologies have identified a host of gene transcripts, microRNAs, and proteins that are differentially regulated by TGF-β in normal and malignant MECs. Although these analyses have yet to decipher the precise sequelae necessary to elicit oncogenic TGF-β signaling, these studies have nonetheless offered several unique in-

[1]These authors provided equal contribution to this work.

Address correspondence to William P. Schiemann, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, 2103 Cornell Road Cleveland, OH 44106, USA. Tel: 216-844-8797; Fax: 216-844-7832; E-mail: william.schiemann@case.edu

sights into the role of TGF-β in mediating the development and progression of breast cancers. For instance, gene expression signatures associated with the TGF-β signaling system been linked to the acquisition of epithelial-mesenchymal transition (EMT) and stem cell-like phenotypes exhibited by breast cancer cells (112,118,123), as well as to their ability to disseminate to the bone (57,58,146) and lung (95) in response to TGF-β. Additional molecular profiling analyses have identified gene signatures capable of predicting the organotropic spread and clinical outcomes of patients with metastatic breast cancer (10, 77,78), thereby solidifying TGF-β as a major driver of metastatic breast cancer.

The mammary gland is comprised of two major compartments: (i) the epithelium, which consists of luminal and myoepithelial cells that make up the ductal structures, and (ii) the stroma, which houses fibroblasts, adipocytes, endothelial, and immune cells, as well as extracellular matrix (ECM) proteins and connective tissue elements. Collectively, both mammary gland compartments function in a coordinated manner to maintain cell and tissue homeostasis, and to suppress mammary tumorigenesis. In contrast, developing neoplasms harbor activated stromal compartments accompanied by inflammatory and fibrotic reactions that enhance tumor development and metastatic progression, as well as predict for poor clinical outcomes of breast cancer patients (11,19,133). In addition to its established functions in normal and malignant MECs, TGF-β is also recognized as a major player involved in regulating the composition and activation of tumor microenvironments, particularly during tumor progression and metastatic dissemination (11,133). Indeed, aberrant upregulation of TGF-β expression positively correlates with enhanced breast cancer progression, angiogenesis, and metastasis, all of which contribute to poor clinical outcomes in patients with late-stage disease (11). Likewise, tumor reactive stroma plays an essential role in dictating whether TGF-β functions as a tumor suppressor or a tumor promoter in developing mammary neoplasms (8,11,130,133). In the succeeding sections, we review recent findings detailing the complex and multifaceted role of TGF-β within mammary tumor microenvironments, including its regulation of (i) autonomous responses by carcinoma cells; (ii) angiogenesis by endothelial cells; (iii) immunosurveillance by infiltrating immune cells; and (iv) activation of cancer-associated fibroblasts (Fig. 1).

TGF-β SIGNALING

TGF-β is the prototypic member of a large family of evolutionary conserved cytokines that includes the activins, bone morphogenetic proteins, growth differentiation factors, Nodal, and inhibins (127). Mammals express three genetically distinct TGF-β ligands (e.g., TGF-β 1–3), whose mature and biologically active forms are ~97% identical and exhibit virtually indistinguishable actions in vitro (14,97). Individual TGF-β molecules play important roles during embryonic development and tissue morphogenesis, and in maintaining cellular and tissue homeostasis in adults (73). TGF-β signaling is initiated by its binding to three high-affinity receptors, TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). TβR-I and TβR-II both harbor Ser/Thr protein kinases in their cytoplasmic domains that are essential for the activation of intracellular signaling by TGF-β (35,73) (Fig. 2). Although TβR-III lacks intrinsic enzymatic activity, this polypeptide is typically the most abundant receptor for TGF-β and functions as an accessory molecule that modulates cellular responses to TGF-β. The expression of TβR-III is essential for the ability of TGF-β to suppress tumor formation, particularly in the breast, ovary, prostate, lung, pancreas, kidney, and endometrium (39). The binding of TGF-β to TβR-II allows for the subsequent recruitment, transphosphorylation, and activation of TβR-I by TβR-II. Activated TβR-I, in turn binds, phosphorylates, and stimulates the latent transcription factors, Smad2 and Smad3, which rapidly form higher order complexes with the common Smad, Smad4 (35,73). The resulting heteromeric Smad2/3/4 complexes accumulate in the nucleus where they regulate gene expression in a cell- and context-specific manner (35,73,117) (Fig. 2). The activation of Smads 2, 3, and 4 by TGF-β is referred to as "canonical TGF-β signaling" and these events are modulated in all subcellular compartments by numerous effector molecules (138). Besides its ability to activate canonical Smad2/3/4 signaling, TGF-β also regulates cell behavior through its activation of a variety of Smad2/3-independent pathways, which are collectively referred to as "noncanonical TGF-β signaling." Included in this ever expanding list of noncanonical TGF-β effectors are the (i) MAP kinases, ERK1/2, p38MAPK, and JNK; (ii) cell survival mediators, PI3K, AKT1/2, and mTOR; (iii) inflammatory mediators, NF-κB, Cox-2, and prostaglandins; (iv) small GTP-binding proteins, Ras, RhoA, Rac1, and Cdc42; and (v) nonreceptor protein tyrosine kinases, Src, FAK, and Abl (56,97).

Collectively, both branches of the TGF-β signaling system coalesce in generating the pleiotropic activities of TGF-β in distinct cell lineages. Importantly, imbalances between the canonical and noncanonical TGF-β signaling systems have been associated with disease development in humans, including cancers of the breast (130,132). Along these lines, early hypotheses to explain cancer development postulated tumors

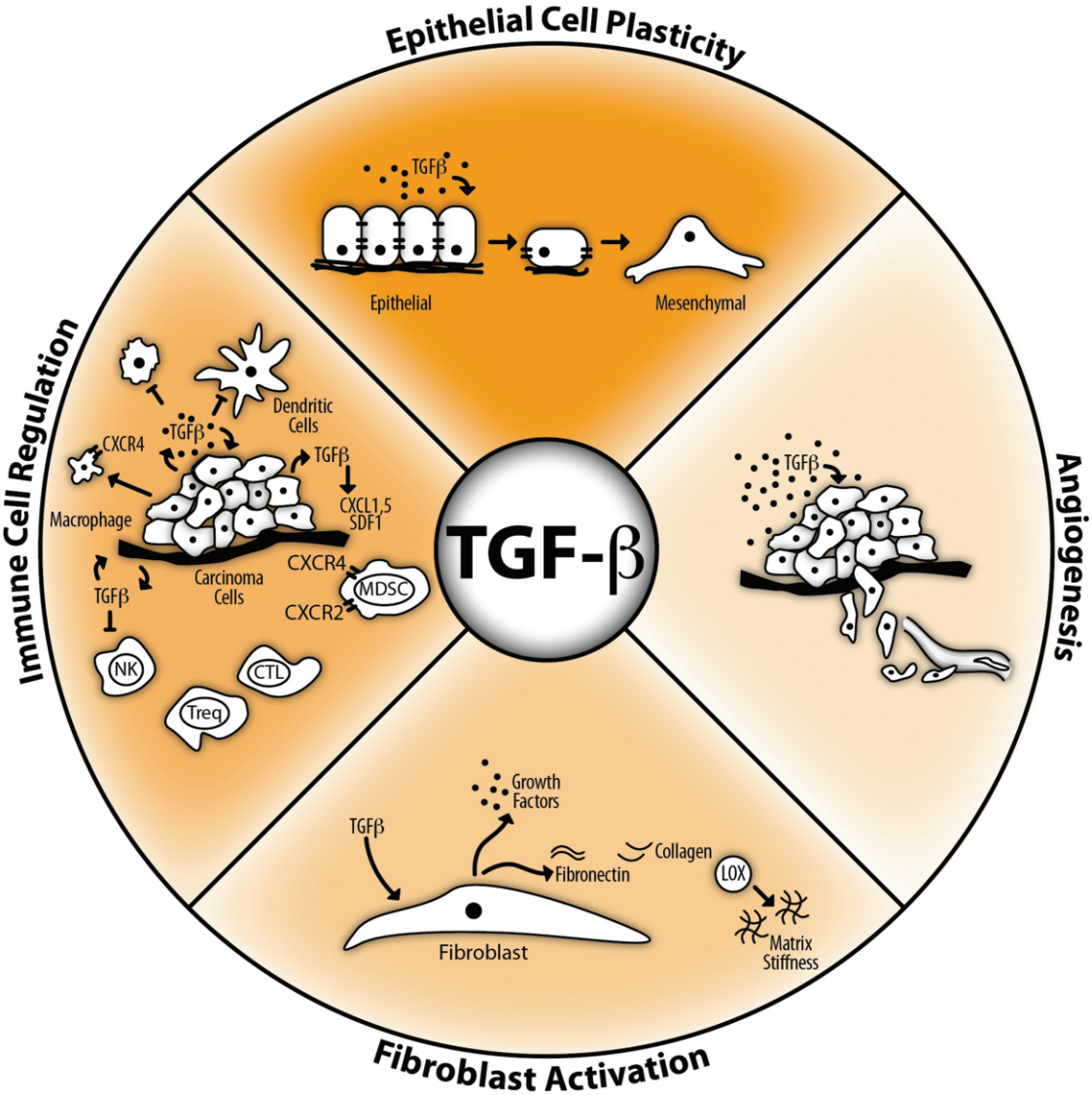


Figure 1. TGF-β is a master regulator of MEC plasticity and microenvironmental homeostasis. TGF-β induces malignant MECs to undergo EMT, leading to the acquisition of highly migratory, invasive, and metastatic phenotypes. TGF-β is also a potent inducer of tumor angiogenesis, which significantly enhances the growth and metastasis of late-stage mammary tumors. Through its ability to inhibit host immunosurveillance, TGF-β also plays an essential role in conferring immune privilege to developing and progressing breast cancers. Finally, TGF-β stimulates fibroblasts to synthesize and secret a variety of growth factors, cytokines, and ECM molecules that collectively create a tumor promoting microenvironment.

as being a collection of homogenous carcinoma cells, whose entire evolution and pathophysiology could be comprehended simply by elucidating the cell-autonomous properties of these clonal neoplasms. This idea has now given way to the view that tumor growth is in many respects reminiscent of that of developing organs, albeit in a highly dysfunctional and disorganized manner (108). Because virtually every cell in the human body is capable of both producing and responding to TGF-β (14), and because TGF-β is a major driver of metastatic progression in mammary tumors (125), it stands to reason that a true understanding of the "TGF-β paradox" will only be realized by first deciphering the functions of TGF-β in all specialized cell types within the tumor microenvironment, and by determining how these events collectively impact the development and progression of breast cancers in response to TGF-β. The role of TGF-β in regulating the activities of distinct stroma cell types is discussed in the succeeding sections (Fig. 1).

TGF-β AND MECs

TGF-β Expression and MECs

Examination of mice engineered to lack the expression of either TGF-β1, 2, or 3 suggest that the

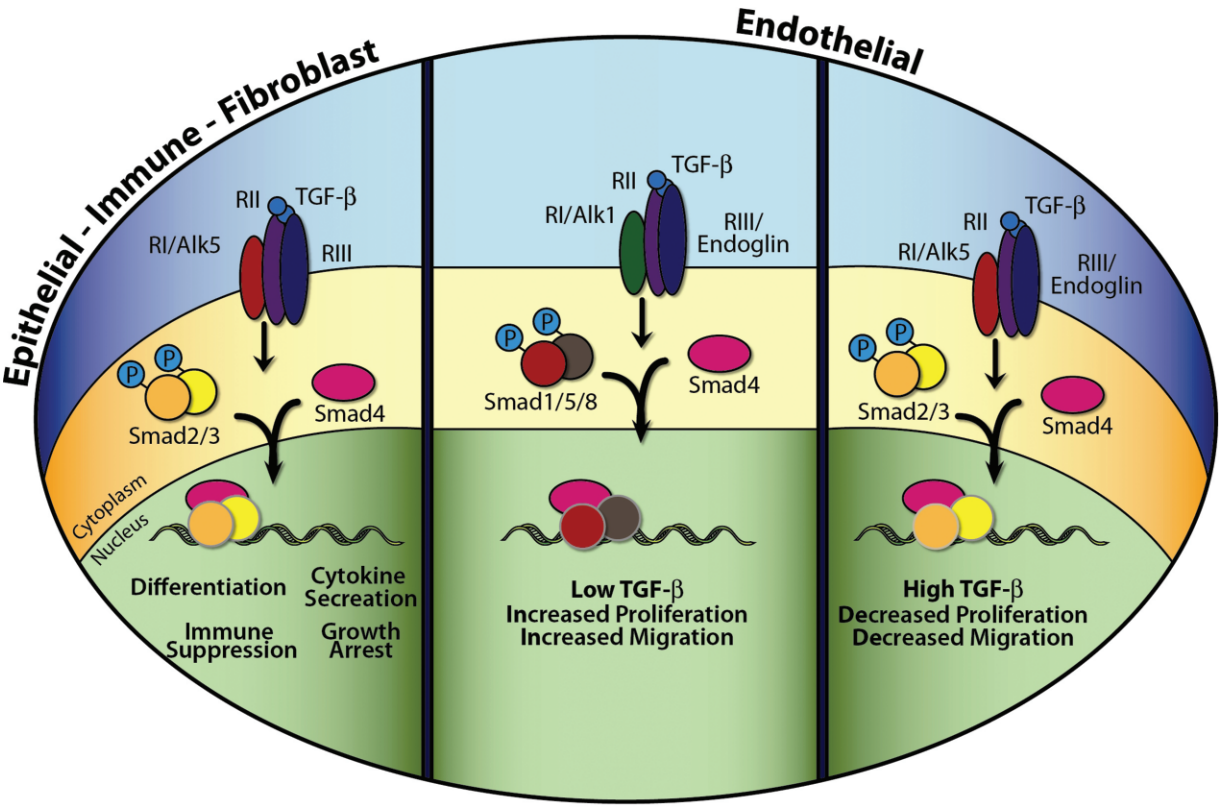


Figure 2. Schematic of canonical TGF-β signaling within distinct cell types located in tumor microenvironments. TGF-β predominantly activates a Smad2/3-based pathway in fibroblasts, epithelial, and immune cells (left panel), and in endothelial cells subjected to high TGF-β concentrations (right panel). In contrast, endothelial cells subjected to low TGF-β concentrations activate a Smad1/5/8-based pathway (middle panel). In general, TGF-β in the extracellular space binds either to TβR-III or endoglin, both of which present TGF-β to TβR-II. In some cells, TGF-β can bind directly to TβR-II independent of TβR-III or endoglin expression on the cell membrane. TβR-II bound to TGF-β then recruits, transphosphorylates, and activates the TβR-I isozymes, ALK-5 and ALK-1. Activated TβR-I/ALK-5 or TβR-I/ALK-1 then phosphorylate and activate Smad2/3 or Smad1/5/8, respectively, which then form heteromeric complexes with Smad4 that readily accumulate in the nucleus to regulate changes in gene expression in a cell- and context-specific manner.

activity of these cytokines are not required for embryonic development of the mammary gland. However, during the branching morphogenesis reactions that take place in postnatal mammary glands, all three TGF-β ligands are expressed and can suppress terminal end bud formation (102). During pregnancy, TGF-β2 and TGF-β3 are highly expressed in alveolar and ductal structures, while little-to-no TGF-β1 expression is detected in these same structures (109). After weaning, the expression of TGF-β3 is rapidly induced during the initial stages of mammary gland involution (34), which subsequently gives way to the elevated expression of TGF-β1 and TGF-β2 as glandular regression progresses and eventually resolves (34,109,114). Despite the fact that all TGF-β isoforms are functionally active in normal mammary tissues, the upregulated expression of TGF-β1 is most commonly associated with mammary tumorigenesis (27), and as such, the function of this TGF-β isoform will be highlighted throughout the remainder of this review.

The use of mouse models has greatly enhanced our understanding of the role of TGF-β and its signaling system in epithelial cells. For example, homozygous deletion of TGF-β1 elicits embryonic lethality in ~50% of the developing pups, while those that survive to term rapidly succumb to massive inflammatory reactions that develop in the heart, lungs, and salivary glands. Homozygous deletion of TGF-β2 or TGF-β3 both elicit perinatal lethality due to multiple developmental defects associated with aberrant EMT reactions during organogenesis and tissue morphogenesis. Along these lines, genetic inactivation of Smad2, Smad4, TβR-I, and TβR-II are all incompatible with life due to defects in mesoderm formation (Smad2), gastrulation (Smad4), and vascular development (TβR-I and TβR-II). In contrast, homozygous deletion of Smad3 and TβR-III result in viable mice that exhibit retarded growth rates and increased incidence of colon cancer due in part to altered immune function (Smad3), and osteoporotic lesions (TβR-III) (18,20). Collectively, these and numerous additional

studies have helped to define the essential role of TGF-β signaling in regulating organ development and immune privilege.

Transgenic mouse models have also played a valuable role in elucidating the functions of TGF-β during mammary tumorigenesis. For instance, mammary gland-specific expression of a constitutively active TGF-β1 mutant results in mammary ductal hypoplasia (64), as well as inhibits the formation of lobular-alveolar structures and the production of milk proteins (54). In addition, crossing MMTV-TGF-β1 mice onto a MMTV-TGF-α background significantly lengthens the latency of tumors induced by 7,12-dimethylbenz[*a*]anthracene (101). Taken together, these studies identify TGF-β as an inhibitory molecule coupled to the prevention of mammary tumorigenesis, particularly during the initial stages of neoplastic development. However, the paradoxical and tumor-promoting activities of TGF-β have also been observed in transgenic mouse models. Indeed, although crossing MMTV-TGF-β1 mice onto a MMTV-c-Neu background fails to alter the latency of tumor formation, this same genetic condition greatly enhances the acquisition of invasive and metastatic phenotypes due in part to the upregulated expression of vimentin in mammary carcinoma cells (82). Likewise, conditional expression of TGF-β1 in MMTV-PyMT-driven mammary tumors failed to alter their proliferative indices and size, but did elicit dramatic elevations in pulmonary metastasis (83). Collectively, these studies illustrate the dichotomy of TGF-β function between early and late-stage mammary tumors.

TGF-β Function in MECs

Our understanding of how MECs respond to TGF-β has also been aided by the transgenic expression constitutively active and dominant-negative versions of the receptors for TGF-β. For instance, mammary gland-specific expression of a truncated and nonfunctional TβR-II mutant (i.e., MMTV-DNIIR) elicits alveolar hyperplasia and excessive MEC differentiation in virgin animals (45), as well as accelerates glandular development and delays involution in their pregnant counterparts (44). Importantly, crossing MMTV-DNIIR mice onto either a MMTV-TGF-β or MMTV-Neu background significantly decreases tumor latency and reduces carcinoma cell invasion and pulmonary metastasis (44,119). Accordingly, crossing a constitutively active TβR-I receptor [i.e., MMTV-TβR-I(AAD)] onto a MMTV-Neu background significantly delays the rate of tumor formation and suppresses pulmonary metastasis (119). Thus, these findings reinforce the notion that TGF-β signaling is essential in both suppressing mammary tumor formation and promoting metastatic progression. Along these lines, conditional and specific deletion of TβR-II in the mammary epithelium (i.e., Tgfbr2MGKO) also elicits alveolar hyperplasia, as well as increased MEC apoptosis in hyperplastic tissues (37). Paradoxically, crossing Tgfbr2MGKO (i.e., TβR-II-deficient) mice onto a MMTV-PyMT background shortens tumor latency and, surprisingly, enhances the metastatic abilities of carcinoma cells rendered unresponsive to TGF-β (13,37). Finally, systemic administration (81) or transgenic expression (145) of a soluble Fc:TβR-II fusion protein, which antagonizes TGF-β signaling by binding and sequestering TGF-β, inhibits the survival, motility, and metastasis of mammary tumors in mice, thereby highlighting the differences between systemic and local actions of TGF-β in developing mammary tumors. Collectively, these intriguing findings demonstrate the plasticity present in the TGF-β signaling system as mammary carcinoma cells develop and progress to metastasis, events that are clearly dependent upon the differential activities of TGF-β in early versus late-stage carcinomas, as well as in the neighboring stromal compartment.

TGF-β and MEC Plasticity

TGF-β is well known for its ability to promote metastatic progression through the induction of EMT in MECs. This transdifferentiation process results in polarized MECs acquiring apolar and highly motile fibroblastoid-like phenotypes (125,138). The process of EMT is characterized by (i) changes in cytoskeletal architecture and intracellular organelle redistribution; (ii) loss of cell polarity due to downregulation of epithelial cell markers (e.g., E-cadherin, ZO-1, and β4 integrin); (iii) upregulation of fibroblastoid markers (e.g., vimentin, N-cadherin, α-smooth muscle actin); and (iv) elevated expression of invasion promoting factors [e.g., MMP-9, fibronectin; see (125,138)]. Recently, the process of EMT has been categorized into three distinct subtypes: (i) type 1 EMT, which represents the transdifferentiation process that occurs during embryogenesis and tissue morphogenesis; (ii) type 2 EMT, which is associated with tissue regeneration during wound healing, fibrotic reactions, and inflammation; and (iii) type 3 EMT, which represents the plasticity exhibited by carcinoma cells that enables them acquire invasive, metastatic, and stem cell-like phenotypes (55). In fact, EMT programs not only enhance the ability of carcinoma cells to invade locally as a means to exit the primary tumor, but also facilitate their survival in the circulation and ability to reinitiate proliferative programs at distant sites of metastasis (95,139,140). At present the contributions of the tumor microenvironment in coupling TGF-β

to EMT programs remains an important question for future research. Readers desiring additional information pertaining the molecular mechanisms whereby TGF-β induces EMT in normal and malignant MECs are directed to several recent comprehensive reviews (125,138).

TGF-β AND ENDOTHELIAL CELLS

TGF-β and Cell Junctions

Adhesive intercellular junctions between endothelial cells are formed by the actions of adherens junctions and tight junctions, which establish and maintain cell–cell contacts, as well as promote the transfer of intracellular signals between cells (90). Although the general organization of adherens and tight junctions in the endothelium is similar to those of epithelial cells, there are nonetheless some cell type-specific differences. For example, adherens junctions in epithelial cells are comprised primarily of the transmembrane protein, epithelial cadherin (E-Cad), which is connected to the actin cytoskeleton via α- and β-catenins (88). The associations between E-cad and TGF-β are well-studied during EMT programs (125,138), where TGF-β inactivates E-Cad function by (i) repressing the synthesis of E-Cad transcripts, and (ii) delocalizing and internalizing E-Cad proteins from the cell membrane, an event coupled to a loss of Rac1 activity (122). Actin cytoskeletal rearrangements engendered by TGF-β become apparent through the activation of RhoA, which reduces cell adhesion and elicits cell migration and invasion (7,107). In contrast to epithelial adherens junctions, those present in endothelial cells contain claudin-5, platelet/endothelial cell adhesion molecule (PECAM-1), and vascular endothelial (VE) cadherin (VE-Cad), which interacts directly or indirectly with multiple intracellular partners, including β-catenin, plakoglobin (γ-catenin), p120 catenin, and the endothelial-specific receptor protein tyrosine phosphatase, VE-PTP (3, 25). At present, the connections between TGF-β and VE-Cad in regulating endothelial cell biology remain to be fully elucidated. However, the VE-Cad has recently been shown to facilitate the maximal response of endothelial cells to TGF-β (111). Indeed, the expression and clustering of VE-Cad maximizes the coupling of TGF-β to antimigration and antiproliferation signals in endothelial cells. Mechanistically, VE-Cad interacts physically with and facilitates the assembly of TGF-β receptors into active signaling complexes, leading to enhanced Smad phosphorylation and gene transcription (111). Along these lines, tyrosine phosphorylation of VE-Cad regulates the ability of TGF-β to increase the paracellular permeability of vascular endothelial cells (116). Interestingly, malignant MECs that are undergoing EMT have been observed to upregulate their expression of VE-Cad. Indeed, elevated VE-Cad expression enhances the ability of breast cancer cells to proliferate in response to TGF-β, as well as to activate mammary tumor angiogenesis (62). Thus, these findings establish VE-Cad as a novel mediator of TGF-β signaling in mammary carcinoma cells and their supporting endothelial cells, suggesting that chemotherapeutic targeting of VE-Cad may provide a novel two-pronged approach to alleviate oncogenic TGF-β signaling in breast cancers.

In contrast to adherens junctions, tight junctions are formed by the actions of claudins, occludins, and junctional adhesion molecules (JAMs), which connect to the actin cytoskeleton by binding to a number of scaffolding proteins, including zonula occludens (ZO-1, -2, and -3), AF6/Afadin, PAR3 (partitioning-defective 3), and others (3,42). The association between TGF-β and Par6 (partitioning-defective 6) has been described in endocardial cells undergoing EMT (135). Par6 also plays an important role in controlling the formation of tight junctions, generation of apical-basolateral polarity, and the initiation of polarized cell migration (17). Par6 interacts physically with TGF-β receptors and can be phosphorylated by TβR-II, leading to the formation of Par6:Smurf1 complexes that promote the ubiquitination and degradation of RhoA (94). These results suggest that Par6 plays an important role in controlling the dynamics between tight junctions and TGF-β signaling in endothelial and epithelial cells.

TGF-β and Vascular Morphogenesis

Efficient tumor growth is absolutely dependent on its ability to secure a dependable supply of nutrients and oxygen, as well as a route to dispose of metabolic waste (2). To satisfy these essential needs, developing tumors synthesize a neovasculature system through the process of angiogenesis, which encompasses endothelial cell proliferation, migration, tubulogenesis, and anastomosis (53). Increased TGF-β expression has been positively associated with poor prognosis and increased tumor growth due the activation of angiogenic programs. Likewise, administration of anti-TGF-β agents has been shown to reduce tumor angiogenesis and, consequently, to inhibit tumor growth and progression (51). Thus, TGF-β likely plays a significant role in stimulating angiogenesis in late-stage mammary tumors. Along these lines, the engineering of mice that fail to express TGF-β or its receptors has revealed important roles for the TGF-β signaling system during vascular development (15,20). Indeed,

homozygous deletion of TGF-β1 in mice results in embryo lethality due to defective yolk sac vasculogenesis (26,46). Interestingly, the vascular abnormalities by TGF-β1 deletion were only observed in specific genetic backgrounds, suggesting the involvement of additional genetic modifiers coupled to vascular development in mice harboring defects in their TGF-β signaling systems. Additionally, genetic inactivation of the TGF-β receptors TβR-II, TβR-I (also called ALK-5), or ALK-1 results in embryonic lethality at E10.5 due to vascular defects, indicating an important role for these receptors in normal endothelial cell function (63,91,92). Along these lines, homozygous deletion in mice of the accessory receptor, endoglin, elicits embryonic lethality at E11.5 that reflects cardiovascular and angiogenic defects (66). Collectively, these findings implicate TGF-β as an essential mediator of vasculogenesis during embryonic development and tissue morphogenesis.

Consistent with the aforementioned conclusion, TGF-β also governs the expression of a variety of genes in endothelial cells, including collagens I, IV, and V, fibronectin, and the fibronectin receptor, integrin α5β1 (29,75,99). TGF-β also induces the expression of PDGF-B, which is important for the recruitment of pericytes during vessel maturation (41). Interestingly, whereas normal vessels are tightly associated with pericytes and benefit from their mechanical and physiological support, tumor vessels typically exhibit noticeably reduced levels of these auxiliary cells, leading to aberrant paracrine signaling networks between pericytes and their underlying endothelial cells (5,51,105). A proangiogenic function for TGF-β and its activation of ALK-1 is further supported by their induction of the transcription factor, Id1, which mediates endothelial cell proliferation and migration (47,48,89). Additionally, activation of ALK-1 readily promotes the angiogenesis, growth, and progression of tumors (24), and, as such, pharmacological inactivation of ALK-1 signaling (e.g., ALK-1-Fc fusion protein) significantly reduces the angiogenesis and growth of pancreatic and breast carcinomas, including that induced by TGF-β, VEGF, and bFGF (24,79). Thus, targeted chemotherapies against ALK-1 may represent a novel class of antitumor agents capable of inhibiting tumor progression by alleviating tumor angiogenesis.

