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The Role of c-Myc and miRNAs on EMT and the TGF-beta switch in Primary Intermediate Basal Cells
Isolated From Prostate Cancer

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14. ABSTRACT We aimed to further understand the role of c-myc and the microRNA let-7 in TGF-beta induced EMT and invasion of primary prostate cancer cells. Overexpression of c-myc in combination with TGF-beta failed to induce EMT in our cell lines. Thus, we have completed mechanistic studies with cells stably transfected with ras effector mutant constructs and constitutively activated MEK1 and MEK2 to show that ras activation of the raf-MEK2 pathway works in concert with TGF-beta to promote EMT and invasion in early stage prostate cancer cells. EMT was characterized by the adoption of a spindle shaped morphology, loss of cell-cell junctions, decreased E-cadherin and increased expression of the prometastatic genes vimentin, fibronectin, fsp-1, HMGA2 MMP-2, MMP-9, Slug and Twist2. We also discovered that EGF signaling down regulates expression of the tumor suppressor let-7a miRNA and upregulates its negative regulator, Lin28B. Activation of this pathway appeared to allow for expression of Let-7a targets which play a key role in EMT, including ras and HMGA2. Additionally, overexpression of pre-let-7a, inhibited the ability of cells to undergo EMT and become invasive. We conclude that cells require both TGF-beta stimulation and let-7a down regulation via ras-raf-MEK2 to undergo EMT and transform to an invasive phenotype. Our studies thus far have provided novel insights into the mechanisms regulating EMT and invasion in prostate cancer and assign a novel function to the tumor suppressor microRNA let-7a.					
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INTRODUCTION:

Despite recent advances in prostate cancer diagnostics and treatment, the long term survival rate for men diagnosed with prostate cancer has not changed significantly. Understanding the steps involved in progression of prostate cancer to invasive disease are critical for the development of better diagnostics to identify tumors with high metastatic potential and therapeutics which can potentially block metastases from forming. As such, this project attempts to delineate essential and novel mechanisms involved in prostate cancer invasion. In normal epithelia the extracellular cytokine TGF-beta acts as a tumor suppressor by repressing mitogenic signals. In advanced carcinomas, however, the cytostatic response (growth arrest) to TGF-beta is often lost and TGF-beta acts to induce epithelial to mesenchymal (EMT) transition, promoting tumor invasion and metastasis. Targeting the TGF-beta signaling pathway to prevent invasion, however, has serious concerns. Notably, doing so might inhibit TGF-beta's growth suppressive functions and thus promote additional tumor development. Therefore, novel pathways regulating the invasive functions of TGF-beta stimulation must be identified.

We initially aimed to further understand the role of c-myc and the role of the expression of the microRNA let-7 play in TGF-beta induced EMT and invasion. To answer these questions we set to perform a series of mechanistic studies in a set of primary prostate cancer cells isolated from patients with increasing Gleason score. The purpose of the proposed work was to assign novel functions to the oncogene c-myc and the tumor suppressor miRNA let-7, showing for the first time that these genes serves as a master regulators of the invasive activities of the TGF-beta signaling network. While our initial results did indicate that c-myc expression was required for TGF-beta induced EMT, we also observed that c-myc over-expression was not sufficient to induce EMT in response to TGF-beta treatment. As part of our original Aim 1 we proposed that myc over-expression might not be sufficient to induce EMT in early stage prostate cancer cells treated with TGF-beta alone and suggested that we attempt to understand the role of ras signaling, as myc is known to be activated downstream of ras (Compere et al., 1989). We therefore amended our statement of work to investigate further the role of ras signaling in regulating cellular responses in prostate cancer to TGF-beta. We found that ras signaling through MAPK reduces Let-7a expression and drives Erk2 into the nucleolus which is sufficient for TGF-beta induced EMT. Furthermore, we found that this pathway does not regulate resistance to TGF-beta's growth suppressive functions indicating that this pathway may represent an attractive therapeutic target for inhibiting EMT and prostate cancer invasion and metastasis. Moreover our results indicated that expression of HMGA2, a Let-7a target gene may represent a novel diagnostic to identify prostate cancers with a higher metastatic potential.

BODY:

We successfully accomplished all research and writing related activities regarding TASK 1 in our statement of work and have published a manuscript with the data (Amatangelo et al., 2012).

1a) IBC-10a and -20a cells were transduced with pBABE:c-myc, pBABE:empty pLKO;shRNA-c-myc and pLKO:scrambled vector constructs. PC3-ML cells were transduced with pBABE:empty pLKO;shRNA-c-myc and pLKO:scrambled vector constructs.

1b) IBC-10a and -20a cells were also stably transduced with pBABE:Ras-V12, pBABE:Ras-V12C40, pBABE:RasV12G37, pBABE:RasV12S35, pBABE:MEK1