The aforementioned findings clearly implicate TGF-β as a potent inducer of tumor angiogenesis; however, this designation remains controversial given the findings in the scientific literature that link TGF-β signaling to angiostatic programs that transpire in context-specific manner (48,96). For example, whereas expression of constitutively active ALK-1 induces angiogenesis in mouse embryonic endothelial cells (48), similar expression of constitutively active TβR-I (ALK-5) inhibits angiogenesis in human umbilical vein endothelial cells (93). Along these lines, the ability of TGF-β to differentially regulate endothelial cell proliferation and migration may reflect changes in the microenvironmental balance between TGF-β and additional angiogenic factors. In fact, low TGF-β concentrations are known to promote bFGF- and VEGF-mediated endothelial cell proliferation and sprouting, while administration of high TGF-β concentrations prevents these events from occurring (48, 99,115) (Fig. 2). In addition, inhibiting ALK-5 activity readily uncouples TGF-β from activating canonical Smad2/3 signaling. However, a recent study observed Smads 2 and 3 to mediate diametrically opposed activities in developing mammary tumors. Indeed, whereas the activation of Smad2 was found to inhibit mammary tumor angiogenesis, growth, and metastasis, Smad3 activation was linked to the oncogenic activity of TGF-β and its stimulation of mammary tumor angiogenesis and metastasis (100). Thus, the selective inactivation of Smad3 may provide a novel and effective means to prevent tumor angiogenesis stimulated by TGF-β. Likewise, administering neutralizing antibodies against endoglin inhibits VEGF-mediated endothelial cell sprouting in vitro, and mammary tumor growth in mice (52,128,136).

Collectively, these findings emphasize the delicate balance that fine tunes and orchestrates the signaling systems that determine whether TGF-β couples to the induction or suppression of tumor angiogenesis. Future studies need identify the microenvironmental factors that govern the angiogenic or angiostatic activities of TGF-β, as well as assess their clinical relevance as potential therapeutic targets or diagnostic biomarkers for breast cancer patients.

TGF-β AND THE IMMUNE SYSTEM

An essential function of TGF-β within tumor microenvironments lies in its ability to suppress immunosurveillance by inhibiting the functions of infiltrating immune cells operant in mediating tumoricidal activities, and to facilitate the recruitment of macrophages and monocytes that enhance metastatic progression (12). Along these lines, we defined a novel TAB1:xIAP:TAK1:IKKβ:NF-κB signaling axis coupled to the production of proinflammatory cytokines in breast cancer cells (85–87). A major effect of this noncanonical TGF-β effector system results in the elevated expression of Cox-2 and its synthesis of PGE2, which promotes breast cancer progression, EMT, and metastasis via autocrine activation of EP2 receptors (131). The overall importance of TGF-β in

regulating immune cell function is underscored by the fact that mice deficient in TGF-β1 expression readily develop lethal multifocal inflammatory disease (60,61). Likewise, genetic inactivation of Smad3 impairs T-cell responsiveness, as well as elicits chemotaxis defects in neutrophils, T cells, and B cells (144). Here we highlight the specific activities of TGF-β on T cells, macrophages, and myeloid-derived suppressor cells (MDSCs) that enhance the development and progression of mammary tumors.

TGF-β Suppresses T Cell Immunosurveillance

$CD8^+$ cytolytic T lymphocytes (CTLs) play a critical role in mediating the clearance of tumor cells. Tumor development and progression is bolstered by the ability of TGF-β to suppress the proliferation, immunosurveillance, and cytolytic activities of $CD8^+$ CTLs. In fact, engineering mouse fibrosarcoma cells to overexpress TGF-β1 enhanced tumor growth by suppressing CTL-mediated tumor rejection (134). Additionally, specific abrogation of TGF-β signaling in T cells mediated by their enforced expression of a nonfunctional TβR-II mutant (i.e., truncated TβR-II) enabled mice to mount an effective immune response capable of eradicating melanoma growth and metastasis (43). Mechanistically, TGF-β inhibits the production of IL-2, represses the expression of c-Myc and cyclins D2 and E, and stimulates the expression of the CDK inhibitors, p15, p21, and p27. The net effect of these events results in a significant decrease in T-cell proliferation and response (67,126,141). Along these lines, TGF-β also represses the ability of T cells to transcribe a variety of apoptosis inducing factors, including perforin, granzymes A and B, FAS ligand, and interferon-γ (1,16,129). Moreover, TGF-β stimulation of $CD8^+$ T cells enhances their production and secretion of IL-17, which activates survival signaling in carcinoma cells (84). Unlike $CD8^+$ T cells, TGF-β has no effect on the proliferation of $CD4^+$ T cells, but instead functions to inhibit their differentiation (43). TGF-β also inactivates the tumoricidal activities of CTLs by inducing the selection and expansion of Tregs, which suppress granule release by activated CTLs (69,74). Similar to $CD8^+$ CTLs, natural killer (NK) cells play an essential role in suppressing tumor formation by targeting tumor cells for destruction. The ability of NK cells to kill carcinoma cells depends upon the activation of NKp30 and NKG2D receptors, whose expression is readily downregulated by TGF-β as a means to inactivate the cytolytic activities of NK cells (4,80,110). Indeed, systemic attenuation of TGF-β signaling increases immune-mediated clearance of tumor cells in vivo, presumably due to the unveiling of normal CTL and NK cell tumoricidal activity (59,129).

Besides its ability to directly inhibit the functions of CTLs and NK cells, TGF-β also suppresses T-cell activity via an indirect mechanism involving the actions of neutrophils and dendritic cells. For instance, TGF-β functions as a potent chemoattractant for neutrophils (106) and inhibits their ability to recognize and destroy Fas ligand (FasL) that is abundantly expressed on carcinoma cells. As such, tumor-infiltrating CTLs undergo apoptosis upon contacting FasL-expressing carcinoma cells, an event that confers these cells immune privilege and promotes their metastatic progression (50). Dendritic cells function in initiating immune responses by presenting antigens to T cells, B cells, and NK cells (12). Interestingly, administering TGF-β to dendritic cells inhibits their maturation and production of the proinflammatory cytokines, IL-1 and IL-12, thereby failing to mount an effective antitumor CTL response (40). Taken together, these studies suggest that measures capable of reducing TGF-β levels within tumor microenvironments will significantly improve the CTL activity and tumor clearance by T cells.

TGF-β and Monocytes and Macrophages

Generally speaking, the recruitment of monocytes and macrophages to tumor microenvironments is associated with enhanced tumor progression (22,70,71). TGF-β is a potent inducer of IL-1 and IL-6 expression by monocytes, as well as a powerful stimulator of their differentiation into macrophages (36). Likewise, TGF-β readily attenuates the effector and cytotoxic functions of macrophages that normally target carcinoma cells for destruction (49). Finally, the activation of resting monocytes by TGF-β stimulates their chemotaxis and infiltration into tumor microenvironments where they (i) promote carcinoma progression by stimulating ECM degradation necessary for tumor angiogenesis, invasion, and metastasis, and (ii) create an immunosuppressive environment through their release of TGF-β (68,103,137).

TGF-β and MDSC Recruitment

The preceding sections highlighted the importance of tumor-associated macrophages, monocytes, and neutrophils in promoting tumor development and metastatic progression (12,23). More recently, immature $Gr\text{-}1^+CD11b^+$ myeloid cells, which are also known as myeloid-derived suppressor cells (MDSCs), have been shown to possess robust immunosuppressive activities (38,142). Indeed, genetic inactivation of TβR-II in mammary carcinoma cells elicits tumor infiltration of $Gr\text{-}1^+CD11b^+$ cells in part via the activation of SDF-1/CXCR4 and CXCL5/CXCR2 chemokine signaling axes. Upon gaining entry into mammary tumor

microenvironments, MDSCs readily inhibit the function of dendritic cells, NK cells, and B and T lymphocytes (12,143). Additionally, MDSCs also secrete high levels of (i) matrix metalloproteinases, which aids breast cancer cell dissemination from the primary tumor, and (ii) TGF-β, which further suppresses host immune response and promotes acquisition of invasive and metastatic phenotypes by breast cancer cells (142). Because MDSCs localize to the invasive front of breast cancers (13,142) and aid in establishing premetastatic niches during breast cancer dissemination (30), it stands to reason that chemotherapeutic targeting of MDSCs may provide a novel opportunity to improve the clinical course of breast cancer patients by simultaneously improving host immune surveillance and inhibiting metastatic progression.

TGF-β AND STROMAL FIBROBLASTS

TGF-β and Cancer-Associated Fibroblasts

Besides its ability to govern the activities and behaviors of epithelial, endothelial, and hematopoietic cell lineages, TGF-β is also a master regulator of the proliferation and differentiation status of fibroblasts, including those in the mammary tumor microenvironment (9,104). Indeed, TGF-β present in tumor microenvironments induces fibroblasts to secret a variety of growth factors, cytokines, and ECM proteins that act in a coordinated fashion to either suppress or promote tumor development in the adjacent epithelium (11,132). Similar to the differential gene expression profiles exhibited between normal and malignant MECs, recent microarray analyses have identified distinct gene expression signatures that readily distinguish normal mammary fibroblasts from their cancer-associated counterparts (120). Importantly, TGF-β was identified as a prominent protein downregulated in tumor-derived fibroblasts, suggesting that diminished TGF-β production by stromal cells engenders a tumor-promoting microenvironment (120). In support of this supposition, conditional deletion of TβR-II specifically in fibroblasts significantly expands the stromal compartments of the prostate and forestomach due in part to disruptions in paracrine signaling networks between fibroblasts and epithelial cells. Ultimately, these aberrant events culminate in the generation intraepithelial neoplasia in the prostate and invasive squamous cell carcinoma in the forestomach (6). Similar genetic inactivation of TβR-II specifically in mammary fibroblasts also expands their abundance in the mammary gland, as well as increases the turnover of adjacent ductal epithelial cells. Interestingly, transplanting TβR-II-deficient fibroblasts under the renal capsule with mammary carcinoma cells greatly increases their growth and invasion relative to that mediated by TβR-II-expressing fibroblasts. The elevated malignancy exhibited by transplanted mammary carcinoma cells reflects their activation of several receptor tyrosine kinases (RTKs), including EGFR, ErbB2, RON, and c-Met. Importantly, TβR-II deficiency in mammary fibroblasts increases their production and secretion of the cognate ligands for these RTKs (e.g., TGF-β, MSP, and HGF) (21). Recently, loss of a single TβR-II allele was determined to enhance the accumulation of fibroblasts, as well as increase the aggressiveness and metastasis of MMTV-PyMT tumors in mice (33). Collectively, these findings indicate that TGF-β signaling in fibroblasts functions to suppress the activation of paracrine signaling networks that promote tumorigenesis in adjacent MECs epithelial cells.

TGF-β and Fibroblast Transdifferentiation

Besides its ability to regulate the activation and proliferation of fibroblasts, TGF-β also promotes the transdifferentiation of fibroblasts into myofibroblasts, which is defined immunohistochemically by their expression of α-smooth muscle actin (α-SMA) and vimentin (76). Fibroblast transdifferentiation is reminiscent of EMT reactions that occur in normal and malignant epithelial cells stimulated with TGF-β, and, as such, transdifferentiated myofibroblasts are highly abundant in invasive breast cancers compared to their in situ counterparts. Moreover, myofibroblasts typically localize to the invasive front of mammary tumors, suggesting an important role for transdifferentiated fibroblasts during metastatic progression (28,113). In fact, tumor-associated myofibroblasts are the predominant cell type responsible for eliciting desmoplastic reactions in mammary tumors as they become palpable. Moreover, desmoplastic reactions lead to the formation of mechanically rigid tumor microenvironments that drive metastatic progression and predict for poor clinical outcomes in breast cancer patients (28,30–32,65). Future studies need to fully characterize the autocrine and paracrine signaling systems that exist between myofibroblasts and their reactive stromal constituents in promoting the oncogenic activities of TGF-β and its stimulation of metastatic progression.

TGF-β, Fibroblasts, and ECM Protein Production

Fibroblasts and myofibroblasts within the tumor microenvironment are the largest producers of ECM components, of which collagen I and fibronectin are the most abundant proteins. TGF-β stimulates the expression and secretion of both of these ECM components (97,125), particularly during fibrotic reactions

coupled to desmoplasia and increased mechanical tension within tumor microenvironments. The exaggerated rigidity exhibited in tumor microenvironments contributes to metastatic progression in mammary tumors and reflects the elevated crosslinking of a variety of ECM components, most notably collagen and elastin (19,32,65,98). Indeed, lysyl oxidases (LOXs) comprise a five-member gene family of copper-dependent amine oxidases that catalyze the crosslinking of collagens and elastin in the ECM, leading to increased tissue tension and stiffness (19,32,65, 98). We observed TGF-β to induce LOX expression in normal and malignant MECs, and in triple-negative breast cancers produced in mice (124). LOX also functions in recruiting Gr-1$^+$CD11b$^+$ cells to premetastatic niches where they produce MMPs, thereby enhancing the invasion and recruitment of bone marrow derived cells (BMDCs) and metastatic breast cancer cells to these secondary sites of metastasis (30). Importantly, we observed differences in ECM tension to alter the response of MECs to TGF-β, such that exposing metastatic breast cancer cells to compliant microenvironments can partially reestablish the cytotoxic activities of TGF-β even in late-stage mammary tumors (124). Future studies need to identify the effectors of mechanotransduction operant in mediating the oncogenic activities of TGF-β in cancers of the breast, as well as to determine the potential of these molecules to serve as novel chemotherapeutic targets and diagnostic markers of mammary tumor development.

CONCLUSIONS

Studies performed over the last 30 years have clearly established TGF-β as a potent tumor suppressor in normal MECs and early stage mammary tumors, whose progression to aggressive disease states is accompanied by the acquisition of oncogenic activity by TGF-β (130,132). This incredible duality in TGF-β function represents a significant challenge to the development of targeted TGF-β chemotherapeutics designed to accentuate the cytostatic functions of TGF-β, while simultaneously attenuating its oncogenic activities in neoplastic mammary tissues. This challenge is further complicated by the fact that TGF-β exerts tumor cell autonomous activities, as well as induces cell- and context-specific activities within individual cell types housed in adjacent tumor microenvironments. Overcoming this challenge will require concerted efforts to map the genetic and epigenetic events that confer TGF-β with oncogenic activities, and to determine the relative extent to which these events derive from aberrancies within mammary carcinoma cells, from within their stromal compartment, or from within both cellular compartments. The emerging evidence presented here highlights the essential role played by tumor microenvironments to influence the pathophysiology of cancer cells and their response to TGF-β. As such, developing novel chemotherapeutics aimed at targeting specific cell types within reactive tumor microenvironments may provide an effective means alleviate the oncogenic activities of TGF-β in patients harboring metastatic breast cancers.

ACKNOWLEDGMENTS

We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), the Department of Defense (BC084651); and Case Comprehensive Cancer Center and the University Hospitals Seidman Cancer Center. M.A.T. was supported by the Department of Defense (BC093128).

REFERENCES

1. Ahmadzadeh, M.; Rosenberg, S. A. TGF-β1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. J. Immunol. 174:5215–5223; 2005.
2. Albig, A. R.; Schiemann, W. P. Fibulin-5 function during tumorigenesis. Future Oncol. 1:23–35; 2005.
3. Bazzoni, G.; Dejana, E. Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. Physiol. Rev. 84:869–901; 2004.
4. Bellone, G.; Aste-Amezaga, M.; Trinchieri, G.; Rodeck, U. Regulation of NK cell functions by TGF-β1. J. Immunol. 155:1066–1073; 1995.
5. Bergers, G.; Song, S. The role of pericytes in blood-vessel formation and maintenance. Neuro. Oncol. 7: 452–464; 2005.
6. Bhowmick, N. A.; Chytil, A.; Plieth, D.; Gorska, A. E.; Dumont, N.; Shappell, S.; Washington, M. K.; Neilson, E. G.; Moses, H. L. TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303:848–851; 2004.
7. Bhowmick, N. A.; Ghiassi, M.; Bakin, A.; Aakre, M.; Lundquist, C. A.; Engel, M. E.; Arteaga, C. L.; Moses, H. L. Transforming growth factor-β mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell. 12: 27–36; 2001.
8. Bhowmick, N. A.; Moses, H. L. Tumor-stroma

interactions. Curr. Opin. Genet. Dev. 15:97–101; 2005.
9. Bhowmick, N. A.; Neilson, E. G.; Moses, H. L. Stromal fibroblasts in cancer initiation and progression. Nature 432:332–337; 2004.
10. Bierie, B.; Chung, C. H.; Parker, J. S.; Stover, D. G.; Cheng, N.; Chytil, A.; Aakre, M.; Shyr, Y.; Moses, H. L. Abrogation of TGF-β signaling enhances chemokine production and correlates with prognosis in human breast cancer. J. Clin. Invest. 119:1571–1582; 2009.
11. Bierie, B.; Moses, H. L. Tumour microenvironment: TGFβ: The molecular Jekyll and Hyde of cancer. Nat. Rev. Cancer 6:506–520; 2006.
12. Bierie, B.; Moses, H. L. Transforming growth factor β (TGF-β) and inflammation in cancer. Cytokine Growth Factor Rev. 21:49–59; 2010.
13. Bierie, B.; Stover, D. G.; Abel, T. W.; Chytil, A.; Gorska, A. E.; Aakre, M.; Forrester, E.; Yang, L.; Wagner, K. U.; Moses, H. L. Transforming growth factor-β regulates mammary carcinoma cell survival and interaction with the adjacent microenvironment. Cancer Res. 68:1809–1819; 2008.
14. Blobe, G. C.; Schiemann, W. P.; Lodish, H. F. Role of transforming growth factor β in human disease. N. Engl. J. Med. 342:1350–1358; 2000.
15. Bobik, A. Transforming growth factor-βs and vascular disorders. Arterioscler Thromb. Vasc. Biol. 26: 1712–1720; 2006.
16. Bonig, H.; Banning, U.; Hannen, M.; Kim, Y. M.; Verheyen, J.; Mauz-Korholz, C.; Korholz, D. Transforming growth factor-β1 suppresses interleukin-15-mediated interferon-γ production in human T lymphocytes. Scand. J. Immunol. 50:612–618; 1999.
17. Bose, R.; Wrana, J. L. Regulation of Par6 by extracellular signals. Curr. Opin. Cell Biol. 18:206–212; 2006.
18. Brown, K. A.; Pietenpol, J. A.; Moses, H. L. A tale of two proteins: Differential roles and regulation of Smad2 and Smad3 in TGF-β signaling. J. Cell. Biochem. 101:9–33; 2007.
19. Butcher, D. T.; Alliston, T.; Weaver, V. M. A tense situation: Forcing tumour progression. Nat. Rev. Cancer 9:108–122; 2009.
20. Chang, H.; Brown, C. W.; Matzuk, M. M. Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocr. Rev. 23:787–823; 2002.
21. Cheng, N.; Bhowmick, N. A.; Chytil, A.; Gorksa, A. E.; Brown, K. A.; Muraoka, R.; Arteaga, C. L.; Neilson, E. G.; Hayward, S. W.; Moses, H. L. Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-β-, MSP- and HGF-mediated signaling networks. Oncogene 24:5053–5068; 2005.
22. Condeelis, J.; Pollard, J. W. Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. Cell 124:263–266; 2006.
23. Coussens, L. M.; Werb, Z. Inflammation and cancer. Nature 420:860–867; 2002.
24. Cunha, S. I.; Pardali, E.; Thorikay, M.; Anderberg, C.; Hawinkels, L.; Goumans, M. J.; Seehra, J.; Heldin, C. H.; ten Dijke, P.; Pietras, K. Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J. Exp. Med. 207:85–100; 2010.
25. Dejana, E.; Orsenigo, F.; Molendini, C.; Baluk, P.; McDonald, D. M. Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees. Cell Tissue Res. 335:17–25; 2009.
26. Dickson, M. C.; Martin, J. S.; Cousins, F. M.; Kulkarni, A. B.; Karlsson, S.; Akhurst, R. J. Defective haematopoiesis and vasculogenesis in transforming growth factor-β1 knock out mice. Development 121: 1845–1854; 1995.
27. Dunker, N.; Krieglstein, K. Targeted mutations of transforming growth factor-β genes reveal important roles in mouse development and adult homeostasis. Eur. J. Biochem. 267:6982–6988; 2000.
28. Elenbaas, B.; Weinberg, R. A. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp. Cell Res. 264:169–184; 2001.
29. Enenstein, J.; Waleh, N. S.; Kramer, R. H. Basic FGF and TGF-β differentially modulate integrin expression of human microvascular endothelial cells. Exp. Cell Res. 203:499–503; 1992.
30. Erler, J. T.; Bennewith, K. L.; Cox, T. R.; Lang, G.; Bird, D.; Koong, A.; Le, Q. T.; Giaccia, A. J. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15:35–44; 2009.
31. Erler, J. T.; Bennewith, K. L.; Nicolau, M.; Dornhofer, N.; Kong, C.; Le, Q. T.; Chi, J. T.; Jeffrey, S. S.; Giaccia, A. J. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 440:1222–1226; 2006.
32. Erler, J. T.; Weaver, V. M. Three-dimensional context regulation of metastasis. Clin. Exp. Metastasis 26:35–49; 2009.
33. Fang, W. B.; Jokar, I.; Chytil, A.; Moses, H. L.; Abel, T.; Cheng, N. Loss of one Tgfbr2 allele in fibroblasts promotes metastasis in MMTV: Polyoma middle T transgenic and transplant mouse models of mammary tumor progression. Clin. Exp. Metastasis 28:351–366; 2011.
34. Faure, E.; Heisterkamp, N.; Groffen, J.; Kaartinen, V. Differential expression of TGF-beta isoforms during postlactational mammary gland involution. Cell Tissue Res. 300:89–95; 2000.
35. Feng, X. H.; Derynck, R. Specificity and versatility in TGF-β signaling through Smads. Annu. Rev. Cell Dev. Biol. 21:659–693; 2005.
36. Fontana, A.; Constam, D. B.; Frei, K.; Malipiero, U.; Pfister, H. W. Modulation of the immune response by transforming growth factor β. Int. Arch. Allergy Immunol. 99:1–7; 1992.
37. Forrester, E.; Chytil, A.; Bierie, B.; Aakre, M.; Gorska, A. E.; Sharif-Afshar, A. R.; Muller, W. J.; Moses, H. L. Effect of conditional knockout of the type II TGF-β receptor gene in mammary epithelia

on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. Cancer Res. 65:2296–2302; 2005.
38. Gabrilovich, D. I.; Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. Nat. Rev. Immunol. 9:162–174; 2009.
39. Gatza, C. E.; Oh, S. Y.; Blobe, G. C. Roles for the type III TGF-β receptor in human cancer. Cell Signal. 22:1163–1174; 2010.
40. Geissmann, F.; Revy, P.; Regnault, A.; Lepelletier, Y.; Dy, M.; Brousse, N.; Amigorena, S.; Hermine, O.; Durandy, A. TGF-β1 prevents the noncognate maturation of human dendritic Langerhans cells. J. Immunol. 162:4567–4575; 1999.
41. Gerhardt, H.; Golding, M.; Fruttiger, M.; Ruhrberg, C.; Lundkvist, A.; Abramsson, A.; Jeltsch, M.; Mitchell, C.; Alitalo, K.; Shima, D.; Betsholtz, C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. 161:1163–1177; 2003.
42. Gonzalez-Mariscal, L.; Tapia, R.; Chamorro, D. Crosstalk of tight junction components with signaling pathways. Biochim. Biophys. Acta 1778:729–756; 2008.
43. Gorelik, L.; Flavell, R. A. Immune-mediated eradication of tumors through the blockade of transforming growth factor-β signaling in T cells. Nat. Med. 7: 1118–1122; 2001.
44. Gorska, A. E.; Jensen, R. A.; Shyr, Y.; Aakre, M. E.; Bhowmick, N. A.; Moses, H. L. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-β receptor exhibit impaired mammary development and enhanced mammary tumor formation. Am. J. Pathol. 163:1539–1549; 2003.
45. Gorska, A. E.; Joseph, H.; Derynck, R.; Moses, H. L.; Serra, R. Dominant-negative interference of the transforming growth factor β type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. Cell Growth Differ. 9:229–238; 1998.
46. Goumans, M. J.; Mummery, C. Functional analysis of the TGFβ receptor/Smad pathway through gene ablation in mice. Int. J. Dev. Biol. 44:253–265; 2000.
47. Goumans, M. J.; Valdimarsdottir, G.; Itoh, S.; Lebrin, F.; Larsson, J.; Mummery, C.; Karlsson, S.; ten Dijke, P. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. Mol. Cell 12:817–828; 2003.
48. Goumans, M. J.; Valdimarsdottir, G.; Itoh, S.; Rosendahl, A.; Sideras, P.; ten Dijke, P. Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J. 21:1743–1753; 2002.
49. Haak-Frendscho, M.; Wynn, T. A.; Czuprynski, C. J.; Paulnock, D. Transforming growth factor-β1 inhibits activation of macrophage cell line RAW 264.7 for cell killing. Clin. Exp. Immunol. 82:404–410; 1990.
50. Hahne, M.; Rimoldi, D.; Schroter, M.; Romero, P.; Schreier, M.; French, L. E.; Schneider, P.; Bornand, T.; Fontana, A.; Lienard, D.; Cerottini, J.; Tschopp, J. Melanoma cell expression of Fas(Apo-1/CD95) ligand: Implications for tumor immune escape. Science 274:1363–1366; 1996.
51. Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: The next generation. Cell 144:646–674; 2011.
52. Hawinkels, L. J.; Kuiper, P.; Wiercinska, E.; Verspaget, H. W.; Liu, Z.; Pardali, E.; Sier, C. F.; ten Dijke, P. Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res. 70:4141–4150; 2010.
53. Holderfield, M. T.; Hughes, C. C. Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. Circ. Res. 102:637–652; 2008.
54. Jhappan, C.; Geiser, A. G.; Kordon, E. C.; Bagheri, D.; Hennighausen, L.; Roberts, A. B.; Smith, G. H.; Merlino, G. Targeting expression of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J. 12:1835–1845; 1993.
55. Kalluri, R.; Weinberg, R. A. The basics of epithelial-mesenchymal transition. J. Clin. Invest. 119:1420–1428; 2009.
56. Kang, J. S.; Liu, C.; Derynck, R. New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol. 19:385–394; 2009.
57. Kang, Y.; He, W.; Tulley, S.; Gupta, G. P.; Serganova, I.; Chen, C. R.; Manova-Todorova, K.; Blasberg, R.; Gerald, W. L.; Massague, J. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc. Natl. Acad. Sci. USA 102: 13909–13914.
58. Kang, Y.; Siegel, P. M.; Shu, W.; Drobnjak, M.; Kakonen, S. M.; Cordon-Cardo, C.; Guise, T. A.; Massague, J. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549; 2003.
59. Kontani, K.; Kajino, K.; Huangi, C. L.; Fujino, S.; Taguchi, O.; Yamauchi, A.; Yokomise, H.; Ogasawara, K. Spontaneous elicitation of potent antitumor immunity and eradication of established tumors by administration of DNA encoding soluble transforming growth factor-β II receptor without active antigen-sensitization. Cancer Immunol. Immunother. 55: 579–587; 2006.
60. Kulkarni, A. B.; Huh, C. G.; Becker, D.; Geiser, A.; Lyght, M.; Flanders, K. C.; Roberts, A. B.; Sporn, M. B.; Ward, J. M.; Karlsson, S. Transforming growth factor β1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. USA 90:770–774; 1993.
61. Kulkarni, A. B.; Karlsson, S. Transforming growth factor-β1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. Am. J. Pathol. 143:3–9; 1993.
62. Labelle, M.; Schnittler, H. J.; Aust, D. E.; Friedrich, K.; Baretton, G.; Vestweber, D.; Breier, G. Vascular endothelial cadherin promotes breast cancer progression via transforming growth factor β signaling. Cancer Res. 68:1388–1397; 2008.
63. Larsson, J.; Goumans, M. J.; Sjostrand, L. J.; van

Rooijen, M. A.; Ward, D.; Leveen, P.; Xu, X.; ten Dijke, P.; Mummery, C. L.; Karlsson, S. Abnormal angiogenesis but intact hematopoietic potential in TGF-β type I receptor-deficient mice. EMBO J. 20: 1663–1673; 2001.
64. Lebrin, F.; Deckers, M.; Bertolino, P.; Ten Dijke, P. TGF-β receptor function in the endothelium. Cardiovasc. Res. 65:599–608; 2005.
65. Levental, K. R.; Yu, H.; Kass, L.; Lakins, J. N.; Egeblad, M.; Erler, J. T.; Fong, S. F.; Csiszar, K.; Giaccia, A.; Weninger, W.; Yamauchi, M.; Gasser, D. L.; Weaver, V. M. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139:891–906; 2009.
66. Li, D. Y.; Sorensen, L. K.; Brooke, B. S.; Urness, L. D.; Davis, E. C.; Taylor, D. G.; Boak, B. B.; Wendel, D. P. Defective angiogenesis in mice lacking endoglin. Science 284:1534–1537; 1999.
67. Li, M. O.; Wan, Y. Y.; Sanjabi, S.; Robertson, A. K.; Flavell, R. A. Transforming growth factor-β regulation of immune responses. Annu. Rev. Immunol. 24: 99–146; 2006.
68. Lin, E. Y.; Li, J. F.; Gnatovskiy, L.; Y., D.; Zhu, L.; Grzesik, D. A.; Qian, H.; Xue, X. N.; Pollard, J. W. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. 66: 11238–11246; 2006.
69. Lopez, M.; Aguilera, R.; Perez, C.; Mendoza-Naranjo, A.; Pereda, C.; Ramirez, M.; Ferrada, C.; Aguillon, J. C.; Salazar-Onfray, F. The role of regulatory T lymphocytes in the induced immune response mediated by biological vaccines. Immunobiology 211: 127–136; 2006.
70. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. Nature 454:436–444; 2008.
71. Mantovani, A.; Schioppa, T.; Porta, C.; Allavena, P.; Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev. 25:315–322; 2006.
72. Massague, J. TGFβ in cancer. Cell 134:215–230; 2008.
73. Massague, J.; Gomis, R. R. The logic of TGFβ signaling. FEBS Lett. 580:2811–2820; 2006.
74. Mempel, T. R.; Pittet, M. J.; Khazaie, K.; Weninger, W.; Weissleder, R.; von Boehmer, H.; von Andrian, U. H. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity 25:129–141; 2006.
75. Merwin, J. R.; Anderson, J. M.; Kocher, O.; Van Itallie, C. M.; Madri, J. A. Transforming growth factor β1 modulates extracellular matrix organization and cell–cell junctional complex formation during in vitro angiogenesis. J. Cell Physiol. 142:117–128; 1990.
76. Micke, P.; Ostman, A. Tumour-stroma interaction: Cancer-associated fibroblasts as novel targets in anticancer therapy? Lung Cancer 45:S163–175; 2004.
77. Minn, A. J.; Gupta, G. P.; Siegel, P. M.; Bos, P. D.; Shu, W.; Giri, D. D.; Viale, A.; Olshen, A. B.; Gerald, W. L.; Massague, J. Genes that mediate breast cancer metastasis to lung. Nature 436:518–524; 2005.
78. Minn, A. J.; Kang, Y.; Serganova, I.; Gupta, G. P.; Giri, D. D.; Doubrovin, M.; Ponomarev, V.; Gerald, W. L.; Blasberg, R.; Massague, J. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J. Clin. Invest. 115:44–55; 2005.
79. Mitchell, D.; Pobre, E. G.; Mulivor, A. W.; Grinberg, A. V.; Castonguay, R.; Monnell, T. E.; Solban, N.; Ucran, J. A.; Pearsall, R. S.; Underwood, K. W.; Seehra, J.; Kumar, R. ALK1-Fc inhibits multiple mediators of angiogenesis and suppresses tumor growth. Mol. Cancer Ther. 9:379–388; 2010.
80. Moretta, A.; Biassoni, R.; Bottino, C.; Mingari, M. C.; Moretta, L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol. Today 21:228–234; 2000.
81. Muraoka, R. S.; Dumont, N.; Ritter, C. A.; Dugger, T. C.; Brantley, D. M.; Chen, J.; Easterly, E.; Roebuck, L. R.; Ryan, S.; Gotwals, P. J.; Koteliansky, V.; Arteaga, C. L. Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. J. Clin. Invest. 109:1551–1559; 2002.
82. Muraoka, R. S.; Koh, Y.; Roebuck, L. R.; Sanders, M. E.; Brantley-Sieders, D.; Gorska, A. E.; Moses, H. L.; Arteaga, C. L. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor β1. Mol. Cell Biol. 23: 8691–8703; 2003.
83. Muraoka-Cook, R. S.; Kurokawa, H.; Koh, Y.; Forbes, J. T.; Roebuck, L. R.; Barcellos-Hoff, M. H.; Moody, S. E.; Chodosh, L. A.; Arteaga, C. L. Conditional overexpression of active transforming growth factor β1 in vivo accelerates metastases of transgenic mammary tumors. Cancer Res. 64:9002–9011; 2004.
84. Nam, J. S.; Terabe, M.; Kang, M. J.; Chae, H.; Voong, N.; Yang, Y. A.; Laurence, A.; Michalowska, A.; Mamura, M.; Lonning, S.; Berzofsky, J. A.; Wakefield, L. M. Transforming growth factor β subverts the immune system into directly promoting tumor growth through interleukin-17. Cancer Res. 68: 3915–3923; 2008.
85. Neil, J. R.; Johnson, K. M.; Nemenoff, R. A.; Schiemann, W. P. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis 29: 2227–2235; 2008.
86. Neil, J. R.; Schiemann, W. P. Altered TAB1:IκB kinase interaction promotes transforming growth factor β-mediated nuclear factor-κB activation during breast cancer progression. Cancer Res. 68:1462–1470; 2008.
87. Neil, J. R.; Tian, M.; Schiemann, W. P. X-linked inhibitor of apoptosis protein and its E3 ligase activity promote transforming growth factor-β-mediated nuclear factor-κB activation during breast cancer progression. J. Biol. Chem. 284:21209–21217; 2009.
88. Niessen, C. M. Tight junctions/adherens junctions: Basic structure and function. J. Invest. Dermatol. 127: 2525–2532; 2007.

89. Nishiyama, K.; Takaji, K.; Kataoka, K.; Kurihara, Y.; Yoshimura, M.; Kato, A.; Ogawa, H.; Kurihara, H. Id1 gene transfer confers angiogenic property on fully differentiated endothelial cells and contributes to therapeutic angiogenesis. Circulation 112:2840–2850; 2005.
90. Nyqvist, D.; Giampietro, C.; Dejana, E. Deciphering the functional role of endothelial junctions by using in vivo models. EMBO Rep. 9:742–747; 2008.
91. Oh, S. P.; Seki, T.; Goss, K. A.; Imamura, T.; Yi, Y.; Donahoe, P. K.; Li, L.; Miyazono, K.; ten Dijke, P.; Kim, S.; Li, E. Activin receptor-like kinase 1 modulates transforming growth factor-β1 signaling in the regulation of angiogenesis. Proc. Natl. Acad. Sci. USA 97:2626–2631; 2000.
92. Oshima, M.; Oshima, H.; Taketo, M. M. TGF-β receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev. Biol. 179: 297–302; 1996.
93. Ota, T.; Fujii, M.; Sugizaki, T.; Ishii, M.; Miyazawa, K.; Aburatani, H.; Miyazono, K. Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells. J. Cell Physiol. 193:299–318; 2002.
94. Ozdamar, B.; Bose, R.; Barrios-Rodiles, M.; Wang, H. R.; Zhang, Y.; Wrana, J. L. Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science 307:1603–1609; 2005.
95. Padua, D.; Zhang, X. H.; Wang, Q.; Nadal, C.; Gerald, W. L.; Gomis, R. R.; Massague, J. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133:66–77; 2008.
96. Pardali, E.; Goumans, M. J.; ten Dijke, P. Signaling by members of the TGF-β family in vascular morphogenesis and disease. Trends Cell Biol. 20:556–567; 2010.
97. Parvani, J. G.; Taylor, M. A.; Schiemann, W. P. Noncanonical TGF-β signaling during mammary tumorigenesis. J. Mammary Gland Biol. Neoplasia 16:127–146; 2011.
98. Paszek, M. J.; Zahir, N.; Johnson, K. R.; Lakins, J. N.; Rozenberg, G. I.; Gefen, A.; Reinhart-King, C. A.; Margulies, S. S.; Dembo, M.; Boettiger, D.; Hammer, D. A.; Weaver, V. M. Tensional homeostasis and the malignant phenotype. Cancer Cell 8:241–254; 2005.
99. Pepper, M. S. Transforming growth factor-β: Vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev. 8:21–43; 1997.
100. Petersen, M.; Pardali, E.; van der Horst, G.; Cheung, H.; van den Hoogen, C.; van der Pluijm, G.; Ten Dijke, P. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene 29:1351–1361; 2010.
101. Pierce, Jr., D. F.; Gorska, A. E.; Chytil, A.; Meise, K. S.; Page, D. L.; Coffey, Jr., R. J.; Moses, H. L. Mammary tumor suppression by transforming growth factor β1 transgene expression. Proc. Natl. Acad. Sci. USA 92:4254–4258; 1995.
102. Pollard, J. W. Tumour-stromal interactions. Transforming growth factor-β isoforms and hepatocyte growth factor/scatter factor in mammary gland ductal morphogenesis. Breast Cancer Res. 3:230–237; 2001.
103. Pollard, J. W. Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer 4:71–78; 2004.
104. Radisky, D. C.; Przybylo, J. A. Matrix metalloproteinase-induced fibrosis and malignancy in breast and lung. Proc. Am. Thorac. Soc. 5:316–322; 2008.
105. Raza, A.; Franklin, M. J.; Dudek, A. Z. Pericytes and vessel maturation during tumor angiogenesis and metastasis. Am. J. Hematol. 85:593–598; 2010.
106. Reibman, J.; Meixler, S.; Lee, T. C.; Gold, L. I.; Cronstein, B. N.; Haines, K. A.; Kolasinski, S. L.; Weissmann, G. Transforming growth factor β1, a potent chemoattractant for human neutrophils, bypasses classic signal-transduction pathways. Proc. Natl. Acad. Sci. USA 88:6805–6809; 1991.
107. Ridley, A. J.; Hall, A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389–399; 1992.
108. Rizki, A.; Bissell, M. J. Homeostasis in the breast: It takes a village. Cancer Cell 6:1–2; 2004.
109. Robinson, S. D.; Silberstein, G. B.; Roberts, A. B.; Flanders, K. C.; Daniel, C. W. Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. Development 113:867–878; 1991.
110. Rook, A. H.; Kehrl, J. H.; Wakefield, L. M.; Roberts, A. B.; Sporn, M. B.; Burlington, D. B.; Lane, H. C.; Fauci, A. S. Effects of transforming growth factor β on the functions of natural killer cells: Depressed cytolytic activity and blunting of interferon responsiveness. J. Immunol. 136:3916–3920; 1986.
111. Rudini, N.; Felici, A.; Giampietro, C.; Lampugnani, M.; Corada, M.; Swirsding, K.; Garre, M.; Liebner, S.; Letarte, M.; ten Dijke, P.; Dejana, E. VE-cadherin is a critical endothelial regulator of TGF-β signalling. EMBO J. 27:993–1004; 2008.
112. Scheel, C.; Eaton, E. N.; Li, S. H.; Chaffer, C. L.; Reinhardt, F.; Kah, K. J.; Bell, G.; Guo, W.; Rubin, J.; Richardson, A. L.; Weinberg, R. A. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell States in the breast. Cell 145:926–940; 2011.
113. Schurch, W. The myofibroblast in neoplasia. Curr. Top. Pathol. 93:135–148; 1999.
114. Serra, R.; Crowley, M. R. TGF-β in mammary gland development and breast cancer. Breast Dis. 18:61–73; 2003.
115. Serrati, S.; Margheri, F.; Pucci, M.; Cantelmo, A. R.; Cammarota, R.; Dotor, J.; Borras-Cuesta, F.; Fibbi, G.; Albini, A.; Del Rosso, M. TGFβ1 antagonistic peptides inhibit TGFβ1-dependent angiogenesis. Biochem. Pharmacol. 77:813–825; 2009.
116. Shen, W.; Li, S.; Chung, S. H.; Zhu, L.; Stayt, J.; Su, T.; Couraud, P. O.; Romero, I. A.; Weksler, B.; Gillies, M. C. Tyrosine phosphorylation of VE-cadherin and claudin-5 is associated with TGF-β1-induced perme-

ability of centrally derived vascular endothelium. Eur. J. Cell Biol. 90:323–332; 2011.
117. Shi, Y.; Massague, J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113: 685–700; 2003.
118. Shipitsin, M.; Campbell, L. L.; Argani, P.; Weremowicz, S.; Bloushtain-Qimron, N.; Yao, J.; Nikolskaya, T.; Serebryiskaya, T.; Beroukhim, R.; Hu, M.; Halushka, M. K.; Sukumar, S.; Parker, L. M.; Anderson, K. S.; Harris, L. N.; Garber, J. E.; Richardson, A. L.; Schnitt, S. J.; Nikolsky, Y.; Gelman, R. S.; Polyak, K. Molecular definition of breast tumor heterogeneity. Cancer Cell 11:259–273; 2007.
119. Siegel, P. M.; Shu, W.; Cardiff, R. D.; Muller, W. J.; Massague, J. Transforming growth factor β signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. Proc. Natl. Acad. Sci. USA 100:8430–8435; 2003.
120. Singer, C. F.; Gschwantler-Kaulich, D.; Fink-Retter, A.; Haas, C.; Hudelist, G.; Czerwenka, K.; Kubista, E. Differential gene expression profile in breast cancer-derived stromal fibroblasts. Breast Cancer Res. Treat. 110:273–281; 2008.
121. Sotiriou, C.; Pusztai, L. Gene-expression signatures in breast cancer. N. Engl. J Med. 360:790–800; 2009.
122. Takaishi, K.; Sasaki, T.; Kotani, H.; Nishioka, H.; Takai, Y. Regulation of cell–cell adhesion by Rac and Rho small G proteins in MDCK cells. J. Cell Biol. 139:1047–1059; 1997.
123. Taube, J. H.; Herschkowitz, J. I.; Komurov, K.; Zhou, A. Y.; Gupta, S.; Yang, J.; Hartwell, K.; Onder, T. T.; Gupta, P. B.; Evans, K. W.; Hollier, B. G.; Ram, P. T.; Lander, E. S.; Rosen, J. M.; Weinberg, R. A.; Mani, S. A. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc. Natl. Acad. Sci. USA 107: 15449–15454; 2010.
124. Taylor, M. A.; Amin, J. D.; Kirschmann, D. A.; Schiemann, W. P. Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor-β signaling in breast cancer cells. Neoplasia 13:406–418; 2011.
125. Taylor, M. A.; Parvani, J. G.; Schiemann, W. P. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells. J. Mammary Gland Biol. Neoplasia 15:169–190; 2010.
126. Teicher, B. A. Transforming growth factor-β and the immune response to malignant disease. Clin. Cancer Res. 13:6247–6251; 2007.
127. ten Dijke, P.; Arthur, H. M. Extracellular control of TGFβ signalling in vascular development and disease. Nat. Rev. Mol. Cell Biol. 8:857–869; 2007.
128. ten Dijke, P.; Goumans, M. J.; Pardali, E. Endoglin in angiogenesis and vascular diseases. Angiogenesis 11:79–89; 2008.
129. Thomas, D. A.; Massague, J. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 8:369–380; 2005.
130. Tian, M.; Neil, J. R.; Schiemann, W. P. Transforming growth factor-β and the hallmarks of cancer. Cell Signal. 23:951–962; 2011.
131. Tian, M.; Schiemann, W. P. PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J. 24: 1105–1116; 2009.
132. Tian, M.; Schiemann, W. P. The TGF-β paradox in human cancer: An update. Future Oncol. 5:259–271; 2009.
133. Tlsty, T. D.; Coussens, L. M. Tumor stroma and regulation of cancer development. Annu. Rev. Pathol. 1: 119–150; 2006.
134. Torre-Amione, G.; Beauchamp, R. D.; Koeppen, H.; Park, B. H.; Schreiber, H.; Moses, H. L.; Rowley, D. A. A highly immunogenic tumor transfected with a murine transforming growth factor type β1 cDNA escapes immune surveillance. Proc Natl. Acad. Sci. USA 87:1486–1490; 1990.
135. Townsend, T. A.; Wrana, J. L.; Davis, G. E.; Barnett, J. V. Transforming growth factor-β-stimulated endocardial cell transformation is dependent on Par6c regulation of RhoA. J. Biol. Chem. 283:13834–13841; 2008.
136. Venkatesha, S.; Toporsian, M.; Lam, C.; Hanai, J.; Mammoto, T.; Kim, Y. M.; Bdolah, Y.; Lim, K. H.; Yuan, H. T.; Libermann, T. A.; Stillman, I. E.; Roberts, D.; D'Amore, P. A.; Epstein, F. H.; Sellke, F. W.; Romero, R.; Sukhatme, V. P.; Letarte, M.; Karumanchi, S. A. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat. Med. 12:642–649; 2006.
137. Wang, J.; Guan, E.; Roderiquez, G.; Calvert, V.; Alvarez, R.; Norcross, M. A. Role of tyrosine phosphorylation in ligand-independent sequestration of CXCR4 in human primary monocytes-macrophages. J. Biol. Chem. 276:49236–49243; 2001.
138. Wendt, M. K.; Allington, T. M.; Schiemann, W. P. Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol. 5:1145–1168; 2009.
139. Wendt, M. K.; Smith, J. A.; Schiemann, W. P. Transforming growth factor-β-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene 29: 6485–6498; 2010.
140. Wendt, M. K.; Taylor, M. A.; Schiemann, B. J.; Schiemann, W. P. Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. Mol. Biol. Cell 22:2423–2435; 2011.
141. Wrzesinski, S. H.; Wan, Y. Y.; Flavell, R. A. Transforming growth factor-β and the immune response: Implications for anticancer therapy. Clin. Cancer Res. 13:5262–5270; 2007.
142. Yang, L.; Huang, J.; Ren, X.; Gorska, A. E.; Chytil, A.; Aakre, M.; Carbone, D. P.; Matrisian, L. M.; Richmond, A.; Lin, P. C.; Moses, H. L. Abrogation of TGFβ signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell 13:23–35; 2008.

143. Yang, L.; Pang, Y.; Moses, H. L. TGF-β and immune cells: An important regulatory axis in the tumor microenvironment and progression. Trends Immunol. 31:220–227; 2010.
144. Yang, X.; Letterio, J. J.; Lechleider, R. J.; Chen, L.; Hayman, R.; Gu, H.; Roberts, A. B.; Deng, C. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J. 18:1280–1291; 1999.
145. Yang, Y. A.; Dukhanina, O.; Tang, B.; Mamura, M.; Letterio, J. J.; MacGregor, J.; Patel, S. C.; Khozin, S.; Liu, Z. Y.; Green, J.; Anver, M. R.; Merlino, G.; Wakefield, L. M. Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects. J. Clin. Invest. 109: 1607–1615; 2002.
146. Zhang, X. H.; Wang, Q.; Gerald, W.; Hudis, C. A.; Norton, L.; Smid, M.; Foekens, J. A.; Massague, J. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell 16:67–78; 2009.

Oncogene (2012), 1–11
© 2012 Macmillan Publishers Limited All rights reserved 0950-9232/12
www.nature.com/onc

ORIGINAL ARTICLE

TGF-β stimulates Pyk2 expression as part of an epithelial-mesenchymal transition program required for metastatic outgrowth of breast cancer

MK Wendt[1], BJ Schiemann[1], JG Parvani[1], Y-H Lee[1], Y Kang[2] and WP Schiemann[1]

Epithelial-mesenchymal transition (EMT) programs are essential in promoting breast cancer invasion, systemic dissemination and in arousing proliferative programs in breast cancer micrometastases, a reaction that is partially dependent on focal adhesion kinase (FAK). Many functions of FAK are shared by its homolog, protein tyrosine kinase 2 (Pyk2), raising the question as to whether Pyk2 also participates in driving the metastatic outgrowth of disseminated breast cancer cells. In addressing this question, we observed Pyk2 expression to be (i) significantly upregulated in recurrent human breast cancers; (ii) differentially expressed across clonal isolates of human MDA-MB-231 breast cancer cells in a manner predictive for metastatic outgrowth, but not for invasiveness; and (iii) dramatically elevated in *ex vivo* cultures of breast cancer cells isolated from metastatic lesions as compared with cells that produced the primary tumor. We further show that metastatic human and murine breast cancer cells robustly upregulate their expression of Pyk2 during EMT programs stimulated by transforming growth factor-β (TGF-β). Genetic and pharmacological inhibition of Pyk2 demonstrated that the activity of this protein tyrosine kinase was dispensable for the ability of breast cancer cells to undergo invasion in response to TGF-β, and to form orthotopic mammary tumors in mice. In stark contrast, Pyk2-deficiency prevented TGF-β from stimulating the growth of breast cancer cells in 3D-organotypic cultures that recapitulated pulmonary microenvironments, as well as inhibited the metastatic outgrowth of disseminated breast cancer cells in the lungs of mice. Mechanistically, Pyk2 expression was inversely related to that of E-cadherin, such that elevated Pyk2 levels stabilized β1 integrin expression necessary to initiate the metastatic outgrowth of breast cancer cells. Thus, we have delineated novel functions for Pyk2 in mediating distinct elements of the EMT program and metastatic cascade regulated by TGF-β, particularly the initiation of secondary tumor outgrowth by disseminated cells.

Oncogene advance online publication, 18 June 2012; doi:10.1038/onc.2012.230

Keywords: EMT; FAK; metastasis; Pyk2; signal transduction; TGF-β

INTRODUCTION

Transforming growth factor-β (TGF-β) and its induction of epithelial-mesenchymal transition (EMT) are essential mediators underlying the acquisition of invasive and metastatic phenotypes in breast cancers. Recent findings have begun to delineate the molecular mechanisms whereby EMT programs are stimulated by TGF-β, which targets a variety of transcription factors and microRNAs that collectively induce polarized epithelial cells to acquire apolar mesenchymal-like characteristics.[1,2] Along these lines, we recently established the essential function of integrins and focal adhesion complexes in mediating oncogenic TGF-β signaling and its coupling to EMT programs (see Wendt *et al.*[1] and Taylor *et al.*[2]). Indeed, formation of TβR-II:β3 integrin complexes amplifies the transduction through a Src:(focal adhesion kinase) FAK:Grb2:p38 MAPK signaling axis coupled to the initiation of EMT and metastasis in breast cancers (see Wendt *et al.*[1] and Taylor *et al.*[2]). Despite these recent advances, science and medicine still know very little as to how the progression through and eventual emergence from an EMT program impacts the flux through cellular signaling systems. Two recent studies by our group have attempted to address this question and demonstrated that breast cancer cells that have undergone EMT possess a selective advantage to (i) escape the confines of the primary tumor via an EGF-dependent mechanism,[3] and (ii) circumvent the inhibitory actions of E-cadherin (E-cad) in suppressing β1 integrin expression necessary to reinitiate proliferative programs and metastatic outgrowth.[4] Collectively, these findings provided novel insights into the functions of TGF-β and EMT in supporting late events of the metastatic cascade; however, the identity and relative contribution of specific TGF-β effectors in mediating these events remains to be fully elucidated.

Protein tyrosine kinase 2 (Pyk2; also known as CAKB, FAK2, CADTK, FADK2 or RAFTK) is a homolog of FAK and shares an overall amino acid identity of 46%, which increases to 60% within the protein tyrosine kinase domain.[5,6] Despite their structural similarities, FAK and Pyk2 exhibit important functional differences. For instance, FAK expression is ubiquitous, essential for life and localizes primarily to focal adhesion complexes where it is activated by integrins and growth factor receptors. In stark contrast, the expression of Pyk2 is highly restricted, dispensable for life, and localizes primarily to the cytoplasm where it is activated by chemokines and G protein-coupled receptors.[7–11]

[1]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA and [2]Department of Molecular Biology, Princeton University, Princeton, NJ, USA. Correspondence: Dr WP Schiemann, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, 2103 Cornell Road Cleveland, OH 44106, USA.
E-mail: william.schiemann@case.edu
Received 15 February 2012; revised 23 April 2012; accepted 4 May 2012

Although both protein tyrosine kinases are known to regulate cell migration and invasion in a compensatory manner,[12–15] the differences existing in their tissue distribution, subcellular localization, and necessity for survival indicate that FAK and Pyk2 have unique roles in regulating cell and tissue homeostasis.[9,16,17] With respect to breast cancers, we[18] and others[19–22] have identified FAK as a key factor operant in coupling TGF-β to EMT, invasive and metastatic behaviors. However, the expression and activity of Pyk2 has also been implicated in regulating the production and expansion of breast cancer stem cells,[13] as well as in promoting the motility and survival of breast cancer cells.[13,23,24] Herein, we identify Pyk2 as a novel EMT biomarker and found its expression to be robustly upregulated by TGF-β in transdifferentiating breast cancer cells. Thus, the objective of this study was to determine the mechanism whereby Pyk2 contributes to breast cancer metastasis.

RESULTS

Pyk2 expression is elevated in aggressive breast cancers

Although elevations in the expression and activity of FAK are clearly associated with breast cancer progression and metastasis,[18–22] a similar understanding related to the function of Pyk2 during mammary tumorigenesis remains to be elucidated. As such, we performed Oncomine 4.4 analyses and observed Pyk2 expression to be significantly upregulated in H-Ras-transformed mammary epithelial cells, and in invasive human breast carcinomas, particularly those that exhibited recurrence (Table 1). Moreover, Pyk2 expression was significantly reduced in human MDA-MB-231 (MDA-231) metastatic breast cancer cells engineered to lack expression of Coco,[25] a bone morphogenetic protein antagonist (GSE28049; Table 1). Bone morphogenetic protein and TGF-β signaling typically oppose one another, particularly during the induction of EMT programs.[4] Therefore, this finding suggests that expression of Pyk2 may reflect the overall balance of signaling inputs originating from TGF-β and Bone morphogenetic protein.

Along these lines, we monitored Pyk2 expression levels in parental and clonal cell populations of MDA-231 cells (Figure 1a), which were established from a pleural effusion and represent a heterogeneous mixture of aggressive and nonaggressive carcinoma cells.[26] Interestingly, while FAK expression was equivalent across all MDA-231 clonal populations, we observed Pyk2 expression to be highly variable and dissociated from the differential invasiveness exhibited by these clonal populations (Figures 1a and b). However, Pyk2 expression was positively associated with the competency of MDA-231 cells to thrive in 3D-organotypic cultures that mimicked the pulmonary microenvironment. Indeed, Figure 1c shows that Pyk2-positive CP2-MDA-231 cells grew robustly in 3D-cultures, while Pyk2-deficient CP4-MDA-231 cells grew poorly and displayed loosely packed and branched organoid structures that we previously linked to the development of metastatic dormancy (Figure 1d).[3,4] It should be noted that FAK activation has also been implicated as a mediator of metastatic outgrowth.[27] Thus, to gage the extent to which FAK or Pyk2 activity drives the outgrowth of metastatic breast cancer cells, we compared the activities of two related FAK and Pyk2 antagonists, PF562271 (PF-271) and PF573228 (PF-228). Importantly, while PF-271 and PF-228 are equivalent in their ability to inhibit FAK (IC50 of 1.5 and 4 nM, respectively), these compounds differ significantly in their ability to inhibit Pyk2 (IC50 of 14 and 1000 nM, respectively).[28,29] Administration of the dual FAK/Pyk2 inhibitor, PF-271, was significantly more effective at inhibiting the 3D-outgrowth of CP2-MDA-231 cells as compared with equivalent concentrations of the FAK-specific inhibitor, PF-228 (Figures 1d and e). Collectively, these findings implicate elevated Pyk2 expression and activity as a potential mediator of metastatic outgrowth by breast cancer cells.

TGF-β induces Pyk2 expression via Smad4- and Src-dependent pathways

Given the strong correlation between elevated Pyk2 expression and breast cancer progression (Table 1 and Figure 1), we next sought to determine whether and how TGF-β coupled to Pyk2 in metastatic breast cancers. Figure 2a shows that TGF-β readily induced Pyk2 expression in MDA-231 cells propagated in traditional 2D-cultures, and that the magnitude of Pyk2 expression was potentiated by propagating MDA-231 cells in compliant 3D-organotypic cultures. Likewise, we also observed Pyk2 expression to increase as a function of the metastatic ability of derivatives belonging to the murine 4T1 breast cancer progression series (Supplementary Figure 1a).[30] Indeed, the tonic levels of Pyk2 mRNA and protein were significantly higher in 4T1 cells as compared with their less aggressive 4T07 counterparts (Figures 2b and c). Moreover, both cell lines rapidly and dramatically upregulated their synthesis of Pyk2 transcripts and protein when stimulated by TGF-β (Figures 2b and c). Importantly, increases in Pyk2 expression were paralleled by elevations in its phosphorylation status (Supplementary Figure 1b). Furthermore, administration of TβR-I or Src inhibitors to 4T1 cells suppressed their expression of Pyk2, thereby implicating autocrine TGF-β signaling and its activation of Src[31–33] as drivers of elevated Pyk2 expression in metastatic breast cancer cells (Figure 2d). It is interesting to note that TGF-β failed to upregulate Pyk2 expression in nonmetastatic and dormant human MCF-7 cells (Supplementary Figure 1c), but readily did so in our recently established model of EGFR-mediated transformation and EMT-driven metastatic progression of normal murine mammary gland cells (Supplementary Figure 1d).[3,4] Collectively, these findings suggest that the extent

Table 1. Increased Pyk2 expression associates with breast cancer development, disease recurrence and BMP antagonism

Condition	*Pyk2 expression (fold change)*	N-*number*	P-*value*	*Study*
H-Ras transfection of primary human mammary epithelial cells	+ 9.967	GFP control = 10; hRas = 10	1.27E-11	Bild *et al.*[56]
Invasive ductal carcinoma	+ 2.948	Normal breast = 15; invasive ductal BC = 7	0.001	Huang *et al.*[57]
Invasive ductal carcinoma-recurrence at 5 years	+ 2.57	No recurrence = 15; recurrant = 31	0.004	Karnoub *et al.*[58]
MDA-MB-231-shCoco	− 2.61	shscram = 3 shCoco = 3	0.0001	GSE28049

Abbreviations: BMP, bone morphogenetic protein; Pyk2, protein tyrosine kinase 2. Oncomine 4.4 analysis of Pyk2 expression in normal and malignant mammary epithelial cells and tissues revealed significant increases in Pyk2 expression in invasive and recurrent human breast cancers. Analysis of microarray data from MDA-231 cells depleted for the BMP-antagonist, Coco (GSE28049), demonstrated that elevated BMP signaling suppressed Pyk2 expression. shScram, nontargeting shRNA. shCoco, shRNA targeting Coco.

© 2012 Macmillan Publishers Limited

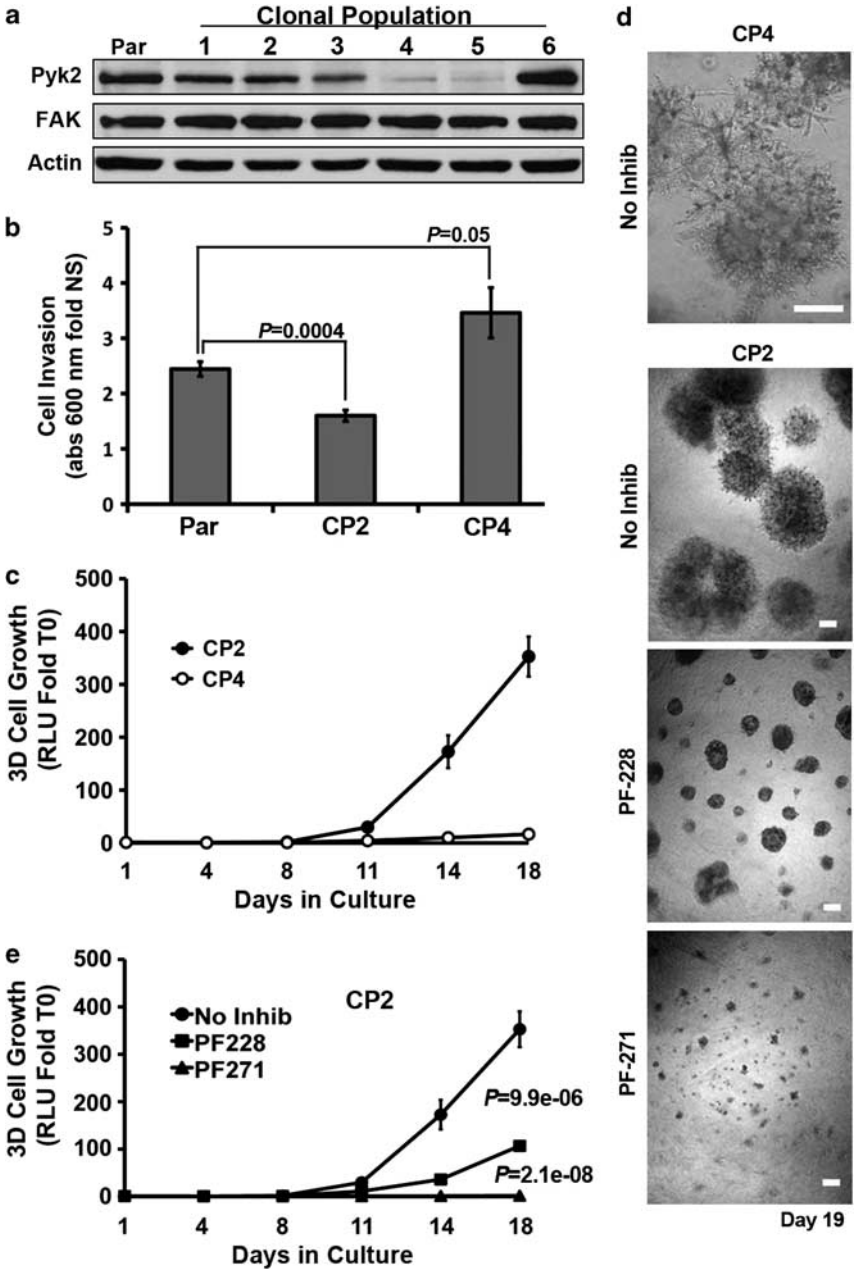


Figure 1. Elevated Pyk2 expression is associated with enhanced 3D-organotypic growth, but not with the invasiveness of MDA-231 cells. (**a**) Immunoblot analysis of parental (Par) and isolated clonal populations of MDA-MB-231 cells showed that Pyk2 expression was highly variable across these cell lines, while that of FAK was ubiquitous and equivalent. (**b**) Pyk2 expression inversely correlated with the invasive potential of MDA-231 clonal populations (CP) CP2 and CP4. Data are the mean (±s.e.) of two-independent experiments completed in triplicate. (**c**) Robust Pyk2 expression in CP2 cells correlates with their enhanced 3D-outgrowth, an event that was lacking in Pyk2-deficient CP4 cells. (**d**) Photomicrographs of CP2 and CP4 cells propagated in 3D-organotypic cultures. Where indicated, CP2 cells were grown in the absence (no Inhib) or presence of the FAK-specific inhibitor, PF573228 (1 μM; PF-228) or the dual FAK/Pyk2 inhibitor, PF562271 (1 μM; PF-271). (**e**) Bioluminescent quantification of CP2 proliferation in 3D-cultures shown in panel (**d**). Data for panels (**c**) and (**e**) are the mean (±s.e.) of three independent experiments completed in triplicate.

to which TGF-β induces Pyk2 expression is directly proportional to the metastatic potential of individual breast cancer cells.

Recent studies have linked chemotherapeutic resistance to the ability of these drugs to elicit an EMT reaction.[34–37] Along these lines, we too found that chronic treatment of 4T07 cells with PF-228 resulted in the generation and expansion of chemoresistant 4T07 cells that displayed an overt EMT phenotype (Supplementary Figure 2a), while similar chronic treatment with PF-271 failed to yield chemoresistant cells capable of thriving in culture (data not shown). However, upregulated Pyk2 expression was not associated with chemotherapy-induced EMT reactions (Supplementary Figure 2b). Similarly, overexpression of the EMT-associated transcription factors Twist or Snail in 4T07 cells had little affect on their expression of Pyk2 in the absence or presence of TGF-β (Supplementary Figure 2c). Taken together, these data suggest that Pyk2 may represent a novel and specific target of TGF-β-induced EMT.

Finally, we sought to delineate the mechanisms whereby TGF-β stimulates the expression of Pyk2. Supplementary Figure 2d shows that the ability of TGF-β to induce Pyk2 expression was blocked by

© 2012 Macmillan Publishers Limited

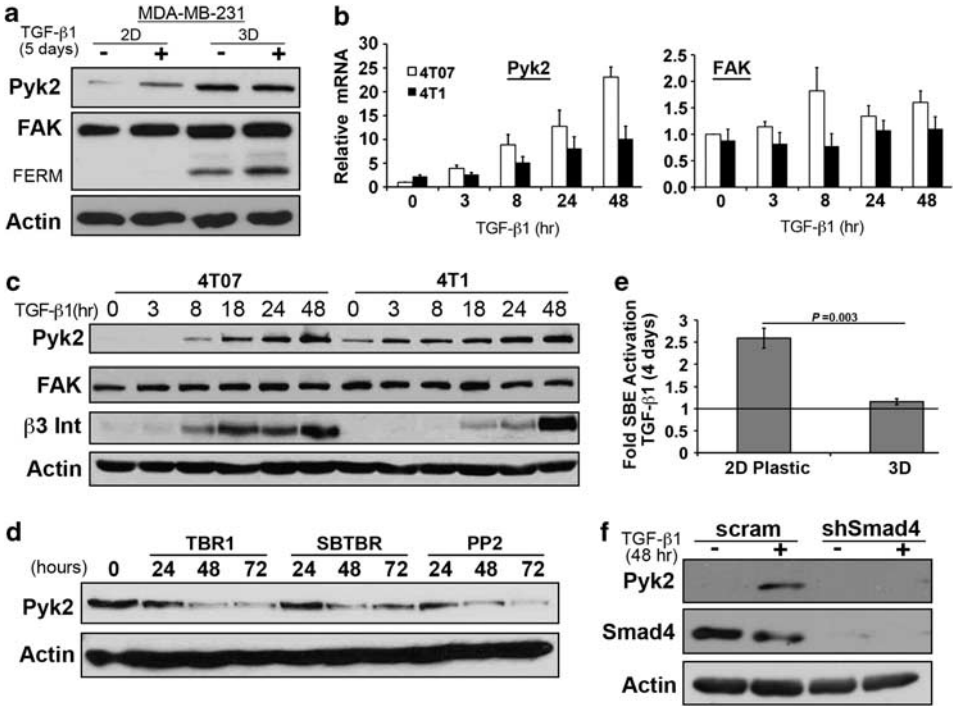


Figure 2. TGF-β induces Pyk2 expression via Src and Smad4 signaling. (**a**) Human MDA-MB-231 breast cancer cells were propagated in traditional 2D-cultures (2D) or 3D-organotypic cultures (3D) for 5 days in the absence or presence of TGF-β1 (5 ng/ml) as indicated. Afterward, differences in the expression levels of Pyk2 and FAK were monitored by immunoblotting. The N-terminally-directed FAK antibody also readily detected the accumulation of the FAK FERM domain as shown. β-actin served as a loading control. (**b**, **c**) 4T07 and 4T1 cells were stimulated with TGF-β1 (5 ng/ml) over a span of 48 h as indicated. Afterward, changes in the levels of Pyk2 and FAK mRNA were quantified by real-time PCR (**b**), or by immunoblotting (**c**). Changes in β3 integrin (β3-Int) expression served as a surrogate marker for EMT, while β-actin served as a loading control. (**d**) 4T1 cells were treated for 0–72 h with the inhibitors against TβR-I (TBR1 or SBTBR) or Src (PP2). Afterward, changes in basal levels of Pyk2 expression were assessed by immunoblot analysis. (**e**) Dual-bioluminescent SBE reporter 4T1 cells were propagated in traditional 2D- or 3D-organotypic cultures for 4 days in the absence or presence of TGF-β1 (5 ng/ml) before monitoring firefly and renilla luciferase activity. Data are the mean (± s.e.) of three independent experiments completed in triplicate. (**f**) Control (scram) or Smad4-depleted (shSmad4) MDA-231-CP4 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h. Smad4-depleted cells failed to upregulate Pyk2. Data in panels (**a**), (**c**), (**d**) and (**f**) are representative of at least two-independent experiments, while those in panel (**b**) are the mean (± s.d.) of two-independent experiments completed in duplicate.

treating 4T07 cells with inhibitors against TβR-I (SB431542 or TβR-I inhibitor II) or Src (PP2), but not by treating these cells with inhibitors against a variety of noncanonical TGF-β effector molecules, including MEK1/2, MAP kinases (p38 MAPK and JNK), AKT1/2, STAT3, MLCK, c-Abl, PI3K, IKK, Rho kinase, GSK3β or Cox-2 (Supplemental Figure 2d). Additionally, the oncogenic activities of TGF-β are aided through its ability to cross-talk with a variety of chemokine and growth factor receptors;[1,2] however, pharmacological inactivation of the receptors for FGF, IGF, EGF and PGE2 failed to significantly alter the coupling of TGF-β to Pyk2 expression in 4T07 cells (Supplementary Figure 2d). Interestingly, the activation of Smad2/3 by TGF-β was abrogated upon propagating the cells in mechanically compliant 3D-organotypic cultures (Figure 2e and Supplementary Figure 3a), an experimental condition that elicited Pyk2 expression independent of TGF-β signaling (Figure 2a). Moreover, inhibiting the activation of Src not only decreased the ability of TGF-β to induce Pyk2 expression, but also diminished its ability to induce the phosphorylation of Smad2/3 (Supplementary Figures 2d and 3b), suggesting a role of Smad2/3/4 signaling in coupling TGF-β to Pyk2 expression. Accordingly, short hairpin RNA-mediated depletion of Smad4 abrogated the coupling of TGF-β to Pyk2 expression (Figure 2f). Taken together, these findings demonstrate the importance of autocrine and paracrine TGF-β signaling to govern Pyk2 expression via the combined actions of a Smad4 and Src signaling axis.

Pyk2 expression is essential for pulmonary tumor formation in mice

Our aforementioned findings suggest that Pyk2 may have a critical role in facilitating breast cancer metastasis. Therefore, we depleted Pyk2 expression in highly metastatic 4T1 cells, which then were engrafted onto the mammary fat pads of Balb/C mice (Figure 3a). Although Pyk2-deficiency failed to affect the growth and latency of primary 4T1 tumors (Figure 3b), this same cellular condition did significantly impair the extent and size to which disseminated 4T1 cells formed macroscopic pulmonary metastases (Figures 3c and d).

We also examined the necessity of Pyk2 in regulating the metastatic ability of 4T07 cells, which dramatically upregulate their expression of this protein tyrosine kinase in response to TGF-β (Figure 2). Although 4T07 cells are capable of forming and exiting a primary orthotopic tumor and being readily detected in the circulatory system of mice, these cells cannot establish proliferating secondary tumors at disseminated organ sites.[3,30] However, when injected into the lateral tail vein of Balb/C mice, 4T07 cells exhibited widespread dissemination and aggressive colonization of multiple tissues, including the brain (data not shown), lung and various skeletal sites (Figure 4a; Supplementary Figure 4a). Importantly, Pyk2 expression was uniformly upregulated in *ex vivo* cultures of 4T07 metastases as compared their parental counterparts (Figure 4b; Supplementary Figure 4b). Moreover, rendering 4T07 cells deficient in Pyk2 expression (Supplementary

© 2012 Macmillan Publishers Limited

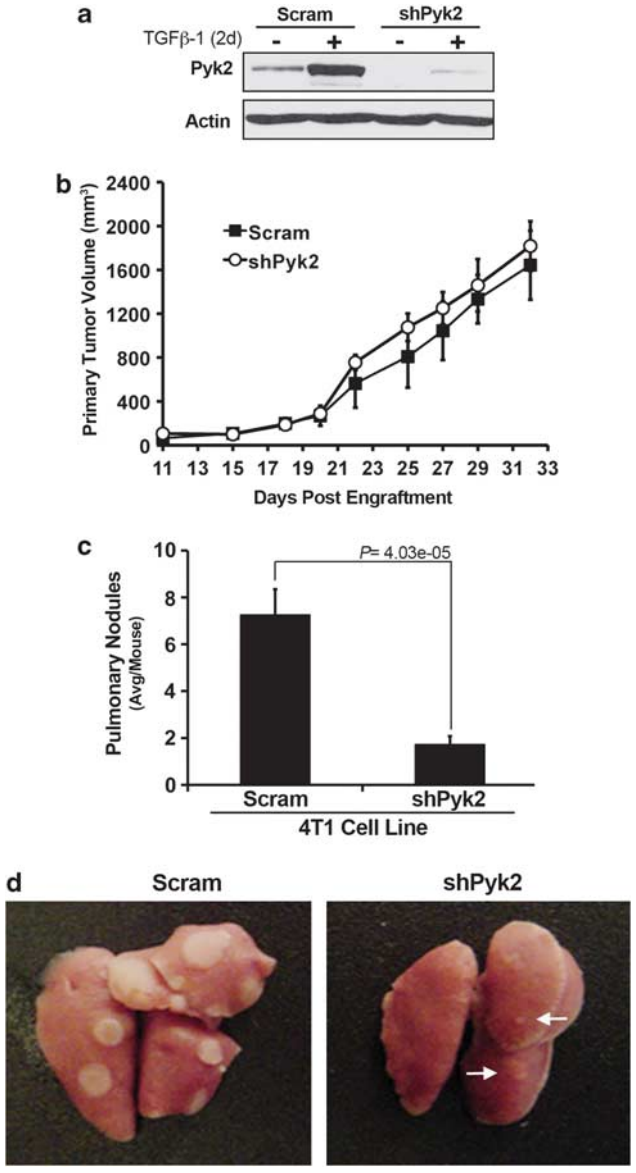


Figure 3. Depletion of Pyk2 inhibits pulmonary metastasis from an orthotropic mammary tumor. (**a**) Control (scram) and Pyk2-depleted (shPyk2) 4T1 cells were stimulated with TGF-β1 for 48 h and analyzed for the presence of Pyk2. (**b**) Control and Pyk2-depleted 4T1 cells (1×10^4 cells) were engrafted onto the mammary fat pad of female Balb/C mice ($n = 5$ mice per group) and primary tumor size was determined at the indicated time points. (**c**) Five weeks after 4T1 cell engraftment, the mice were euthanized and pulmonary metastases were quantified. (**d**) Shown are representative lungs and metastatic foci isolated from mice described in panels (**b**) and (**c**). Arrows indicate the small metastatic foci formed by Pyk2-depleted 4T1 cells.

Figure 4b) elicited a significant reduction in the growth of secondary tumor lesions in the lung and other organ sites (Figures 4c and d), as well as greatly extended the overall survival of tumor bearing mice (Figure 4e). Thus, Pyk2 expression and activity function to mediate 4T1 pulmonary metastases.

Pyk2 expression is dispensable for breast cancer cell invasion stimulated by TGF-β

We next sought to establish the specific role of Pyk2 during breast cancer metastasis stimulated by TGF-β. Figure 5a shows that TGF-β treatment of 4T07 cells resulted in a robust rearrangement of the actin cytoskeletal system. Interestingly, administration of PF-228 (FAK) or PF-271 (FAK/Pyk2) both elicited the accumulation of filopodia and formation of actin stress fibers in a TGF-β-independent manner (Figure 5a). However, despite the morphological similarities observed in these static photomicrographs, time-lapse microscopy showed that administration of PF-228 inhibited the motility of 4T07 cells by preventing their ability to turnover focal adhesion complexes (Supplementary Movies 1–3).[29,38,39] In stark contrast, we observed EMT induced by TGF-β to significantly enhance the invasion (Figure 5b) and migration (Supplementary Movie 2) of 4T07 cells. Interestingly, pharmacological inhibition of FAK (PF-228) or FAK/Pyk2 (PF-271) similarly prevented 4T07 cells from acquiring invasive phenotypes in response to TGF-β (Figure 5b), an event reminiscent of the failure of TGF-β to induce the invasion of FAK-deficient breast cancer cells.[18] Collectively, these findings suggest that FAK, but not Pyk2, is responsible for coupling TGF-β to breast cancer cell invasion, and as such, predict that targeted inactivation of Pyk2 would be ineffective in altering breast cancer cell invasion stimulated by TGF-β. To test this hypothesis, we utilized two-independent short hairpin RNAs to deplete the expression of Pyk2 in 4T07 cells, whose invasive response to TGF-β was indistinguishable from that displayed by their parental counterparts (Figure 5c). Collectively, these findings demonstrate that Pyk2 expression is dispensable for TGF-β stimulation of breast cancer invasion, a process dependent upon FAK-mediated turnover of focal adhesion complexes (Supplementary Movies 1–3).[18]

Upregulated Pyk2 expression couples EMT to metastatic outgrowth stimulated by TGF-β

We recently showed that EMT can circumvent breast cancer dormancy and initiate proliferative programs operant in mediating metastatic outgrowth.[4] This scenario is reminiscent of the ability of Pyk2 to enable disseminated breast cancer cells to form macroscopic secondary tumors in the lungs and other tissues in mice (Figures 3 and 4). Given these parallels, we sought to address the hypothesis that upregulated Pyk2 expression is encompassed in an EMT signature operant in driving metastatic outgrowth. Figure 6a shows that TGF-β stimulation of EMT (Post-EMT) significantly increased the outgrowth of 4T07 cells in pulmonary 3D-organotypic cultures.[4,27,40,41] Dual inactivation of FAK and Pyk2 by administration of PF-271 was more effective in diminishing TGF-β-mediated 4T07 outgrowth as compared with singular inactivation of FAK by PF-228 (Figure 6a). In light of the dramatic increase in Pyk2 expression observed in Post-EMT 4T07 cells (Figure 2), this finding suggests that the significantly greater effectiveness of PF-271 to inhibit 4T07 organoid outgrowth relative to that mediated by PF-228 likely reflects the preferential targeting of PF-271 to inactivate the upregulated pool of Pyk2 in Post-EMT cells. Accordingly, while Pyk2-deficiency did not alter the 2D morphology of the 4T07 cells, this cellular condition did (i) dramatically enhance their formation of branched organoids in 3D-cultures (Supplementary Figure 5a and Figure 6b), a phenotype we have previously associated with mammary gland differentiation and metastatic dormancy;[3,4] and (ii) significantly impair their growth in 3D-cultures in response to TGF-β (Figure 6c). Finally, we observed TGF-β stimulation of EMT to bestow 4T07 cells with a dramatic outgrowth advantage in the lungs of mice, a reaction that was absolutely dependent upon Pyk2 expression (Figures 6d and e). Taken together, our findings clearly establish the importance of upregulated Pyk2 expression in coupling oncogenic TGF-β signaling to the metastatic outgrowth of breast cancer cells.

Pyk2 is required for TGF-β stimulation of an outgrowth proficient phenotype

We next sought to determine the relationship of Pyk2 in coordinating the mutually exclusive expression patterns exhibited

© 2012 Macmillan Publishers Limited

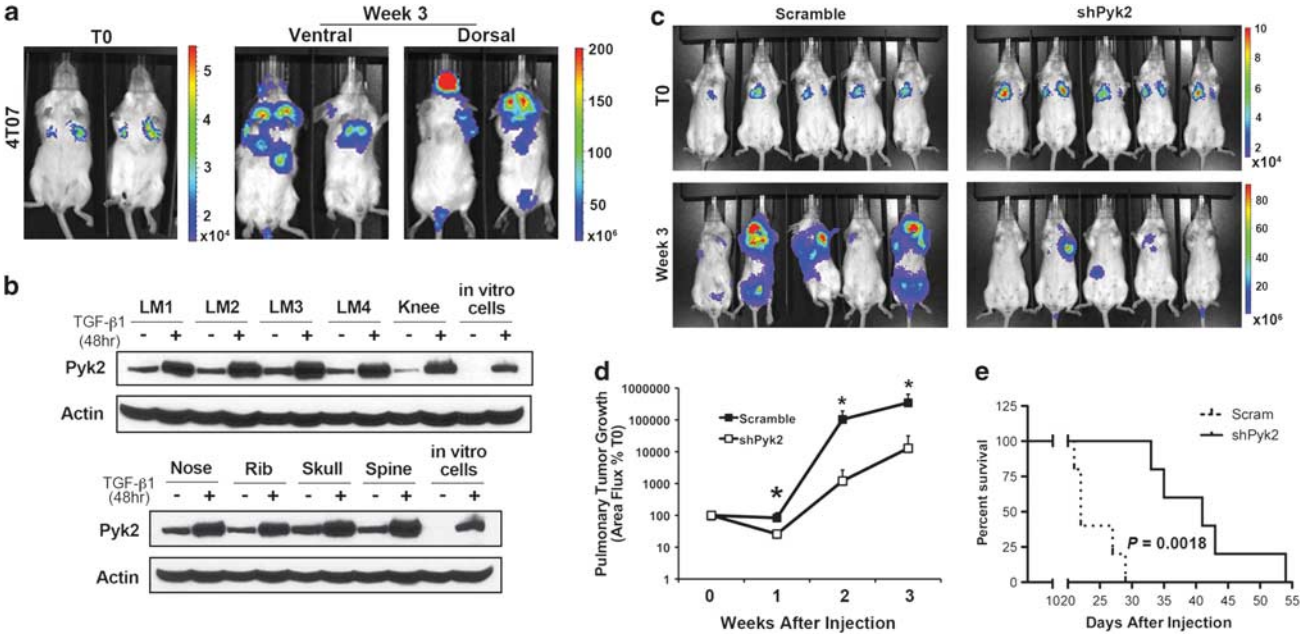


Figure 4. Pyk2 expression is essential for pulmonary tumor formation in mice. (**a**) Luciferase-expressing 4T07 cells were injected into the lateral tail vein of Balb/C mice (1×10^5 cells/mouse). Shown are bioluminescent images of representative mice ($n = 40$) captured at the time of injection (T0) and 3 weeks later, at which point secondary 4T07 tumor formation was widespread. (**b**) 4T07 metastases as described in panel (**a**) were isolated from the indicated locations (LM, lung metastasis) and subcultured *ex vivo* in the absence or presence of TGF-β1 (5 ng/ml) for 48 h before monitoring changes in Pyk2 expression by immunoblot analyses. β-actin served as a loading control. (**c**) Shown are bioluminescent images of representative mice ($n = 5$) injected with control (scramble) or Pyk2-depleted (shPyk2) 4T07 cells immediately after their inoculation into the lateral tail vein (T0), and again 3 weeks later. (**d**) Data are the mean (± s.e.; $n = 5$ mice per group) area flux values normalized to the injected values (T0) over a span of 3 weeks (*$P < 0.05$). (**e**) Pyk2-deficiency significantly extended the overall survival of mice inoculated with 4T07 cells as described in panel (**c**).

by E-cad and β1 integrin in outgrowth-incompetent (for example, E-cad high, β1 integrin low) or -proficient (for example, E-cad low, β1 integrin high) breast cancer cells.[4] In doing so, we observed Pyk2-deficiency in 4T07 cells to elicit a dramatic upregulation in E-cad expression and prevent TGF-β from inducing that of β1 integrin (Figure 7a). Despite their elevated basal expression of E-cad, Pyk2-deficient 4T07 cells remained competent to down-regulate E-cad expression (Figure 7a) and stimulate Smad2/3 phosphorylation (Supplementary Figure 5b) in response to TGF-β. Thus, unlike FAK,[18] Pyk2 expression appeared dispensable for general TGF-β signaling, but was essential in coupling TGF-β to β1 integrin expression in outgrowth proficient breast cancer cells. Accordingly, ectopic expression of wild-type E-cad, but not that of a dominant-negative mutant lacking its extracellular domain (ΔE-Ecad),[42,43] prevented TGF-β from inducing Pyk2 expression in a manner that correlated with the ability of wild-type E-cad to repress the expression of β1 integrin in metastatic D2.A1 breast cancer cells (Figure 7b).[4] Moreover, when propagated in 3D-organotypic cultures, 4T07 cells simultaneously downregulated their expression of E-cad and upregulated that of Pyk2 (Figure 7c).[4] Importantly, the ability of Pyk2-deficient 4T07 cells to maintain E-cad expression destabilized β1 integrin expression under pulmonary growth conditions (Figure 7c), an event that we[4] and others[27,41] have shown to prevent the initiation of metastatic outgrowth. Accordingly, β1 integrin-deficiency elicited an upregulation of E-cad expression that abrogated the ability of TGF-β to induce Pyk2 expression (Figure 7d). Taken together, these findings highlight the dynamic events required for TGF-β and EMT to initiate the pulmonary outgrowth of disseminated breast cancer cells, a process culminating in diminished E-cad expression, upregulated Pyk2 expression and stabilized β1 integrin expression (Figure 8).

DISCUSSION

The switch in TGF-β function from that of a tumor suppressor to a promoter of metastasis is largely thought to reflect the processes associated with EMT. Indeed, EMT elicits the physical interaction of TGF-β receptors with integrins,[31,33] growth factor receptors[3] and molecules housed within focal adhesion complexes, including FAK and p130Cas.[18,44] Collectively, these events coalesce in amplifying the invasive potential of breast cancer cells via stimulation several different signaling pathways, including p38 MAPK, NF-κB and Cox-2 (see Taylor *et al.*[2] and Parvani *et al.*[45]). Indeed, based on structural similarities that exist between FAK and Pyk2,[5,6] we anticipated Pyk2 to have an essential function in driving breast cancer cell invasion stimulated by TGF-β. However, disrupting Pyk2 activity through pharmacological or genetic means failed to alter TGF-β signaling and its stimulation of breast cancer cell invasion, events that are clearly reliant upon FAK (Figure 5).[18] Instead, we defined a novel positive-feedback loop that enables TGF-β to overcome systemic dormancy and initiate the metastatic outgrowth of disseminated breast cancer cells by simultaneously regulating the expression of E-cad, Pyk2 and β1 integrin (Figure 8).

Pyk2 expression varied across clonal populations of human MDA-231 breast cancer cells. Moreover, the level of Pyk2 expression directly correlated with the ability of these cells to thrive in pulmonary 3D-organotypic cultures, but not with their ability to invade through Matrigel (Figure 1). Accordingly, we observed Pyk2 expression to be selectively and specifically upregulated in *ex vivo* cultures of macroscopic breast cancer metastases (Figure 2). Moreover, TGF-β-induced EMT significantly enhanced the production of pulmonary tumors, an event that was lacking in Pyk2-deficient cells. Mechanistically, the ability of TGF-β to induce Pyk2 transpires through both Smad4-dependent and -independent pathways. For instance, we previously observed

© 2012 Macmillan Publishers Limited

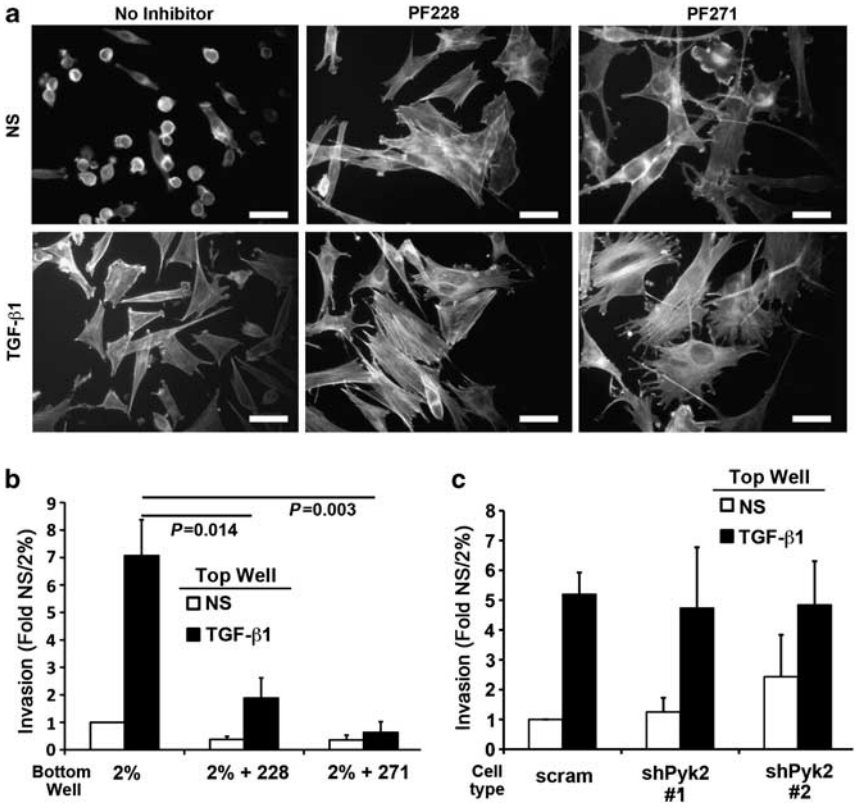


Figure 5. Pyk2 is dispensable for breast cancer cell invasion stimulated by TGF-β. (**a**) 4T07 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h in the absence or presence of PF573228 (1 μM; PF-228) or PF562271 (1 μM; PF-271). Afterward, alterations in the actin cytoskeleton were visualized by fluorescent-phalloidin staining. (NS, no stimulation). Shown are representative photomicrographs of three independent experiments. (**b**) 4T07 cells were induced to invade through Matrigel by 2% serum for 48 h in the absence or presence of TGF-β1 (5 ng/ml). Where indicated, PF-228 (1 μM) or PF-271 (1 μM) was added to the bottom well. (**c**) Control (scram) and Pyk2-deficient 4T07 cells were allowed to invade through Matrigel membranes for 48 h as described in panel (**b**). Data in panels (**b**) and (**c**) were normalized to serum-induced invasion in the absence of TGF-β1 and are the mean (± s.e.) of three independent experiments completed in triplicate.

Smad3/4-based signaling to be robust in primary mammary tumors and greatly diminished in their resultant pulmonary metastases.[46] Our current findings indicate that the differential activation of Smad3/4 by TGF-β is due in part to alterations in tissue compliance, such that Smads are strongly stimulated in biomechanically rigid microenvironments and weakly stimulated in their biomechanically compliant counterparts. Thus, our findings imply that metastatic breast cancers must upregulate their expression of Pyk2 before exiting the rigid primary tumor and subsequent blunting of their capacity to activate Smad3/4 upon seeding within the compliant pulmonary microenvironment. As such, it is tempting to speculate that Pyk2 directs an intricate Smad-based EMT program that must be fully operational at the time of metastatic dissemination to avoid succumbing to pulmonary dormancy. Finally, it is important to note that these Pyk2- and EMT-dependent events operant in initiating pulmonary outgrowth must ultimately give way to mesenchymal-epithelial transition (MET), which is required to sustain macroscopic outgrowth of breast metastases.[4,47]

Our findings clearly link Smad4 to increased Pyk2 expression stimulated by TGF-β (Figure 2); however, the simple activation of Smad4 by TGF-β was insufficient to universally elevate Pyk2 levels in all breast cancer cells, particularly those that possess low-grade and nonmetastatic phenotypes (for example, MCF-7 and 67NR cells; Supplementary Figure 1). For instance, we previously observed metastatic 4T1 cells to poorly activate Smad3/4 as compared with their weakly tumorigenic 67NR counterparts,[44] which failed to elevate Pyk2 expression in response to TGF-β (Supplementary Figure 1). Likewise, re-expression of Smad4 in Smad4-null MDA-MB-468 cells also failed to couple TGF-β to the expression of Pyk2 (data not shown). These findings suggest that metastatic breast cancer cells selectively express additional Smad4 transcriptional regulators and/or activate alternative effector pathways required to mediate TGF-β stimulation of Pyk2 expression. We previously established Src as an essential factor in mediating the oncogenic activities of TGF-β and its stimulation of breast cancer EMT and metastasis.[18,48] Along these lines, we show herein that pharmacological inactivation of Src not only decreased the coupling of TGF-β to Pyk2 expression, but also its stimulation of Smad2/3 phosphorylation, thereby potentially implicating Src as an upstream effector operant in coupling TGF-β to the activation of Smads and Pyk2. However, at present, we cannot discount a direct inhibitory effect of PP2 on the activity of TGF-β receptors.[49] Nonetheless, it is interesting to note that Smad4 and Src are both essential in mediating the metastasis of breast cancer cells to bone,[50,51] which provides a biomechanically rigid microenvironment capable of supporting strong Smad-dependent signaling.[52] Collectively, these studies and the findings presented herein implicate Pyk2 as an important mediator of breast cancer metastasis, particularly their ability to reinitiate proliferative programs at sites of secondary tumor formation.

Our conclusion that Pyk2 and E-cad expression are directly related is supported by a recent study that observed over-expression of Pyk2 to elicit the downregulation of E-cad.[14] We show herein that TGF-β controls these interdependent activities, events that coalesce in the stabilization of β1 integrin and production of an outgrowth proficient phenotype (Figure 8).[4] Precisely how these effectors reinitiate proliferative programs

© 2012 Macmillan Publishers Limited

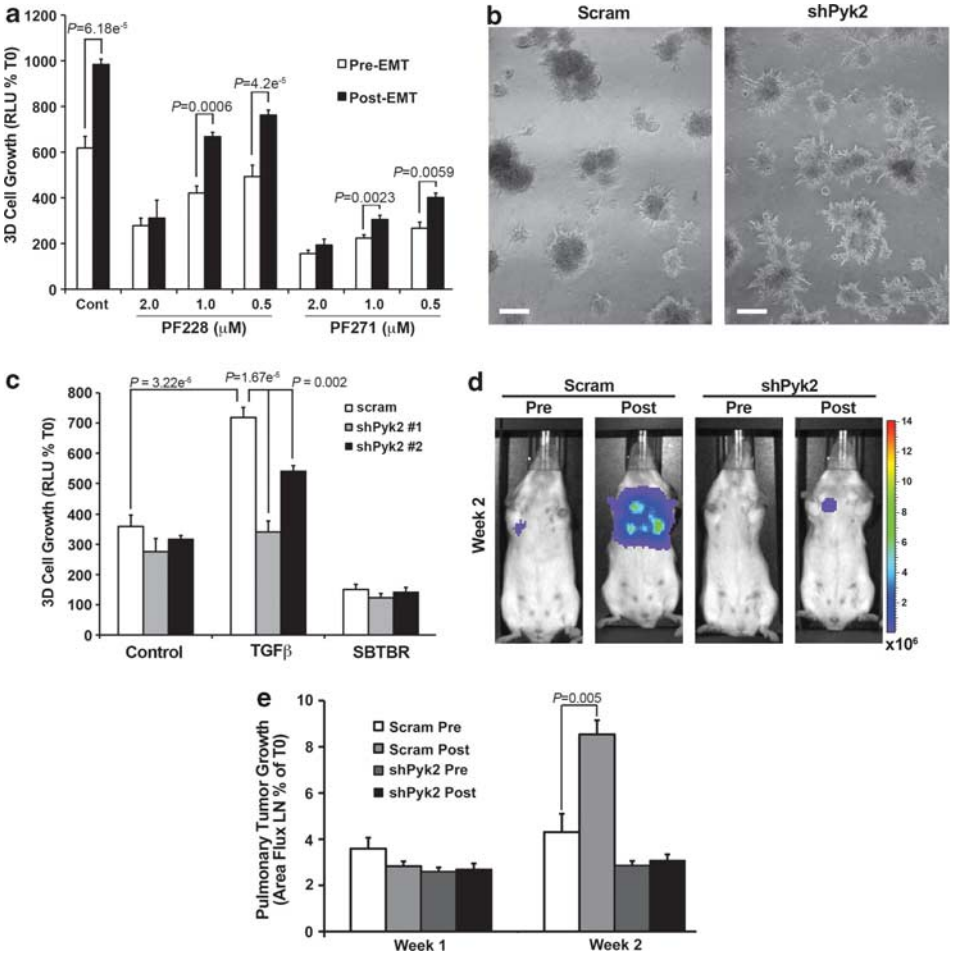


Figure 6. Upregulated Pyk2 expression couples EMT to metastatic outgrowth stimulated by TGF-β. (**a**) 4T07 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h to induce an EMT reaction (Post-EMT). Afterward, Pre- and Post-EMT 4T07 populations were propagated in 3D-organotypic cultures for 4 days in the presence of increasing concentrations of PF-228 or PF-271 as indicated. Changes in 3D organoid outgrowth were quantified by bioluminescence. (**b**) Control (scram) and Pyk2-deficient (shPyk2) 4T07 cells were propagated in 3D-organotypic cultures for 5 days, at which point alterations in organoid morphology were visualized by phase contrast microscopy (× 100). (**c**) Control (scram) and Pyk2-deficient (shPyk2) 4T07 cells were propagated in 3D-organotypic cultures for 4 days in the absence or presence of TGF-β1 (5 ng/ml) or the TβR-I inhibitor, SB431542 (SBTBR; 10 μM) as shown. Changes in organoid outgrowth were quantified by bioluminescence. Data in panels (**a**) and (**c**) are the mean (± s.e.) of three independent experiments completed in triplicate. (**d**) Pre- and Post-EMT control (scram) or Pyk2-deficient (shPyk2) 4T07 cells were inoculated into the lateral tail vein (5×10^4 cells/mouse). Shown are bioluminescent images of representative mice 2 weeks after injection. (**e**) Data are the mean (± s.e.; $n = 5$ mice per group) area flux values normalized to the injected values (T0) for panel (**d**).

in disseminated breast cancer cells remains incompletely understood. However, FAK[53] and Pyk2[54] have been detected in the nucleus of malignant cells where they promote Mdm2-mediated ubiquitination and degradation of p53, thereby facilitating enhanced cell proliferation and survival. In response to TGF-β administration, we recently detected both the full-length and FERM domains of Pyk2 and FAK in the nuclei of several human and murine triple-negative breast cancer cell lines (data not shown). Thus, it is tempting to speculate that the activation of β1 integrin promotes the outgrowth of disseminated breast cancer cells via a p53 pathway that relies in part on nuclear localization of Pyk2. Studies are currently underway to address these questions, and to determine the extent to which cytoplasmic and nuclear Pyk2 drive metastatic progression stimulated by TGF-β.

MATERIALS AND METHODS

Cell lines and reagents

Murine D2-HAN (D2.OR and D2.A1) and 4T1 derivatives (67NR, 168FARN, 4T07, 66c14 and 4T1) cells were obtained from Dr Fred Miller (Wayne State University, Detroit, MI, USA), while human MDA-MB-231 cells were purchased from the ATCC (Manassas, VA, USA). All human and murine breast cancer cells were cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep as described previously.[3] Bioluminescent 4T07 and MDA-MB-231 cells were engineered to stably express firefly luciferase as described.[3,18] Dual-bioluminescent SBE-reporter cells were generated by stably transfecting 4T1 cells with pcDNA3.1-Hygro (Invitrogen, Carlsbad, CA, USA) that constitutively-expressed renilla luciferase under control of the CMV promoter. Afterward, the resulting hygromycin-resistant 4T1 cell populations were stably transfected with pGL4.20-Puro (Promega, Madison, WI, USA) that expressed firefly luciferase under control of the synthetic SBE (4xSmad-binding element) promoter. Clonal MDA-MB-231 cell populations CP1-3 were constructed by transfection with pNifty-CMV-luciferase and clonal isolation under antibiotic selection, while CP4-6 (SCP2-4) were kindly provided by the lab of Dr Joan Massague.[55] MDA-231-SCP-2 cells that lacked Smad4 were previously described.[50] Cellular depletion of β1 integrin and Pyk2 expression was achieved by VSVG lentiviral transduction of pLKO.1 short hairpin RNA vectors (Open Biosystems, Lafayette, CO, USA; Supplementary Table 1) as described.[4,18] Ectopic expression of wild-type or a dominant-negative E-cad mutant lacking its extracellular domain (ΔE-Ecad)[42,43] was accomplished as described.[4] Finally, time-lapse microscopy was performed using a Leica DMI6000 over a span of 24 h with the resulting images being captured once every 10 min.

© 2012 Macmillan Publishers Limited

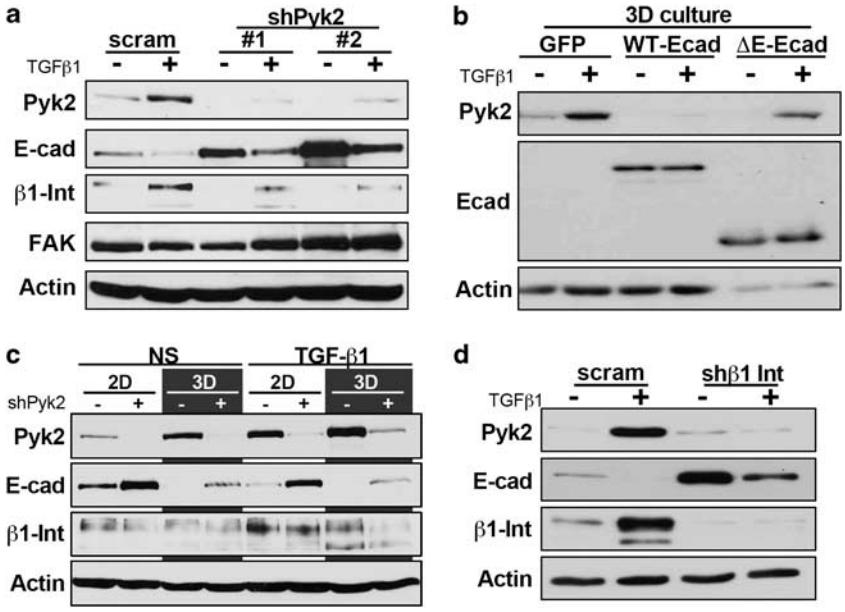


Figure 7. Pyk2 is essential in generating an outgrowth proficient phenotype. (**a**) Control (scram) and Pyk2-deficient (shPyk2#1 and #2) 4T07 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h, at which point altered expression of Pyk2, FAK, β1 Integrin and E-cad were monitored by immunoblot analyses. (**b**) Parental (GFP), wild-type (WT) or ΔE-E-cad-expressing D2.A1 cells were propagated in the absence or presence of TGF-β1 (5 ng/ml) in 3D-organotypic cultures for 72 h, at which point differences in Pyk2 expression were monitored by immunoblot analyses. (**c**) Control and Pyk2-depleted 4T07 cells were propagated for 5 days in the absence or presence of TGF-β1 (5 ng/ml) in traditional 2D-cultures (2D) or in 3D-organotypic cultures (3D). Afterward, changes in Pyk2, E-cad, β1 Integrin and β-actin were monitored by immunoblotting. (**d**) Control (scram) and β1 Integrin-deficient (shβ1 Int) 4T07 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h, at which point altered expression of Pyk2, E-cad, β1 Integrin and were monitored by immunoblot analyses. Data in panels (**a**–**d**) are representative of at least two-independent experiments.

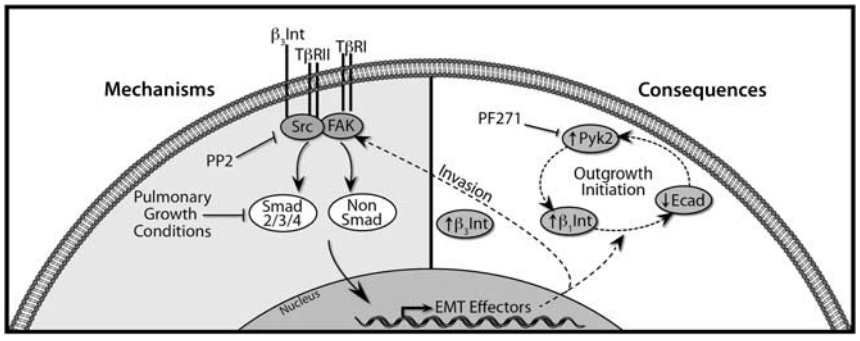


Figure 8. Schematic depicting the formation of '*Oncogenic TGF-β Signaling Complexes*' comprised of β3 integrin,[32] Src[48] and FAK,[18] whose activation is required for TGF-β aberrant signaling and the initiation of EMT programs (left). The consequences of these reactions results in the regulation of several factors that contribute to increased cellular invasiveness, including the upregulation of β3 Integrin and downregulation of E-cad. Subsequent to seeding at a secondary site, downregulated E-cad expression, upregulated Pyk2 expression and the stabilization of β1 integrin are necessary to escape systemic dormancy.[4] Collectively, this interdependent relationship between E-cad, Pyk2 and β1 integrin functions in EMT-initiated pulmonary outgrowth by metastatic breast cancers (right).

In vivo bioluminescent imaging of tumor growth and metastasis

Parental (scrambled short hairpin RNA) and Pyk2-deficient 4T07 cells were injected into the lateral tail vein of 4- (Figure 3) or 14- (Figure 5) week old Balb/C mice. Alternatively, parental and Pyk2-deficient 4T1 cells (1×10^4 cells) were engrafted onto the mammary fat pad of 4-week old Balb/C mice. All bioluminescent images were captured on a Xenogen IVIS-200 (Caliper Life Sciences, Hopkinton, MA, USA), and pulmonary tumor development was assessed by weekly bioluminescent imaging as described.[3,18,44] Upon completion of these studies, the mice were euthanized and their metastases were rapidly resected, enzymatically dissociated, and immediately subcultured *ex vivo* in Zeocin-containing media for 5–7 days, at which point the cells were collected and prepared for immunoblot analysis. Where indicated, metastatic 4T1 lung nodules were quantified by three individuals who were blinded to experimental conditions. All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee for Case Western Reserve University.

Immunoblot assays

Lysates generated from 2D- and 3D-cultures were prepared as described previously.[4] Antibodies used herein are described in the Supplementary Table 2.

3D-organotypic growth assays

Human and murine breast cancer were diluted in complete media supplemented with 5% Cultrex (Trevigen, Gaithersburg, MD, USA) and seeded onto solidified Cultrex cushions (50 μl/well) contained in 96-well

© 2012 Macmillan Publishers Limited

plates (1×10^4 cells/cm^2). Longitudinal bioluminescent growth assays were performed as described.[3,4] Pharmacological inhibitors used herein are described in the Supplementary Table 3.

Cell biological assays

The ability of TGF-β1 (5 ng/ml) to alter serum-induced invasion of MDA-231 and 4T07 cells was analyzed using a Matrigel-coated transwell assay as described.[31] For real-time PCR analysis, 4T07 cells were stimulated with TGF-β1 (5 ng/ml) for varying lengths of time, at which point total RNA was isolated using RNeasy Plus Kit (Qiagen, Valencia, CA, USA). Afterward, total RNA was reverse transcribed using the iScript cDNA Synthesis System (BioRad), and semi-quantitative real-time PCR was conducted using iQ SYBR Green (BioRad, Hercules, CA, USA) as described previously.[18] The oligonucleotide primer pairs used are provided in Supplementary Table 1. Lastly, direct phalloidin-fluorescence to visualize the actin cytoskeleton was accomplished as described previously.[18,44]

Statistical analyses

Statistical values were defined using an unpaired Student's *t*-test, where a P value <0.05 was considered significant. Statistically significant difference in the overall survival of mice bearing parental or Pyk2-deficient 4T07 tumors was analyzed using a log-rank test. P values for all experiments are indicated.

CONFLICT OF INTEREST

The authors declare not conflict of interest.

ACKNOWLEDGEMENTS

We thank Pfizer for generously providing the small molecule inhibitors against FAK and Pyk2. Members of the Schiemann Laboratory are thanked for critical reading of the manuscript. WPS was supported in part by grants from the National Institutes of Health (CA129359), the Susan G Komen for the Cure Foundation (BCTR0706967), the Department of Defense (BC084561), and pilot funding from the Case Comprehensive Cancer Center (P30 CA043703), while MKW was supported by a fellowship from the American Cancer Society (PF-09-120-01).

REFERENCES

1 Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-β. *Future Oncol* 2009; **5**: 1145–1168.
2 Taylor MA, Parvani JG, Schiemann WP. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. *J Mammary Gland Biol Neoplasia* 2010; **15**: 169–190.
3 Wendt MK, Smith JA, Schiemann WP. Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. *Oncogene* 2010; **29**: 6485–6498.
4 Wendt MK, Taylor MA, Schiemann BJ, Schiemann WP. Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. *Mol Biol Cell* 2011; **22**: 2423–2435.
5 Lipinski CA, Loftus JC. Targeting Pyk2 for therapeutic intervention. *Expert Opin Ther Targets* 2010; **14**: 95–108.
6 Schaller MD. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci* 2010; **123**: 1007–1013.
7 Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N *et al.* Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995; **377**: 539–544.
8 Okigaki M, Davis C, Falasca M, Harroch S, Felsenfeld DP, Sheetz MP *et al.* Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc Natl Acad Sci USA.* 2003; **100**: 10740–10745.
9 Schaller MD, Sasaki T. Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. *J Biol Chem* 1997; **272**: 25319–25325.
10 Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM *et al.* Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 1995; **376**: 737–745.
11 Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* 1996; **383**: 547–550.
12 Zrihan-Licht S, Fu Y, Settleman J, Schinkmann K, Shaw L, Keydar I *et al.* RAFTK/Pyk2 tyrosine kinase mediates the association of p190 RhoGAP with RasGAP and is involved in breast cancer cell invasion. *Oncogene* 2000; **19**: 1318–1328.
13 Fan H, Guan JL. Compensatory function of Pyk2 protein in the promotion of focal adhesion kinase (FAK)-null mammary cancer stem cell tumorigenicity and metastatic activity. *J Biol Chem* 2011; **286**: 18573–18582.
14 Sun CK, Ng KT, Lim ZX, Cheng Q, Lo CM, Poon RT *et al.* Proline-rich tyrosine kinase 2 (Pyk2) promotes cell motility of hepatocellular carcinoma through induction of epithelial to mesenchymal transition. *PLoS One* 2011; **6**: e18878.
15 Weis SM, Lim S-T, Lutu-Fuga KM, Barnes LA, Chen XL, Gothert JR *et al.* Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J Cell Biol* 2008; **181**: 43–50.
16 Sieg DJ, Ilic D, Jones KC, Damsky CH, Hunter T, Schlaepfer DD. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *Embo J.* 1998; **17**: 5933–5947.
17 Nakamura K, Yano H, Schaefer E, Sabe H. Different modes and qualities of tyrosine phosphorylation of Fak and Pyk2 during epithelial-mesenchymal transdifferentiation and cell migration: analysis of specific phosphorylation events using site-directed antibodies. *Oncogene* 2001; **20**: 2626–2635.
18 Wendt M, Schiemann W. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. *Breast Cancer Res* 2009; **11**: R68.
19 Judson PL, He X, Cance WG. Van Le L. Overexpression of focal adhesion kinase, a protein tyrosine kinase, in ovarian carcinoma. *Cancer* 1999; **86**: 1551–1556.
20 Kim SJ, Park JW, Yoon JS, Mok JO, Kim YJ, Park HK *et al.* Increased expression of focal adhesion kinase in thyroid cancer: immunohistochemical study. *J Korean Med Sci* 2004; **19**: 710–715.
21 Lightfoot Jr HM, Lark A, Livasy CA, Moore DT, Cowan D, Dressler L *et al.* Upregulation of focal adhesion kinase (FAK) expression in ductal carcinoma in situ (DCIS) is an early event in breast tumorigenesis. *Breast Cancer Res Treat* 2004; **88**: 109–116.
22 Carelli S, Zadra G, Vaira V, Falleni M, Bottiglieri L, Nosotti M *et al.* Up-regulation of focal adhesion kinase in non-small cell lung cancer. *Lung Cancer* 2006; **53**: 263–271.
23 Behmoaram E, Bijian K, Jie S, Xu Y, Darnel A, Bismar TA *et al.* Focal adhesion kinase-related proline-rich tyrosine kinase 2 and focal adhesion kinase are co-overexpressed in early-stage and invasive ErbB-2-positive breast cancer and cooperate for breast cancer cell tumorigenesis and invasiveness. *Am J Pathol* 2008; **173**: 1540–1550.
24 Burdick AD, Ivnitski-Steele ID, Lauer FT, Burchiel SW. PYK2 mediates anti-apoptotic AKT signaling in response to benzo[a]pyrene diol epoxide in mammary epithelial cells. *Carcinogenesis* 2006; **27**: 2331–2340.
25 Avsian-Kretchmer O, Hsueh AJ. Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. *Mol Endocrinol* 2004; **18**: 1–12.
26 Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD *et al.* Genes that mediate breast cancer metastasis to lung. *Nature* 2005; **436**: 518–524.
27 Shibue T, Weinberg RA. Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci USA.* 2009; **106**: 10290–10295.
28 Roberts WG, Ung E, Whalen P, Cooper B, Hulford C, Autry C *et al.* Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res* 2008; **68**: 1935–1944.
29 Slack-Davis JK, Martin KH, Tilghman RW, Iwanicki M, Ung EJ, Autry C *et al.* Cellular Characterization of a Novel Focal Adhesion Kinase Inhibitor. *J Biol Chem* 2007; **282**: 14845–14852.
30 Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* 1992; **52**: 1399–1405.
31 Galliher AJ, Schiemann WP. Beta3 integrin and Src facilitate transforming growth factor-beta mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* 2006; **8**: R42.
32 Galliher AJ, Neil JR, Schiemann WP. Role of transforming growth factor-beta in cancer progression. *Future Oncol* 2006; **2**: 743–763.
33 Galliher-Beckley AJ, Schiemann WP. Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. *Carcinogenesis* 2008; **29**: 244–251.
34 Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S *et al.* A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010; **141**: 69–80.
35 Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J *et al.* Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci USA.* 2010; **107**: 15449–15454.
36 Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A *et al.* Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci USA.* 2009; **106**: 13820–13825.

© 2012 Macmillan Publishers Limited

37 Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V *et al.* A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med* 2009; **15**: 68–74.
38 Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT *et al.* FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol.* 2004; **6**: 154–161.
39 Ilic Dk, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N *et al.* Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995; **377**: 539–544.
40 Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ *et al.* Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Res* 2008; **68**: 6241–6250.
41 Barkan D, El Touny LH, Michalowski AM, Smith JA, Chu I, Davis AS *et al.* Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. *Cancer Res* 2010; **70**: 5706–5716.
42 Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008; **68**: 3645–3654.
43 Dahl U, Sjodin A, Semb H. Cadherins regulate aggregation of pancreatic beta-cells in vivo. *Development* 1996; **122**: 2895–2902.
44 Wendt MK, Smith JA, Schiemann WP. p130Cas is required for mammary tumor growth and transforming growth factor-beta-mediated metastasis through regulation of Smad2/3 activity. *J Biol Chem* 2009; **284**: 34145–34156.
45 Parvani JG, Taylor MA, Schiemann WP, Noncanonical TGF-β. Signaling During Mammary Tumorigenesis. *J Mammary Gland Biol Neoplasia* 2011; **16**: 127–146.
46 Wendt MK, Molter J, Flask CA, Schiemann WP. In vivo Dual Substrate Bioluminesent Imaging. *J Vis Exp* 2011; pii:3245 (doi:10.3791/3245).
47 Korpal M, Ell BJ, Buffa FM, Ibrahim T, Blanco MA, Celia-Terrassa T *et al.* Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat Med* 2011; **17**: 1101–1108.
48 Galliher AJ, Schiemann WP. Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer Res* 2007; **67**: 3752–3758.
49 Ungefroren H, Sebens S, Groth S, Gieseler F. Fandrich F. The Src family kinase inhibitors PP2 and PP1 block TGF-β1-mediated cellular responses by direct and differential inhibition of type I and type II TGF-β receptors. *Curr Cancer Drug Targets* 2011; **11**: 524–535.
50 Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR *et al.* Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci USA.* 2005; **102**: 13909–13914.
51 Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M *et al.* Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell* 2009; **16**: 67–78.
52 Korpal M, Yan J, Lu X, Xu S, Lerit DA, Kang Y. Imaging transforming growth factor-beta signaling dynamics and therapeutic response in breast cancer bone metastasis. *Nat Med* 2009.
53 Lim S-T, Chen XL, Lim Y, Hanson DA, Vo T-T, Howerton K *et al.* Nuclear FAK Promotes Cell Proliferation and Survival through FERM-Enhanced p53 Degradation. *Mol Cell* 2008; **29**: 9–22.
54 Lim ST, Miller NL, Nam JO, Chen XL, Lim Y, Schlaepfer DD. Pyk2 inhibition of p53 as an adaptive and intrinsic mechanism facilitating cell proliferation and survival. *J Biol Chem* 2010; **285**: 1743–1753.
55 Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M *et al.* Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest* 2005; **115**: 44–55.
56 Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D *et al.* Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006; **439**: 353–357.
57 Huang E, Cheng SH, Dressman H, Pittman J, Tsou MH, Horng CF *et al.* Gene expression predictors of breast cancer outcomes. *Lancet* 2003; **361**: 1590–1596.
58 Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; **449**: 557–563.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

© 2012 Macmillan Publishers Limited

Chapter 5
Regulation of TGF-β Signaling and Metastatic Progression by Tumor Microenvironments

Michael K. Wendt and William P. Schiemann

Abstract The TGF-β signaling system comprises a complex and dynamic cascade of molecular interactions that invoke a variety of intracellular and extracellular reactions that coalesce to maintain tissue homeostasis. A rapidly accumulating body of scientific literature clearly demonstrates a conversion in TGF-β function from that of a powerful tumor suppressor in normal epithelium and early-stage carcinomas to that of a prometastatic molecule in their late-stage counterparts. Collectively, this malicious switch in TGF-β behavior is termed the "TGF-β Paradox." Historically, cell autonomous changes that transpire during tumor development and progression have been studied extensively as a means to decipher the "TGF-β Paradox." Although highly informative and intriguing, these findings have yet to unravel the molecular underpinnings of the "TGF-β Paradox," thereby suggesting involvement of additional signaling components and players that originate beyond the confines of developing carcinomas. Indeed, recent studies have been directed at interrogating the microenvironments of developing carcinomas and how changes within this unique cellular niche manifest the "TGF-β Paradox." For instance, tumor microenvironments house an array of essential cellular, structural, and humoral factors that include stromal cells and altered elastic moduli, integrins and their engagement of matrix proteins, hypoxic zones, and a host of cytokines, growth factors, and chemokines that collectively influence the response of carcinoma cells to TGF-β. Here we review recent findings demonstrating the importance of the tumor microenvironment to regulate TGF-β signaling and its stimulation of metastatic progression. In addition, we also highlight recent *in vitro* and *in vivo* scientific advances capable of recapitulating various aspects of the metastatic process and its regulation by TGF-β.

M.K. Wendt • W.P. Schiemann (✉)
Division of General Medical Sciences-Oncology, Case Comprehensive Cancer Center, Case Western Reserve University, Wolstein Research Building, 2103 Cornell Road, Cleveland, OH 44106, USA
e-mail: william.schiemann@case.edu

A. Fatatis (ed.), *Signaling Pathways and Molecular Mediators in Metastasis*,
DOI 10.1007/978-94-007-2558-4_5, © Springer Science+Business Media B.V. 2012

Indeed, incorporating and extending these novel systems to analyses of the "TGF-β Paradox" may offer new inroads in resolving this enigma and improving the overall survival of cancer patients.

Abbreviations

2D	2-Dimensional
3D	3-Dimensional
CSF-1	Colony stimulating factor-1
Dab2	Disabled-2
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
Hgs	Hepatocyte growth factor-regulated tyrosine kinase substrate
IHC	Immunohistochemistry
JNK	c-Jun N-terminal kinase
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MMP	Matrix metalloproteinase
PTK	Protein tyrosine kinase
RBM	Reconstituted basement membrane
SARA	Smad anchor for receptor activation
TAK-1	TGF-β-activated kinase 1
TGF-β	Transforming growth factor-β
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptor
VEGF	Vascular endothelial growth factor

5.1 Introduction

Transforming growth factor-β (TGF-β) is a ubiquitously expressed cytokine that regulates an assortment of biological activities in essentially all cell types and tissues. Besides its role in regulating cell development, differentiation, and survival, TGF-β also inhibits the proliferation of epithelial, endothelial, and hematopoietic cell lineages [1–4]. Interestingly, resistance to TGF-β-mediated cytostasis is a hallmark of neoplastic transformation, which ultimately transform the signals produced by this cytokine into oncogenic activities, particularly enhanced cancer cell invasion

and metastasis [2, 5]. This conversion in TGF-β function is known as the "TGF-β Paradox" [5–7], which underlies the lethality of TGF-β in developing carcinomas, including their acquisition of EMT, metastatic, and chemoresistant phenotypes [5, 8, 9]. The molecular mechanisms whereby TGF-β mediates tumor suppression in normal and neoplastic cells remain to be fully elucidated. Likewise, how TGF-β promotes tumor progression in late-stage carcinomas is even more mysterious. Scientific investigations into TGF-β action have historically applied "cell-centric" approaches to interrogate the "TGF-β Paradox." Indeed, these analyses have relied heavily on the differential expression of gene transcripts, proteins, and microRNAs between normal and malignant cells as a potential means to explain the dichotomous of TGF-β in responsive cells. Although these studies have yielded enormous volumes of data related to potential alterations in the coupling of TGF-β to its downstream effectors [10–12], they have failed to accurately recapitulate the "TGF-β Paradox" and unlock its ability to promote the development and progression of human tumors. In light of these limitations, recent studies are now actively investigating the impact that dynamic microenvironment alterations play in dictating the behaviors of TGF-β during metastatic progression. Here we review recent findings that elucidate novel mechanisms whereby tumor microenvironments manifest the "TGF-β Paradox." Likewise, we also expound the virtues of novel 3-dimensional (3D)-organotypic culture techniques to recapitulate the changing microenvironments encountered by metastatic cancer cells as they disseminate throughout the body to distant locales.

5.2 The TGF-β Signaling System

Mammals express three genetically distinct TGF-β ligands (e.g., TGF-βs 1–3) whose mature and biologically active forms are ~97% identical and exhibit virtually indistinguishable actions *in vitro* [1, 13]. Interestingly, individual TGF-β ligands are expressed in a spatiotemporal manner during embryogenesis and tissue morphogenesis, which contributes to the array of diverse and nonredundant phenotypes displayed by mice lacking distinct TGF-β isoforms [14]. Transmembrane signaling by TGF-β is propagated by its binding to three high-affinity receptors, TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). When expressed, TβR-III is the most abundant TGF-β receptor present on the cell surface where it was originally believed to function solely as an accessory receptor that binds and modulates TGF-β function in responsive cells. However, recent findings implicate TβR-III as an essential mediator of the tumor suppressing activities of TGF-β. Indeed, loss of TβR-III expression associates with disease progression and poor overall survival in patients with cancers of the breast, ovary, prostate, lung, pancreas, kidney, and endometrium [15], suggesting that measures capable of elevating TβR-III expression or activity may circumvent the "TGF-β Paradox" and its initiation of oncogenic TGF-β signaling. Although TβR-III lacks intrinsic enzymatic activity, TβR-I and TβR-II both possess Ser/Thr protein kinases in their cytoplasmic

domains that serve to initiate downstream signaling [2, 3, 16–18]. In fact, the binding of TGF-β to TβR-II enables this polypeptide to transphosphorylate and activate TβR-I, which subsequently binds, phosphorylates, and stimulates the latent transcription factors, Smad2 and Smad3 [2, 3, 19]. Once phosphorylated, Smads 2 and 3 rapidly form higher order complexes with the common Smad, Smad4, with the resulting heterotrimeric complexes that rapidly translocate and accumulate in the nucleus where they cooperate with an ever expanding list of transcriptional activators or repressors to govern gene expression in a gene- and cell-specific manner [2, 3, 16–18]. Changes in cell behavior regulated by the activation of Smad2/3 is referred to as "canonical TGF-β signaling" and is modulated in all subcellular compartments by numerous effector molecules. For example, the specific recruitment and phosphorylation of Smad2/3 by TβR-I is regulated by several adaptor proteins, including SARA (Smad Anchor for Receptor Activation; [20]), Hgs (Hepatocyte Growth factor-regulated tyrosine kinase Substrate; [21]), and Dab2 (Disabled-2; [22]). Incorporation of the inhibitory Smad, Smad7, into active TGF-β receptor complexes displaces Smad2/3 and prevents their phosphorylation and activation by TβR-I [23–25]. Elevated Smad7 expression also inhibits TGF-β signaling through its ability to recruit Smurf E3 ubiquitin ligases, which promote the ubiquitination and degradation of TβR-I [26, 27]. Smad2/3 signaling is also governed in the nucleus by their association with a variety of interacting proteins, including ATF-3, Sp-1, AP-1, Jun/Fos, Stats, and members of the Forkhead family of transcription factors [2, 3, 16–18]. Finally, Smad2/3 signaling is also subject to fine-tuning following their phosphorylation by cellular protein kinases, as well as to inactivation by their dephosphorylation and ubiquitination by phosphatases and E3 ubiquitin ligases, respectively [28]. Figure 5.1 depicts the TGF-β signaling system and the potential role of Smads 2 and 3 in regulating metastatic progression driven by TGF-β. Additional details linking Smad2/3 to tumor metastasis are discussed in the following sections.

TGF-β also couples to a variety of noncanonical signaling systems (i.e., Smad2/3-independent), including ERK1/2, p38 MAPK, JNK, AKT, the small GTP-binding proteins Rho, Rac, and Cdc42 (*see* [5, 9, 17, 29]). The specific sequence of events and molecular mechanisms that couple TGF-β receptors to the activation of these pathways remain to be fully delineated. However, we recently defined a novel signaling axis comprised of αvβ3 integrin:Src:FAK:p130Cas:TβR-II:Grb2 that is critical for TGF-β stimulation of MAP kinases, EMT, and pulmonary metastasis of breast cancer cells [30–32]. Additional evidence suggests that the ability of TGF-β to activate MAPKs can also emanate from TβR-I and its physical interaction with the E3 ubiquitin ligase, TRAF6, which ubiquitinates itself and the MAPK kinase kinase TAK-1 (TGF-β-activated kinase 1) to facilitate JNK and p38 MAPK activation [33, 34]. TGF-β displays a dichotomous connection with NF-κB, whose transcriptional activity is inhibited by TGF-β in normal epithelial cells [35], but is robustly stimulated by this cytokine in their transformed counterparts [36–38]. The inverted coupling of TGF-β to NF-κB in normal and malignant cells may reflect a loss of TβR-III expression, which suppresses NF-κB activity through the formation of TβR-III:β-arrestin-2:NF-κB complexes [39]. Likewise, the coupling

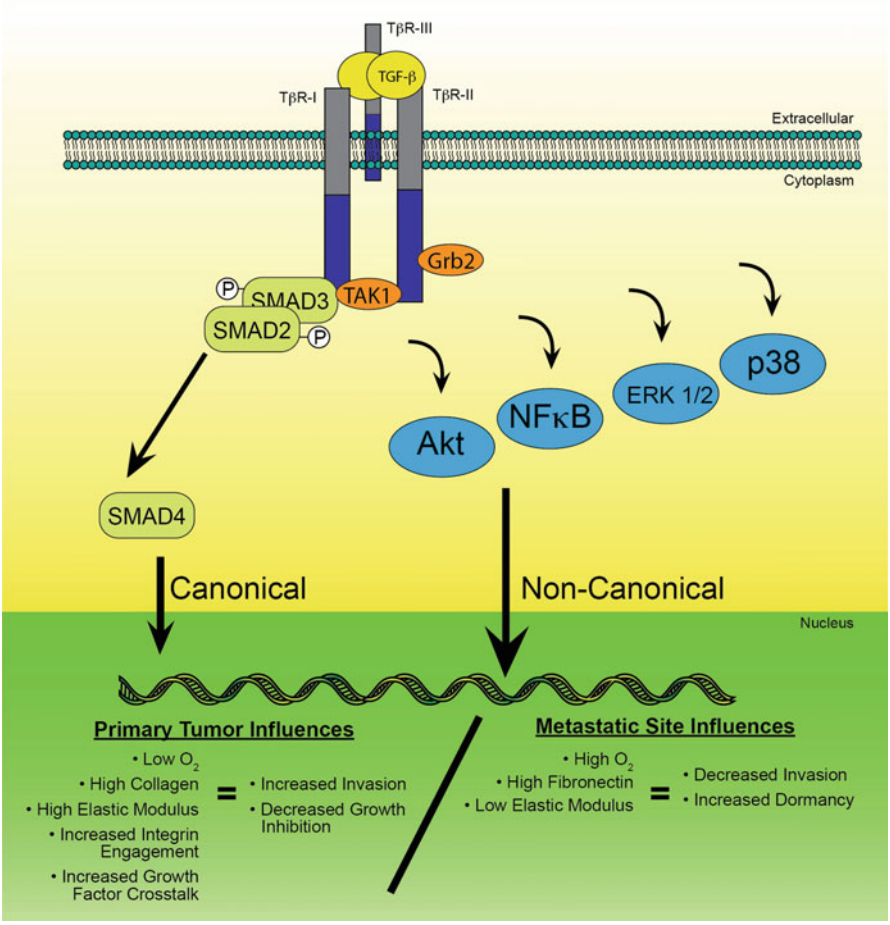


Fig. 5.1 The TGF-β signaling system during metastatic progression. TGF-β initiates transmembrane signaling by binding to its three cell surface receptors, TβR-I, TβR-II, and TβR-III. Once activated, TβR-I phosphorylates Smads 2 and 3, which form hetrotrimeric complexes with Smad4 that translocate to the nucleus to regulate gene expression. The transduction of messages *via* Smad2/3 constitutes the canonical arm of the TGF-β signaling system. Activated TGF-β receptors also stimulate a variety of non-Smad2/3-based messages that constitute the noncanonical arm of the TGF-β signaling system. Also shown are the influences and outcomes of TGF-β signaling within the primary tumor *versus* those at metastatic sites

of TGF-β to epithelial-mesenchymal transition (EMT; see below) is critically reliant on NF-κB activity [38]. We extended these findings to show that mammary tumorigenesis and EMT were both sufficient induce the formation of TβR-I:xIAP:TAB1:TAK1:IKKβ complexes operant in activating NF-κB [40–42], as well as its initiation of an autocrine Cox-2:PGE2:EP2 signaling axis that drives breast cancer metastasis [43]. Interestingly, IKKα also impacts canonical TGF-β signaling by interacting physically with Smad3 to enhance its binding at EMT-regulated

promoters [44]. Finally, the ability of TGF-β to regulate cellular behaviors also transpires through its stimulation of a number of nonreceptor protein tyrosine kinases (PTKs), including focal adhesion kinase (FAK; [45–49]), c-Src [30–32, 50], and c-Abl [51–54]. Collectively, the preferential coupling of TGF-β to its noncanonical effectors appears inappropriately amplified in metastatic cancer cells, thereby generating a signaling imbalance that overrides and/or dampens the tumor suppressing messages transduced by Smad2/3 in human tumors ([46]; Fig. 5.1). Unfortunately, precisely how noncanonical effectors are activated by TGF-β in normal and malignant cells remains to be determined definitively, as does the manner in which these pathways become dysregulated and magnified during the acquisition of metastatic phenotypes by cancer cells. These relationships and their regulation by tumor microenvironments during metastatic progression driven by TGF-β are discussed below.

5.3 EMT

The impact of TGF-β on metastatic progression is strongly linked to EMT, which reflects the ability of immotile, polarized epithelial cells to acquire highly motile, apolar fibroblastoid-like phenotypes [8, 9, 55–57]. More specifically, epithelial cells undergoing EMT exhibit several distinct features, including (i) the loss of cell polarity due to downregulated expression of epithelial cell markers (e.g., E-cadherin, zona occluden-1, and β4 integrin); (ii) cytoskeletal architecture reorganization and intracellular organelle redistribution; (iii) upregulated expression of fibroblast markers (e.g., vimentin, N-cadherin, α-smooth muscle actin); and (iv) elevated invasion factors (e.g., MMP-9, fibronectin) [8, 9, 55–57]. In general, these steps underlie the pathophysiological reactions associated with EMT, which were recently categorized into three distinct subtypes: (i) ***Type 1 EMT***, which reflects the epithelial plasticity associated with embryogenesis and tissue morphogenesis; (ii) ***Type 2 EMT***, which reflects the epithelial plasticity of tissue regeneration and fibrotic reactions; and (iii) ***Type 3 EMT***, which reflects the plasticity of carcinoma cells during metastatic progression [58]. Indeed, the initiation of Type 3 EMT confers carcinoma cells a selective invasive advantage to exit both the primary tumor [45] and the circulation at sites of dissemination [47, 59]. At first blush, this classification scheme acknowledges that the extent to which an EMT reaction transpires and/or resolves likely reflects the overall health and well-being of the epithelium and its immediate microenvironment. Unfortunately, the vast majority of EMT studies employ "cell-centric" approaches to assess the functional consequences of EMT in normal and malignant cells, and as such, the contributions of the microenvironment to regulating epithelial plasticity remains a critical and underexplored question. Despite these limitations and knowledge gaps, recent inroads into how microenvironments influence EMT have been gleaned through the use of novel 3D-organotypic culture systems capable of modeling distinct steps of the metastatic cascade. The impact of these cultures systems on the morphologies and phenotypes associated with Type 3

EMT are discussed below. Readers desiring in-depth summaries pertaining to the molecular mechanisms whereby TGF-β promotes EMT are directed to several recent reviews [8, 9].

5.4 The "TGF-β Paradox" in Animal Models

TGF-β was originally defined and purified 30 years ago by its ability to promote the morphological transformation and anchorage-independent growth of normal rat kidney fibroblasts (NRK-49 cells; [60, 61]). However, ensuing examinations of TGF-β action in epithelial cells quickly established this cytokine as a potent inhibitor of cell cycle progression [62], thus providing the initial framework for the "TGF-β Paradox" in traditional culture systems. Extending these analyses to animal studies quickly confirmed the dichotomous nature of TGF-β in regulating tumorigenesis. Indeed, implantation of slow-release TGF-β pellets into the mammary gland significantly inhibited its growth and morphogenesis in a reversible manner [63]. In contrast, administering neutralizing TGF-β antibodies suppressed breast cancer development in mice in part by enhancing splenic natural killer (NK) cell activity [64]. Likewise, transgenic expression of dominant-negative TβR-II enhanced mammary tumorigenesis in carcinogen-treated mice [65], while transgenic expression [66] or systemic administration [67] of a soluble Fc:TβR-II fusion protein both inhibited metastatic progression in mice. In addition, canonical TGF-β signaling was shown to inhibit the tumorigenicity of normal, premalignant, and malignant breast cancer cells, while stimulating that of highly invasive and metastatic breast cancer cells [68–70]. These findings coalesce to support a model wherein TGF-β drives the metastatic progression of late-stage carcinomas, which can upregulate their secretion of TGF-β by as much as 40-fold as compared to their normal counterparts [71]. Accordingly, conditional expression of active TGF-β1 in established mammary tumors significantly enhanced their metastatic progression without affecting the proliferation or growth of the corresponding primary tumors [72]. Similarly, targeted expression of active TGF-β1 in mouse keratinocytes promoted their proliferation in response to carcinogens, as well as induced their progression to aggressive spindle cell carcinomas [73, 74].

Additional insights into the importance of TGF-β signaling during normal development were gleaned from genetically engineering mice to lack expression of individual TGF-β isoforms and their receptors. For example, homozygous deletion of TGF-β1 results in ~50% of the developing pups to die *in utero* at embryonic day E10.5 [75, 76], while those that survive to term typically succumb to massive inflammatory reactions that result in multifocal infiltration of lymphocytes and macrophages into the heart, lungs, and salivary glands [77]. Homozygous deletion of TGF-β2 elicited perinatal lethality due to multiple developmental defects indicative of aberrant Type I EMT reactions during organogenesis and tissue morphogenesis [78], while disruption of the TGF-β3 locus also produces perinatal lethality resulting from defective EMT that occurred during lung and palate development [79].

Mice lacking TβR-II exhibit yolk sac hematopoiesis and vasculogenesis defects that phenocopy those observed in TGF-β1-deficient mice [80], while mice deficient in TβR-I expression die during midgestation due to placental and yolk sac vascular defects [81]. Collectively, these and numerous additional studies have helped to define the essential role of TGF-β signaling regulating organ development and immune surveillance and privilege [82–85].

Recent technological advancements have enabled the generation of mice that harbor alleles of the TGF-β signaling system that are flanked by LoxP sites (Floxed) as a means to circumvent the embryonic lethality associated with traditional gene targeting approaches. Indeed, conditional deletion of TβR-II in has provided new understanding of its role in regulating cardiac, skull, and palate development [86–88], and in mediating mammary gland involution and wound healing [89, 90]. It is important to note that the biology of TGF-β can largely be divided into two broad categories: regulation of cell cycling *versus* regulation of cell microenvironments (i.e., extracellular matrix (ECM) remodeling, angiogenesis, fibroblast activation, and immune cell infiltration). Thus, besides its ability to induce cytostasis in epithelial cells, TGF-β also governs the behaviors of adjacent fibroblasts and their synthesis and secretion of paracrine factors and ECM molecules that collectively suppress carcinoma development. Indeed, rendering fibroblasts deficient in TβR-II expression led to the formation of prostate intraepithelial neoplasia and invasive carcinoma of the forestomach [91]. Likewise, conditional deletion of TβR-II in mammary gland fibroblasts expanded their abundance and proliferative potential in a manner correlating with abnormal ductal development [92]. Moreover, these same TβR-II-deficient mammary fibroblasts greatly exaggerated the growth and invasion of breast cells simultaneously engrafted under the renal capsule in part through their upregulated expression of TGF-α, MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor) [91–94]. Even more remarkably, similar deletion of TβR-II in mammary carcinoma cells promoted the inappropriate activation of two distinct paracrine signaling axes – i.e., SDF-1:CXCR4 and CXCL5:CXCR2 – whose activation led to the recruitment of immature $GR1^{+}CD11b^{+}$ myeloid cells that drive breast cancer metastasis by inhibiting host tumor immunosurveillance, and by inducing MMP expression [95]. Interestingly, targeted deletion of Smad4 in T cells also elicited carcinoma formation within the gastrointestinal track (e.g., colon, rectum, intestine, and stomach) due to aberrant stromal expansion and signaling [96]. Finally, similar conditional deletion of TβR-I was targeted to the oral cavity of mice, which facilitated carcinogen-induced tumor formation in a manner correlated with constitutive PI3K/AKT activation [97]. Moreover, mice rendered heterozygous null for TβR-I displayed twice as many intestinal tumors as compared to their wild-type counterparts when crossed onto an $Apc^{(Min/+)}$ background [98]. Collectively, these findings highlight the importance of tissue homeostasis and cell microenvironments to facilitate the tumor suppressing activities of TGF-β, as well as establish the pathophysiological basis of the "TGF-β Paradox" in preclinical models of TGF-β-responsive carcinomas. In the succeeding sections, we present recent findings that frame our understanding of the "TGF-β Paradox" and its regulation by the diversity of microenvironments encountered by carcinoma cells during their acquisition of metastatic phenotypes.

5.5 TGF-β Signaling Within Primary Tumor Microenvironments

5.5.1 Mechanotransduction

The use of traditional 2-dimensional (2D) tissue culture systems to compare the behaviors and activities of normal *versus* malignant cells have proven to be wholly insufficient in recapitulating the "TGF-β Paradox." To circumvent this experimental deficiency, researchers have begun employing novel 3D-organotypic cultures to more accurately model and assess how changes within tumor microenvironments impact the functions of TGF-β. For example, culturing normal and malignant mammary epithelial cells (MECs) on top of or embedded into cushions of reconstituted basement membranes (RBMs) has provided numerous insights into how malignant transformation alters acinar development [99]. Although the precise conditions employed in these analyses can impart dramatic alterations in organoid development and behavior [100], it is nonetheless widely accepted that these 3D-organotypic systems more accurately recapitulate the signaling dynamics experienced by MECs and their microenvironments during mammary gland development [101]. It is important to note that tumor development is typically accompanied by intense desmoplastic and fibrotic reactions that enable primary tumors to be palpable in the context of surrounding normal tissue architectures. Moreover, tumor fibrosis results in the formation of mechanically rigid tumor microenvironments that (i) enhance the selection and expansion of developing neoplasms, particularly that of late-stage metastatic tumors, and (ii) predict for poor clinical outcomes in patients with cancers of the colon, ovary, and breast [102–105]. Interestingly, these aberrant cellular activities are highly reminiscent of those attributed to TGF-β [1, 2, 6, 106], whose upregulated expression dictates the composition tumor reactive stroma. In a reciprocal manner, tumor reactive stroma plays an important role during cancer initiation and progression by determining whether TGF-β suppresses or promotes tumor formation [5, 91–94]. In fact, it has been argued that "phenotypes dominate genotypes," a statement referring to the ability of the ECM and cell microenvironments to either suppress or promote tumorigenesis in a manner independent of genotypic alternations in MECs [107]. Dramatic evidence supporting this idea was provided by the findings that normal mice readily developed from (i) blastocysts injected with stable teratoma cells [108], and (ii) enucleated oocytes injected with nuclei isolated from melanoma cells [109]. Unfortunately, a role for TGF-β to impact these events remains surprisingly unexplored. However, infecting chicken embryos with RSV *in ovo* was shown to elicit cellular transformation and tumor formation only in response to tissue wounding, a reaction mediated by TGF-β [110, 111]. Collectively, these findings provide compelling evidence to support the notion that "phenotypes dominate genotypes," as well as implicate TGF-β as a principal player operant in overseeing this phenomenon. As such, inclusion of collagen and other matrices to RBM preparations represented a significant scientific achievement to initiate mechanotransduction in

modeled tumor microenvironments [112, 113]. For instance, elevated collagen concentrations results in integrin clustering and increased activation of focal adhesion complexes, growth factor receptors, and MAP kinase pathways [102–105, 113]. Along these lines, aberrant lysyl oxidase (LOX) activity also associates with cancer progression and the development of desmoplasia [104]. Indeed, elevated expression of LOX family members, particularly LOX, LOXL (LOX-like), and LOXL2, correlates with increased malignancy and the acquisition of invasive/metastatic phenotypes, and with the induction of EMT and the formation of the premetastatic niche [103, 113–118]. In particular, LOX expression is essential for hypoxia-induced metastasis of human MDA-MB-231 breast cancer cells in mice [115, 116]. Moreover, elevated LOX expression in breast cancer is observed most frequently in poorly differentiated, high grade tumors and, consequently, predicts for increased disease recurrence and decreased patient survival [115, 116]. Likewise, LOX expression has recently been validated as a prognostic marker for metastasis development in patients with head and neck cancers [119]. Interestingly, we observed TGF-β to be a potent inducer of LOX expression and activity, an event coupled to the acquisition of oncogenic TGF-β signaling in cancers of the breast [120]. Finally, two-photon intravital imaging analyses demonstrated that cancer cells attempting to exit the primary tumor utilize a paracrine signaling axis comprised of carcinoma-derived colony stimulating factor-1 (CSF-1) and macrophage-derived epidermal growth factor (EGF) that facilitates tumor cell migration and invasion [121–124]. Importantly, TGF-β plays a critical role in establishing this paracrine signaling network through its ability to (i) upregulate CSF-1 receptor expression [121]; (ii) stabilize EGF receptor (EGFR) expression at the cell surface [47]; and (iii) facilitate tumor-infiltration of macrophages *via* a FAK-dependent pathway [45].

The ability of mechanotransduction to alter function and composition of TGF-β signaling modules in normal and malignant cells has recently been linked to signals originating from integrins. For example, we observed TβR-II to interact physically with β3 integrin following its activation by vitronectin or in response to EMT induced by TGF-β [30–32]. Furthermore, the formation of β3 integrin:TβR-II complexes is critically dependent upon the expression and activity of FAK, such that depleting cells of FAK expression not only disrupts this interaction, but also markedly reduces the ability of TGF-β to stimulate breast cancer invasion and metastasis [45]. Along these lines, β1 integrin also interacts physically with TβR-II [30] and is required for its stimulation of EMT and p38 MAPK [125]. Additionally, the FAK effector, p130Cas, can bind and sequester Smad3 to prevent its phosphorylation by TβR-I and subsequent transit to the nucleus [126]. In extending these findings, we found elevated p130Cas expression to mark metastatic progression in breast cancers, as well as to distort the balance of TGF-β signaling from canonical to noncanonical effectors in metastatic MECs [46]. Indeed, genetic depletion of p130Cas was sufficient in drastically reducing the ability of TGF-β to promote breast cancer development and progression [46], presumably by enhancing their sensitivity to apoptotic stimuli [127]. Taken together, these findings highlight the direct influence integrins and focal adhesion complexes possess in promoting tumor progression,

particularly that driven by TGF-β. These studies also emphasize the power of 3D-organotypic models to faithfully recapitulate these events in sensitive and rapid cell-based assays capable of interrogating the "TGF-β Paradox."

The use of collagen to increase matrix rigidity strongly suggests that integrins, particularly those that ligate collagen, have the ability to influence TGF-β signaling. This notion is supported by our recent findings demonstrating that propagating malignant MECs in compliant 3D-organotypic cultures restored the cytostatic activities of TGF-β in highly metastatic 4T1 cells, which normally fail to undergo growth arrest in response to TGF-β in traditional 2D-cultures [32, 41, 51, 128]. Importantly, inclusion of type I collagen in these 3D-organotypic cultures to increase their rigidity/tension and to initiate mechanotransduction dose-dependently uncoupled TGF-β from the regulation of cell cycle progression in 4T1 cells [51]. Conversely, treating 4T1 organoids with the small molecule TβR-I inhibitor, TβR-I Inhibitor II stimulated the growth of 4T1 cells in compliant 3D-organotypic cultures wherein TGF-β functions as a tumor suppressor. However, administering this same pharmacological treatment regimen to tense 3D-organotypic cultures wherein TGF-β functions as a tumor promoter greatly inhibited the growth of the resultant 4T1 organoids [120], thereby validating the first *in vitro* system that recapitulates the "TGF-β Paradox" solely by modulating the tension sensed by malignant MECs. Interestingly, a recent study suggests that dramatic decreases in matrix compliance (i.e., elastic modulus) can serve as the driving force to alter cell signaling. Indeed, increasing matrix rigidity not only induced dramatic differences in cell proliferation, but also suppressed E-cadherin expression in a manner associated with the exaggerated development of EMT phenotypes [129]. Analogous alterations in tumor microenvironments also enabled E-cadherin-negative breast cancer cells to reinitiate expression of this junctional protein during metastatic outgrowth in the lungs of mice [130]. Although a direct participatory role for TGF-β during these events was not addressed, these analyses have nonetheless linked mechanotransduction to the activation of known TGF-β effectors. Moreover, increased matrix rigidity elicits increased TGF-β production [120], presumably *via* augmented integrin-mediated activation of latent TGF-β complexes [131]. Taken together, these studies stress the importance of integrins and matrix rigidity in regulating TGF-β signaling modules, and in enhancing the release of TGF-β from inactive matrix depots.

5.5.2 Hypoxia

It has long been appreciated that tumor development and progression significantly deprives these neoplasms access to sufficient nutrients and oxygen that facilitates their unabated growth, leading to the hypothesis that chemotherapeutic targeting of tumor angiogenesis would prove effective in starving tumors to death [132, 133]. Unfortunately, administering angiostatic agents to cancer patients has offered little improvement in overall patient survival, presumably reflecting the fact that hypoxic tumors house significantly elevated quantities of oncogenic factors that collectively

drive carcinoma cell dissemination to more hospitable environments [134]. Indeed, hypoxic culture conditions are sufficient to produce hyperinvasive and EMT phenotypes in carcinoma cells [135]. Moreover, hypoxia and TGF-β act in concert to induce the expression of vascular endothelial growth factor (VEGF) and the chemokine receptor CXCR4, two molecules essential to the metastatic process [136]. As expected, these oncogenic TGF-β activities are mediated by p38 MAPK [136], and by Smad7, which somewhat counterintuitively actually promoted carcinoma invasion under hypoxic conditions [137]. Collectively, these findings support a paradigm in which the activities of canonical and noncanonical TGF-β signaling systems are balanced under normal homeostatic conditions [138], but suddenly become unbalanced and slanted towards noncanonical TGF-β signaling under hypoxic tumor conditions. Indeed, these findings highlight the intimate relationship between hypoxia and oncogenic TGF-β signaling and their ability to be interrogated mechanistically *via* employment of 3D-organotypic culture systems.

5.5.3 Reactive Tumor Stroma

The cellular and structural composition of tumor microenvironments has a profound impact on tumor progression and TGF-β signaling. As mentioned above, targeted deletion of TβR-II in mammary carcinoma cells facilitated exaggerated infiltration of immature myeloid cells that greatly enhanced tumor invasion and metastasis in part *via* upregulated production of matrix metalloproteinases (MMPs) and TGF-β1 [95]. Interestingly, targeted inactivation of the TGF-β signaling system in fibroblasts [91] or the Smad4 pathway in T cells [96] both elicited carcinoma development due to disruptions of tumor suppressing paracrine signaling networks. Attempts to model these complex microenvironmental events *in vitro* have recently been initiated. For instance, the influence of $CD4^+$ T cells and their production of cytokines, particularly TGF-β, to drive breast cancer metastasis was determined to be dependent upon the development of M2-type macrophages. Indeed, co-culturing M2 macrophages with mammary carcinoma cells in 3D-organotypic cultures led to the production of invasive structures from resulting organoids in a manner indicative of increased carcinoma malignancy [139]. Although the synthesis of protruding branched structures is believed to reflect the extent of metastatic progression, it should be noted that the validity of this assumption remains to be rigorously examined. Thus, while certain metastatic cells do in fact grow as branched structures [140, 141], others readily assume a dense spherical morphology when propagated in 3D-organotypic systems. Indeed, we recently observed nonmetastatic cells to display dysmorphic branching structures that readily disappear during metastatic progression and in response to EMT induced by TGF-β (Fig. 5.2), which also promoted luminal filling of established acinar structures [47, 140]. Collectively, these findings highlight the potential of 3D-organotypic cultures to model distinct cell morphologies and behaviors in a manner that recapitulates defined aspects of tumor:stromal interactions. Extending these systems to include additional stromal

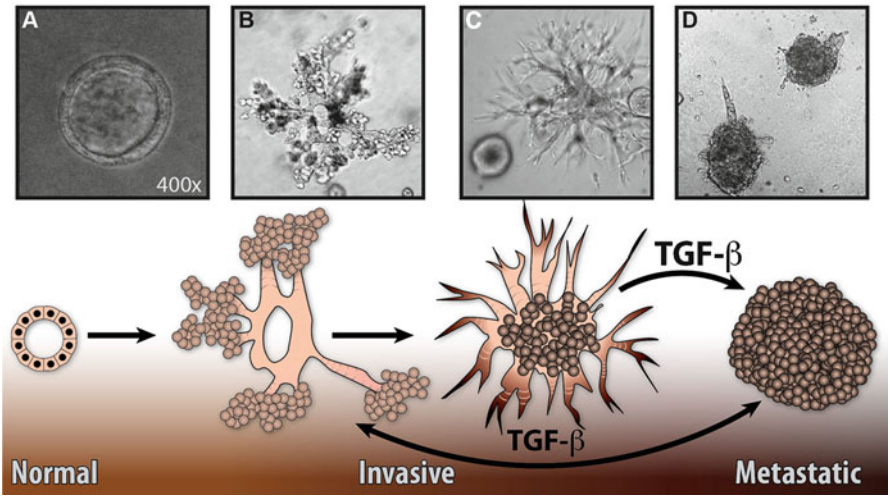


Fig. 5.2 The influence of TGF-β signaling on normal and malignant MEC acinar morphology. Photomicrographs of MECs grown in 3D-organotypic cultures (*left* to *right*): (i) normal MECs grown in the absence (**a**, 400×) or presence (**b**, 100×) of EGF; (ii) nonmetastatic breast cancer cells (**c**, 100×); and (iii) metastatic breast cancer cells (**d**, 100×). Accompanying schematic depicts these morphologies and how TGF-β signaling drives the acquisition of metastatic phenotypes

mediators (e.g., fibroblasts, endothelial, and immune cells) propagated under varying oxygen tensions and elastic moduli will greatly enhance the ability of science and medicine to decipher and manipulate the molecular underpinnings of the "TGF-β Paradox."

5.5.4 Visualizing Metastatic Progression Stimulated by TGF-β

Immunohistochemistry (IHC) is one of the most powerful tools available to pathologists attempting the grade and subtype tumor biopsies. Moreover, as the molecular basis of tumorigenesis continues to unfold, these IHC analyses will become more sophisticated in their ability to accurately diagnose and prognose patient outcomes, as well as to monitor the effectiveness of specific therapeutic regimens. However, employing IHC to gauge the extent of TGF-β function in human tissues is fraught with caveats and misinterpretations because this technique only captures a small snap-shot of the dynamics built into the TGF-β signaling system. Powerful developments in intravital imaging have greatly accelerated our understanding of (i) "how," "when," and "where" cancer cells systemically disseminate, and (ii) the role of TGF-β in mediating discrete steps of the metastatic cascade [45–47]. By employing dual bioluminescent imaging techniques, Korpal et al. [142] demonstrated the essential importance of canonical TGF-β

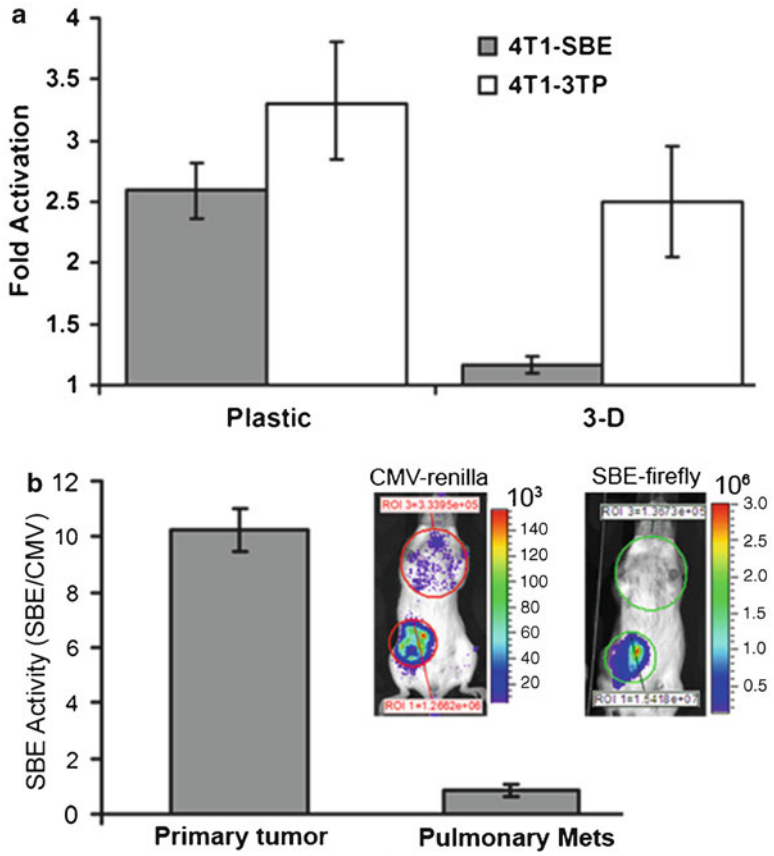


Fig. 5.3 Smad2/3 Signaling is decreased dramatically during the outgrowth of pulmonary metastases**.** (**a**) Metastatic 4T1 breast cancer cells stably expressing CMV-renilla luciferase in conjunction with either SBE (4T1-SBE)- or p3TP (4T1-p3TP)-driven firefly luciferase were propagated (±) TGF-β in 2D- or 3D-cultures. Data are the fold-induction of luciferase activity stimulated by TGF-β. (**b**) 4T1-SBE cells were engrafted onto the mammary fat pad of Balb/C mice. Primary tumor growth and metastasis was assessed 4 weeks later using dual bioluminescent imaging to visualize these events (CMV-renilla), as well as the extent of TGF-β-specific Smad2/3 signaling (SBE-firefly). Data are the mean luciferase ratios of SBE-firefly:CMV-renilla (±SD; $n = 4$)

signaling to promote the establishment of osteolytic bone lesions by metastatic breast cells. Importantly, bisphosphonate administration was shown to be more effective in suppressing osteolytic bone lesions and canonical TGF-β signaling early in the metastatic process as opposed to after these lesions were fully established [142]. These findings suggest that canonical TGF-β signaling is differentially regulated during specific stages of the metastatic cascade. Accordingly, transient activation of Smad2/3 by TGF-β was sufficient in converting the migration of breast cancer cells from cohesive to single cell programs

[143, 144]. Remarkably, the ability of breast cancer metastases to resume proliferative programs within the pulmonary microenvironment required these cells to first inactivate Smad2/3 signaling [143, 144], and as such, it is tempting to speculate that altered elastic moduli govern the coupling of TGF-β to Smad2/3. In support of this supposition, Fig. 5.3 shows that TGF-β becomes selectively uncoupled from the activation of Smad2/3 in (i) compliant 3D-organotypic cultures relative to rigid tissue culture plastic (Fig. 5.3a), and (ii) pulmonary metastases relative to their site of origin (Fig. 5.3b). Collectively, these intriguing findings demonstrate the plasticity present in the TGF-β signaling system as carcinoma cells undergo EMT and metastatic outgrowth, presumably reflecting a shift from canonical (i.e., Smad2/3-based) to noncanonical (i.e., non-Smad2/3-based) signaling that originates from altered mechanotransduction within the tumor microenvironment [9, 46, 47]. Clearly, extending these novel intravital imaging techniques holds great promise to delineate and define the mechanics of the TGF-β signaling during distinct stages of metastatic progression.

5.6 TGF-β Signaling Within the Circulation

After exiting the confines of the primary tumor, disseminated cancer cells are confronted with the challenges of traversing and surviving the nonadherent microenvironments provided by the lymphatic and circulatory systems. In fact, the lack of ECM support, coupled with shear forces encountered in circulation, make this process of the metastatic cascade one of the harshest and deadliest faced by metastatic cells. This notion is bolstered by "experimental metastasis" studies which demonstrated that 95% of inoculated cancer cells die prior to entering a metastatic niche [145, 146]. At present, a direct role for TGF-β in regulating the behaviors of circulating tumor cells remains largely unexplored. However, TGF-β does promote the generation of "mammospheres," which are nonadherent structures enriched for stem-like progenitor cells [147]. These data suggest that TGF-β and its induction of EMT functions to promote the selection and expansion of cancer initiating cells that are ideally suited for exiting the primary tumor and surviving nonadherent conditions. Anoikis is a specialized form of apoptosis exhibited by adherent cells when they are deprived of their normal cell: ECM interactions [148]. Interestingly, anoikis also plays a critical role during acinar development by promoting "nonadherent" luminal cells to undergo apoptosis during organoid hollowing [149]. As mentioned previously, EMT induced by TGF-β prevents acinar hollowing [47, 140], suggesting that TGF-β confers resistance to anoikis in post-EMT cell populations. Along these lines, myofibroblasts can be protected from anoikis following TGF-β-mediated activation of FAK and AKT [48]. Although additional studies are clearly warranted to solidify the relationship between TGF-β and anoikis, these findings do coalesce to support a model wherein circulating tumor cells activated by TGF-β are afforded a selective survival advantage during their rapid and violent transit to distant metastatic sites.

5.7 TGF-β Signaling Within Metastatic Sites

5.7.1 Pulmonary Microenvironment

As mentioned previously, FAK mediates the interaction between integrins and TGF-β receptors [9, 46], as well as promotes the metastatic outgrowth of breast cancer cells in pulmonary tissues [150]. Interestingly, recent evidence demonstrates that the success or failure of breast cancer cells grow in pulmonary microenvironments can be modeled and recapitulated by the sparse plating of cells in 3D-organotypic cultures [150–152]. Moreover, culturing normal MECs in these 3D-organotypic cultures is sufficient to induce acinar formation and milk production [153], strongly suggesting that these culture systems represent suitable surrogates to model the differentiation and development of normal mammary glands [99], as well as metastatic dormancy in isolated low-grade carcinoma cells [151]. The dichotomous nature of these two examples (i.e., acinar development *versus* metastatic dormancy) raises two important questions for future analyses. First, what are the underlying mechanisms that enable a single microenvironment to elicit such varying responses in MECs? And second, what role does TGF-β play in directing these disparate functions of MECs? Although both questions remain unanswered, a recent study described a metastatic gene signature associated with the ability of breast cancers to metastasize to the lungs [154]. Central players to this response were IDs (Inhibitor of Differentiation) 1 and 3, which promoted breast cancer cells to extravasate and colonize the lung [154]. Along these lines, we found TGF-β to strongly suppress the expression of IDs 1 and 3 in normal MECs, but to dramatically induce their expression in late-stage breast cancer cells [106]. Thus, future studies need to gauge the extent to which dysregulated coupling of TGF-β to ID1/3 expression manifests the "TGF-β Paradox," as well as dictates the response of normal and malignant MECs to microenvironmental signals. TGF-β stimulation of angiopoietin-like 4 expression enabled breast cancer cells to specifically extravasate and colonize the lungs, but not the bone [59], suggesting that TGF-β signaling is critical in determining metastatic tropism. Likewise, activation of Smad2/3 by TGF-β was shown to be critical in promoting the invasive exodus of breast cancer cells out of the primary tumor; however, reinitiation of proliferation programs necessary for metastatic colonization of the lungs required the activity of Smad2/3 to be silenced (Fig. 5.3; [143, 144]). Unfortunately, these analyses only employed a "Smad2/3-centric" approach, and in doing so, failed to address the relative contribution of noncanonical TGF-β signaling inputs to the latter steps of metastasis. Given the established and essential function of noncanonical TGF-β signaling to EMT and metastasis [8, 9], we propose a provocative explanation that the inherent plasticity of the TGF-β signaling system enables metastatic cells to actively induce or repress specific branches of this unique signaling system metastatic progression *via* a microenvironment-dependent manner. Future studies need to address this hypothesis using an intricate combination of 3D-organotypic cultures and *in vivo* metastasis models in mice.

5.7.2 Skeletal Microenvironment

Despite the fact that skeletal metastases pose a significant health concern for many cancers, the development of appropriate 3D-organotypic cultures to model the interactions of carcinoma cells with a bone microenvironment have yet to be fully exploited. However, a recent study did employ bone hydroxyapatite (HA) to mineralize polymeric scaffolds which supported the growth of breast cancer cells [155]. Importantly, mineralized microenvironments rendered breast cancer cells more competent to synthesize pro-osteoclastic interleukin-8, as well as more sensitive to bisphosphonate treatment [155]. Because TGF-β induces interleukin-8 expression [156], and bisphosphonates inhibit TGF-β signaling [142], it is tempting to speculate that mineralized microenvironments support oncogenic TGF-β. Accordingly, inhibiting TGF-β signaling *via* administration of the TβR-I inhibitor, SD-208 [157] or a soluble TβR-II molecule [158] both effectively reduced bone metastases. Moreover, Smad4-deficiency or expression of a dominant-negative TβR-II mutant both abrogated breast cancer metastasis to bone in part *via* diminished the expression of PTHrP, IL-11, and CTGF [10, 159, 160]. Collectively, these findings highlight the importance of TGF-β signaling in mediating bone metastases, and more importantly, the role of bone microenvironments to directly impact the pathophysiology of TGF-β. Indeed, the continued refinement of mineralized microenvironmental cultures clearly holds tremendous promise to elucidate the molecular mechanisms whereby skeletal-derived signals manifest the "TGF-β Paradox."

5.8 Summary

Attempts to solve the "TGF-β Paradox" have yet to be actualized since the discovery of this phenomenon nearly 30 years ago. Indeed, the inability of science and medicine to solve the "TGF-β Paradox" reflects their collective failure to develop a sensitive and rapid cell-based assay that fully recapitulates this phenomenon *in vitro*. Here we discussed recent advances in our understanding of the "TGF-β Paradox" and its role in promoting metastatic progression, and in doing so, highlighted the importance of novel 3D-organotypic culture systems to deconstruct the metastatic cascade to better investigate the molecular connections between tumor microenvironments and the TGF-β signaling system. Collectively, these findings coalesce to support the model in Fig. 5.4 that depicts the potential role of ECM and microenvironmental rigidity to alter the response of carcinoma cells to TGF-β. Indeed, we propose that early-stage carcinomas evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-β. The continued growth of the developing neoplasm enhances ECM rigidity by upregulating TGF-β production, which further enhances the demsoplastic process and its inappropriate formation of receptor complexes comprised of integrins, growth factor receptors, and TGF-β receptors. Once formed, these hyperactivated signaling modules greatly

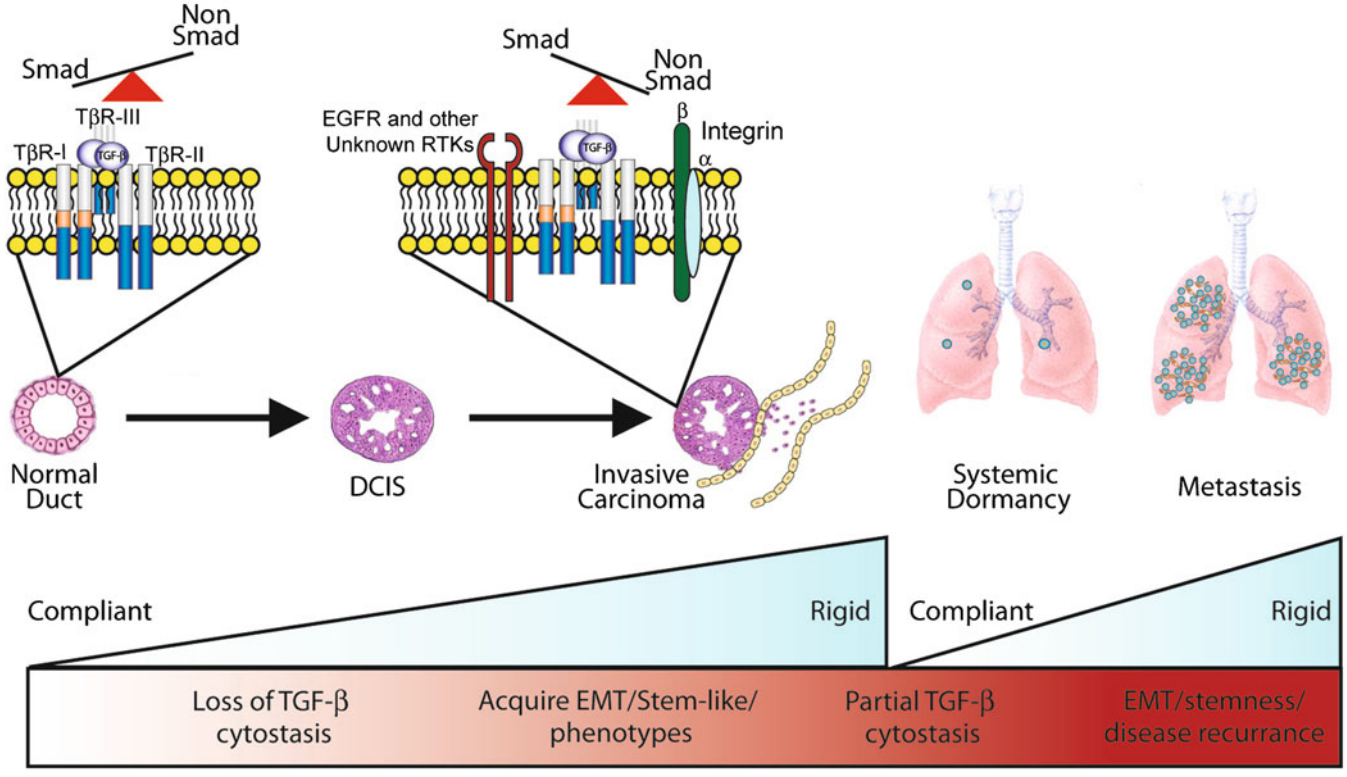


Fig. 5.4 Schematic depicting the role of microenvironmental and ECM rigidity in mediating oncogenic TGF-β signaling. Tumor-initiated carcinomas evolve in compliant microenvironments that favor canonical signaling by TGF-β. Neoplastic growth enhances microenvironmental rigidity, leading to the inappropriate formation of integrin:TβR-II and TGF-β receptor:EGFR receptor complexes and the amplification of noncanonical signaling TGF-β. These unbecoming events culminate in maximal noncanonical TGF-β signaling, leading to the acquisition of EMT, stem-like, and metastatic phenotypes. Deposition of metastatic cells at compliant micrometastatic niches partially restores the cytostatic function of TGF-β, resulting in tumor dormancy. Over time, this microenvironment cycle is repeated, leading to disease recurrence and poor clinical outcomes in patients harboring metastatic disease

amplify the activation of noncanonical effectors by TGF-β, which culminate in the acquisition of EMT, stem-like, and metastatic phenotypes. Arrival of disseminated carcinoma cells to metastatic niches once again places these cells in compliant microenvironments, which partially reinstates the cytostatic activities of TGF-β and initiates micrometastatic dormancy. Over time, this vicious microenvironment cycle is repeated, leading to disease recurrence and poor clinical outcomes in carcinoma patients harboring metastatic disease. The basic tenets of this model clearly are supported by the findings presented herein, and as such, this model should serve as a launching point for future studies aimed at identifying the individual effectors operant in regulating ECM tension and TGF-β function in distinct carcinoma subtypes. The use of advanced 3D-organotypic cultures, together with powerful intravital imaging techniques will undoubtedly delineate the complex interactions whereby tumor microenvironments govern oncogenic TGF-β signaling and its stimulation of metastatic progression. In doing so, we believe that it will one day be possible to manipulate the "TGF-β Paradox" and prevent its pathophysiological manifestations, thereby improving the prognosis and overall survival of patients with metastatic disease.

Acknowledgements We thank members of the Schiemann Laboratory for critical comments and reading of the manuscript. W.P.S. was supported by grants from the National Institutes of Health (CA129359), the Komen Foundation (BCTR0706967), the Department of Defense (BC084651); and the Case Comprehensive Cancer Center and the University Hospitals Seidman Cancer Center. M.K.W. was supported by the American Cancer Society (PF-09-120-01-CS).

References

1. Blobe GC, Schiemann WP, Lodish HF (2000) Role of TGF-β in human disease. N Engl J Med 342:1350–1358
2. Galliher AJ, Neil JR, Schiemann WP (2006) Role of TGF-β in cancer progression. Future Oncol 2:743–763
3. Massague J, Gomis RR (2006) The logic of TGF-β signaling. FEBS Lett 580:2811–2820
4. Siegel PM, Massague J (2003) Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat Rev Cancer 3:807–821
5. Tian M, Schiemann WP (2009) The TGF-β paradox in human cancer: an update. Future Oncol 5:259–271
6. Schiemann WP (2007) Targeted TGF-β chemotherapies: friend or foe in treating human malignancies? Expert Rev Anticancer Ther 7:609–611
7. Rahimi RA, Leof EB (2007) TGF-β signaling: a tale of two responses. J Cell Biochem 102:593–608
8. Taylor MA, Parvani JG, Schiemann WP (2010) The pathophysiology of epithelial-mesenchymal transition induced by TGF-β in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia 15:169–190
9. Wendt MK, Allington TM, Schiemann WP (2009) Mechanisms of the epithelial-mesenchymal transition by TGF-β. Future Oncol 5:1145–1168
10. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549

11. Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, Ponomarev V, Gerald WL, Blasberg R, Massague J (2005) Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J Clin Invest 115:44–55
12. Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, Piek E, Bottinger EP (2001) Genetic programs of epithelial cell plasticity directed by TGF-β. Proc Natl Acad Sci U S A 98:6686–6691
13. Massague J (1998) TGF-β signal transduction. Annu Rev Biochem 67:753–791
14. Chang H, Brown CW, Matzuk MM (2002) Genetic analysis of the mammalian TGF-β superfamily. Endocr Rev 23:787–823
15. Gatza CE, Oh SY, Blobe GC (2010) Roles for the type III TGF-β receptor in human cancer. Cell Signal 22:1163–1174
16. Feng XH, Derynck R (2005) Specificity and versatility in TGF-β signaling through Smads. Annu Rev Cell Dev Biol 21:659–693
17. Moustakas A, Heldin CH (2005) Non-Smad TGF-β signals. J Cell Sci 118:3573–3584
18. Shi Y, Massague J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113:685–700
19. Massague J, Seoane J, Wotton D (2005) Smad transcription factors. Genes Dev 19:2783–2810
20. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGF-β receptor. Cell 95:779–791
21. Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, Hanai JI, Beppu H, Tsukazaki T, Wrana JL, Miyazono K, Sugamura K (2000) Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol 20:9346–9355
22. Hocevar BA, Smine A, Xu XX, Howe PH (2001) The adaptor molecule Disabled-2 links the TGF-β receptors to the Smad pathway. EMBO J 20:2789–2801
23. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MAJ, Wrana JL, Falb D (1997) The MAD-related protein Smad7 associates with the TGF-β receptor and functions as an antagonist of TGF-β signaling. Cell 89: 1165–1173
24. Nakao A, Afrakht M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P (1997) Identification of Smad7, a TGF-β inducible antagonist of TGF-β signalling. Nature 389:631–635
25. Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL, ten Dijke P (1998) Physical and functional interaction of murine and xenopus Smad7 with bone morphogenetic protein receptors and TGF-β receptors. J Biol Chem 273:25364–25370
26. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, Miyazono K (2001) Smurf1 interacts with TGF-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem 276:12477–12480
27. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol Cell 6:1365–1375
28. Itoh S, ten Dijke P (2007) Negative regulation of TGF-β receptor/Smad signal transduction. Curr Opin Cell Biol 19:176–184
29. Kang JS, Liu C, Derynck R (2009) New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol 19:385–394
30. Galliher AJ, Schiemann WP (2006) β3 integrin and Src facilitate TGF-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 8:R42
31. Galliher AJ, Schiemann WP (2007) Src phosphorylates Tyr284 in TGF-β type II receptor and regulates TGF-β stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer Res 67:3752–3758
32. Galliher-Beckley AJ, Schiemann WP (2008) Grb2 binding to Tyr284 in TβR-II is essential for mammary tumor growth and metastasis stimulated by TGF-β. Carcinogenesis 29: 244–251
33. Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE (2008) TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol Cell 31:918–924

34. Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH, Landstrom M (2008) The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol 10:1199–1207
35. Azuma M, Motegi K, Aota K, Yamashita T, Yoshida H, Sato M (1999) TGF-β1 inhibits NF-κB activity through induction of IκB-α expression in human salivary gland cells: a possible mechanism of growth suppression by TGF-β1. Exp Cell Res 250:213–222
36. Arsura M, Panta GR, Bilyeu JD, Cavin LG, Sovak MA, Oliver AA, Factor V, Heuchel R, Mercurio F, Thorgeirsson SS, Sonenshein GE (2003) Transient activation of NF-κB through a TAK1/IKK kinase pathway by TGF-β1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene 22:412–425
37. Park J-I, Lee M-G, Cho K, Park B-J, Chae K-S, Byun D-S, Ryu B-K, Park Y-K, Chi S-G (2003) TGF-β1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF-κB, JNK, and Ras signaling pathways. Oncogene 22:4314–4332
38. Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T (2004) NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest 114:569–581
39. You HJ, How T, Blobe GC (2009) The type III TGF-β receptor negatively regulates NF-κB signaling through its interaction with β-arrestin2. Carcinogenesis 30:1281–1287
40. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP (2008) Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-β through a PGE2-dependent mechanisms. Carcinogenesis 29:2227–2235
41. Neil JR, Schiemann WP (2008) Altered TAB1:IκB kinase interaction promotes TGF-β-mediated NF-κB activation during breast cancer progression. Cancer Res 68:1462–1470
42. Neil JR, Tian M, Schiemann WP (2009) X-linked inhibitor of apoptosis protein and its E3 ligase activity promote TGF-β-mediated NF-κB activation during breast cancer progression. J Biol Chem 284:21209–21217
43. Tian M, Schiemann WP (2010) PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-β signaling during mammary tumorigenesis. FASEB J 24:1105–1116
44. Brandl M, Seidler B, Haller F, Adamski J, Schmid RM, Saur D, Schneider G (2010) IKK(alpha) controls canonical TGF-β-SMAD signaling to regulate genes expressing SNAIL and SLUG during EMT in panc1 cells. J Cell Sci 123:4231–4239
45. Wendt M, Schiemann W (2009) Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-β signaling and metastasis. Breast Cancer Res 11:R68
46. Wendt MK, Smith JA, Schiemann WP (2009) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem 284:34145–34156
47. Wendt MK, Smith JA, Schiemann WP (2010) TGF-β-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene 29: 6485–6498
48. Horowitz JC, Rogers DS, Sharma V, Vittal R, White ES, Cui Z, Thannickal VJ (2007) Combinatorial activation of FAK and AKT by TGF-β1 confers an anoikis-resistant phenotype to myofibroblasts. Cell Signal 19:761–771
49. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE (2003) Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J Biol Chem 278:12384–12389
50. Park SS, Eom YW, Kim EH, Lee JH, Min DS, Kim S, Kim SJ, Choi KS (2004) Involvement of c-Src kinase in the regulation of TGF-β1-induced apoptosis. Oncogene 23:6272–6281
51. Allington TM, Galliher-Beckley AJ, Schiemann WP (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling and tumorigenesis in mammary tumors. FASEB J 23:4231–4243
52. Allington TM, Schiemann WP (2011) The Cain and Abl of epithelial-mesenchymal transition and TGF-β in mammary epithelial cells. Cells Tissues Organs 193:98–113
53. Wang S, Wilkes MC, Leof EB, Hirschberg R (2005) Imatinib mesylate blocks a non-Smad TGF-β pathway and reduces renal fibrogenesis in vivo. FASEB J 19:1–11

54. Wilkes MC, Leof EB (2006) TGF-β activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures. J Biol Chem 281:27846–27854
55. Moustakas A, Heldin CH (2007) Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 98:1512–1520
56. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454
57. Zavadil J, Bottinger EP (2005) TGF-β and epithelial-to-mesenchymal transitions. Oncogene 24:5764–5774
58. Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–1428
59. Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massague J (2008) TGF-β primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133:66–77
60. Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB (1981) New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci U S A 78:5339–5343
61. Moses HL, Branum EL, Proper JA, Robinson RA (1981) Transforming growth factor production by chemically transformed cells. Cancer Res 41:2842–2848
62. Carr BI, Hayashi I, Branum EL, Moses HL (1986) Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type β transforming growth factor. Cancer Res 46:2330–2334
63. Silberstein GB, Daniel CW (1987) Reversible inhibition of mammary gland growth by TGF-β. Science 237:291–293
64. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT (1993) Anti-TGF-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. J Clin Invest 92:2569–2576
65. Bottinger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM (1997) Transgenic mice overexpressing a dominant-negative mutant type II TGF-β receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. Cancer Res 57:5564–5570
66. Yang YA, Dukhanina O, Tang B, Mamura M, Letterio JJ, MacGregor J, Patel SC, Khozin S, Liu ZY, Green J, Anver MR, Merlino G, Wakefield LM (2002) Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects. J Clin Invest 109:1607–1615
67. Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, Easterly E, Roebuck LR, Ryan S, Gotwals PJ, Koteliansky V, Arteaga CL (2002) Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest 109:1551–1559
68. Tian F, Byfield SD, Parks WT, Stuelten CH, Nemani D, Zhang YE, Roberts AB (2004) Smad-binding defective mutant of TGF-β type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 64:4523–4530
69. Tian F, DaCosta BS, Parks WT, Yoo S, Felici A, Tang B, Piek E, Wakefield LM, Roberts AB (2003) Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 63:8284–8292
70. Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM (2003) TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest 112:1116–1124
71. Anzano MA, Roberts AB, De Larco JE, Wakefield LM, Assoian RK, Roche NS, Smith JM, Lazarus JE, Sporn MB (1985) Increased secretion of type β transforming growth factor accompanies viral transformation of cells. Mol Cell Biol 5:242–247
72. Muraoka-Cook RS, Kurokawa H, Koh Y, Forbes JT, Roebuck LR, Barcellos-Hoff MH, Moody SE, Chodosh LA, Arteaga CL (2004) Conditional overexpression of active TGF-β1 in vivo accelerates metastases of transgenic mammary tumors. Cancer Res 64:9002–9011

73. Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ (1996) TGF-β1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell 86:531–542
74. Fowlis DJ, Cui W, Johnson SA, Balmain A, Akhurst RJ (1996) Altered epidermal cell growth control in vivo by inducible expression of TGF-β1 in the skin of transgenic mice. Cell Growth Differ 7:679–687
75. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D et al (1992) Targeted disruption of the mouse TGF-β1 gene results in multifocal inflammatory disease. Nature 359:693–699
76. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S (1993) TGF-β1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A 90:770–774
77. Christ M, McCartney-Francis NL, Kulkarni AB, Ward JM, Mizel DE, Mackall CL, Gress RE, Hines KL, Tian H, Karlsson S et al (1994) Immune dysregulation in TGF-β1-deficient mice. J Immunol 153:1936–1946
78. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T (1997) TGF-β2 knockout mice have multiple developmental defects that are non-overlapping with other TGF-β knockout phenotypes. Development 124:2659–2670
79. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J (1995) Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 11:415–421
80. Oshima M, Oshima H, Taketo MM (1996) TGF-β receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol 179:297–302
81. Larsson J, Goumans MJ, Sjostrand LJ, van Rooijen MA, Ward D, Leveen P, Xu X, ten Dijke P, Mummery CL, Karlsson S (2001) Abnormal angiogenesis but intact hematopoietic potential in TGF-β type I receptor-deficient mice. EMBO J 20:1663–1673
82. Kuruvilla AP, Shah R, Hochwald GM, Liggitt HD, Palladino MA, Thorbecke GJ (1991) Protective effect of TGF-β1 on experimental autoimmune diseases in mice. Proc Natl Acad Sci U S A 88:2918–2921
83. Goey H, Keller JR, Back T, Longo DL, Ruscetti FW, Wiltrout RH (1989) Inhibition of early murine hemopoietic progenitor cell proliferation after in vivo locoregional administration of TGF-β1. J Immunol 143:877–880
84. Bartlett WC, Purchio A, Fell HP, Noelle RJ (1991) Cognate interactions between helper T cells and B cells. VI. TGF-β inhibits B cell activation and antigen-specific, physical interactions between Th and B cells. Lymphokine Cytokine Res 10:177–183
85. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS (1986) Production of TGF-β by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 163:1037–1050
86. Langlois D, Hneino M, Bouazza L, Parlakian A, Sasaki T, Bricca G, Li JY (2010) Conditional inactivation of TGF-β type II receptor in smooth muscle cells and epicardium causes lethal aortic and cardiac defects. Transgenic Res 19:1069–1082
87. Seo HS, Serra R (2009) Tgfbr2 is required for development of the skull vault. Dev Biol 334:481–490
88. Ito Y, Yeo JY, Chytil A, Han J, Bringas P Jr, Nakajima A, Shuler CF, Moses HL, Chai Y (2003) Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. Development 130:5269–5280
89. Bierie B, Gorska AE, Stover DG, Moses HL (2009) TGF-β promotes cell death and suppresses lactation during the second stage of mammary involution. J Cell Physiol 219:57–68
90. Martinez-Ferrer M, Afshar-Sherif AR, Uwamariya C, de Crombrugghe B, Davidson JM, Bhowmick NA (2010) Dermal TGF-β responsiveness mediates wound contraction and epithelial closure. Am J Pathol 176:98–107
91. Bhowmick NA, Neilson EG, Moses HL (2004) Stromal fibroblasts in cancer initiation and progression. Nature 432:332–337

92. Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, Arteaga CL, Neilson EG, Hayward SW, Moses HL (2005) Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks. Oncogene 24:5053–5068
93. Bhowmick NA, Moses HL (2005) Tumor-stroma interactions. Curr Opin Genet Dev 15:97–101
94. Bierie B, Moses HL (2006) Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer 6:506–520
95. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, Moses HL (2008) Abrogation of TGF-β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell 13:23–35
96. Kim BG, Li C, Qiao W, Mamura M, Kasprzak B, Anver M, Wolfraim L, Hong S, Mushinski E, Potter M, Kim SJ, Fu XY, Deng C, Letterio JJ (2006) Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature 441:1015–1019
97. Bian Y, Terse A, Du J, Hall B, Molinolo A, Zhang P, Chen W, Flanders KC, Gutkind JS, Wakefield LM, Kulkarni AB (2009) Progressive tumor formation in mice with conditional deletion of TGF-β signaling in head and neck epithelia is associated with activation of the PI3K/Akt pathway. Cancer Res 69:5918–5926
98. Zeng Q, Phukan S, Xu Y, Sadim M, Rosman DS, Pennison M, Liao J, Yang GY, Huang CC, Valle L, Di Cristofano A, de la Chapelle A, Pasche B (2009) Tgfbr1 haploinsufficiency is a potent modifier of colorectal cancer development. Cancer Res 69:678–686
99. Weaver VM, Fischer AH, Peterson OW, Bissell MJ (1996) The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. Biochem Cell Biol 74:833–851
100. Benton G, George J, Kleinman HK, Arnaoutova IP (2009) Advancing science and technology via 3D culture on basement membrane matrix. J Cell Physiol 221:18–25
101. Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, Lorenz K, Lee EH, Barcellos-Hoff MH, Petersen OW, Gray JW, Bissell MJ (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 1:84–96
102. Butcher DT, Alliston T, Weaver VM (2009) A tense situation: forcing tumour progression. Nat Rev Cancer 9:108–122
103. Erler JT, Weaver VM (2009) Three-dimensional context regulation of metastasis. Clin Exp Metastasis 26:35–49
104. Paszek MJ, Weaver VM (2004) The tension mounts: mechanics meets morphogenesis and malignancy. J Mammary Gland Biol Neoplasia 9:325–342
105. Turley EA, Veiseh M, Radisky DC, Bissell MJ (2008) Mechanisms of disease: epithelial-mesenchymal transition–does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol 5:280–290
106. Tian M, Schiemann WP (2009) Preclinical efficacy of cystatin C to target the oncogenic activity of TGF-β in breast cancer. Transl Oncol 2:174–183
107. Bissell MJ (2007) Modelling molecular mechanisms of breast cancer and invasion: lessons from the normal gland. Biochem Soc Trans 35:18–22
108. Mintz B, Illmensee K (1975) Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci U S A 72:3585–3589
109. Hochedlinger K, Blelloch R, Brennan C, Yamada Y, Kim M, Chin L, Jaenisch R (2004) Reprogramming of a melanoma genome by nuclear transplantation. Genes Dev 18:1875–1885
110. Bissell MJ, Labarge MA (2005) Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? Cancer Cell 7:17–23
111. Dolberg DS, Hollingsworth R, Hertle M, Bissell MJ (1985) Wounding and its role in RSV-mediated tumor formation. Science 230:676–678

112. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM (2005) Tensional homeostasis and the malignant phenotype. Cancer Cell 8:241–254
113. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM (2009) Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139:891–906
114. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT, Giaccia AJ (2009) Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15:35–44
115. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 440:1222–1226
116. Erler JT, Giaccia AJ (2006) Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res 66:10238–10241
117. Sion AM, Figg WD (2006) Lysyl oxidase (LOX) and hypoxia-induced metastases. Cancer Biol Ther 5:909–911
118. Payne SL, Hendrix MJ, Kirschmann DA (2007) Paradoxical roles for lysyl oxidases in cancer–a prospect. J Cell Biochem 101:1338–1354
119. Le QT, Harris J, Magliocco AM, Kong CS, Diaz R, Shin B, Cao H, Trotti A, Erler JT, Chung CH, Dicker A, Pajak TF, Giaccia AJ, Ang KK (2009) Validation of lysyl oxidase as a prognostic marker for metastasis and survival in head and neck squamous cell carcinoma: Radiation Therapy Oncology Group trial 90–03. J Clin Oncol 27:4281–4286
120. Taylor MA, Amin J, Kirschmann DA and Schiemann WP (2011). Lysyl oxidase and hydrogen peroxide promote oncogenic signaling by transforming growth factor-b in mammary epithelial cells. *(submitted)*
121. Patsialou A, Wyckoff J, Wang Y, Goswami S, Stanley ER, Condeelis JS (2009) Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. Cancer Res 69:9498–9506
122. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J (2004) A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64:7022–7029
123. Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, Condeelis JS (2007) Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. Cancer Res 67:3505–3511
124. Roussos ET, Keckesova Z, Haley JD, Epstein DM, Weinberg RA, Condeelis JS (2010) AACR Special Conference on epithelial-mesenchymal transition and cancer progression and treatment. Cancer Res 70:7360–7364
125. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL (2001) TGF-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 12:27–36
126. Kim W, Seok Kang Y, Soo Kim J, Shin NY, Hanks SK, Song WK (2008) The integrin-coupled signaling adaptor p130Cas suppresses Smad3 function in TGF-β signaling. Mol Biol Cell 19:2135–2146
127. Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, Sapino A, Arisio R, Cavallo F, Forni G, Glukhova M, Silengo L, Altruda F, Turco E, Tarone G, Defilippi P (2006) p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-Neu oncogene-dependent breast tumorigenesis. Cancer Res 66:4672–4680
128. Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP (2008) Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-β in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis 29:2243–2251
129. Tilghman RW, Cowan CR, Mih JD, Koryakina Y, Gioeli D, Slack-Davis JK, Blackman BR, Tschumperlin DJ, Parsons JT (2010) Matrix rigidity regulates cancer cell growth and cellular phenotype. PLoS One 5:e12905

130. Chao YL, Shepard CR, Wells A (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. Mol Cancer 9:179
131. Wipff PJ, Rifkin DB, Meister JJ, Hinz B (2007) Myofibroblast contraction activates latent TGF-β1 from the extracellular matrix. J Cell Biol 179:1311–1323
132. Folkman J (1985). Angiogenesis and its inhibitors. *Important Adv Oncol*, 42–62
133. Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186
134. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15:220–231
135. Lester RD, Jo M, Montel V, Takimoto S, Gonias SL (2007) uPAR induces epithelial mesenchymal transition in hypoxic breast cancer cells. J Cell Biol 178:425–436
136. Dunn LK, Mohammad KS, Fournier PG, McKenna CR, Davis HW, Niewolna M, Peng XH, Chirgwin JM, Guise TA (2009) Hypoxia and TGF-β drive breast cancer bone metastases through parallel signaling pathways in tumor cells and the bone microenvironment. PLoS One 4:e6896
137. Heikkinen PT, Nummela M, Jokilehto T, Grenman R, Kahari VM, Jaakkola PM (2010) Hypoxic conversion of SMAD7 function from an inhibitor into a promoter of cell invasion. Cancer Res 70:5984–5993
138. Xu X, Han J, Ito Y, Bringas P Jr, Deng C, Chai Y (2008) Ectodermal Smad4 and p38 MAPK are functionally redundant in mediating TGF-β/BMP signaling during tooth and palate development. Dev Cell 15:322–329
139. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM (2009) CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell 16:91–102
140. Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, Roncari L, Narimatsu M, Bose R, Moffat J, Wong JW, Kerbel RS, O'Malley FP, Andrulis IL, Wrana JL (2009) A role for the TGF-β-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci U S A 106:14028–14033
141. Sodunke TR, Turner KK, Caldwell SA, McBride KW, Reginato MJ, Noh HM (2007) Micropatterns of Matrigel for three-dimensional epithelial cultures. Biomaterials 28: 4006–4016
142. Korpal M, Yan J, Lu X, Xu S, Lerit DA, Kang Y (2009) Imaging TGF-β signaling dynamics and therapeutic response in breast cancer bone metastasis. Nat Med 15:960–966
143. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E (2009) Localized and reversible TGF-β signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol 11:1287–1296
144. Giampieri S, Pinner S, Sahai E (2010) Intravital imaging illuminates TGF-β signaling switches during metastasis. Cancer Res 70:3435–3439
145. Fidler IJ (1970) Metastasis: guantitative analysis of distribution and fate of tumor embolilabeled with 125 I-5-iodo-2′-deoxyuridine. J Natl Cancer Inst 45:773–782
146. Wendt MK, Cooper AN, Dwinell MB (2008) Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. Oncogene 27:1461–1471
147. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704–715
148. Nagaprashantha LD, Vatsyayan R, Lelsani PC, Awasthi S, Singhal SS (2011) The sensors and regulators of cell-matrix surveillance in anoikis resistance of tumors. Int J Cancer 128: 743–752
149. Debnath J (2008) Detachment-induced autophagy during anoikis and lumen formation in epithelial acini. Autophagy 4:351–353
150. Shibue T, Weinberg RA (2009) Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. Proc Natl Acad Sci U S A 106:10290–10295

151. Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, Liu ZY, Costes SV, Cho EH, Lockett S, Khanna C, Chambers AF, Green JE (2008) Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68:6241–6250
152. Barkan D, El Touny LH, Michalowski AM, Smith JA, Chu I, Davis AS, Webster JD, Hoover S, Simpson RM, Gauldie J, Green JE (2010) Metastatic growth from dormant cells induced by a Col-I-enriched fibrotic environment. Cancer Res 70:5706–5716
153. Weigelt B, Bissell MJ (2008) Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. Semin Cancer Biol 18:311–321
154. Gupta GP, Perk J, Acharyya S, de Candia P, Mittal V, Todorova-Manova K, Gerald WL, Brogi E, Benezra R, Massague J (2007) ID genes mediate tumor reinitiation during breast cancer lung metastasis. Proc Natl Acad Sci U S A 104:19506–19511
155. Pathi SP, Kowalczewski C, Tadipatri R, Fischbach C (2010) A novel 3-D mineralized tumor model to study breast cancer bone metastasis. PLoS One 5:e8849
156. Fong YC, Maa MC, Tsai FJ, Chen WC, Lin JG, Jeng LB, Yang RS, Fu WM, Tang CH (2008) Osteoblast-derived TGF-β stimulates IL-8 release through AP-1 and NF-κB in human cancer cells. J Bone Miner Res 23:961–970
157. Mohammad KS, Javelaud D, Fournier PG, Niewolna M, McKenna CR, Peng XH, Duong V, Dunn LK, Mauviel A, Guise TA (2011) The TGF-β receptor I kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. Cancer Res 71(1):175–184
158. Hu Z, Zhang Z, Guise T, Seth P (2010) Systemic delivery of an oncolytic adenovirus expressing soluble TGF-β receptor II-Fc fusion protein can inhibit breast cancer bone metastasis in a mouse model. Hum Gene Ther 21:1623–1629
159. Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR, Manova-Todorova K, Blasberg R, Gerald WL, Massague J (2005) Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc Natl Acad Sci U S A 102:13909–13914
160. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA (1999) TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest 103:197–206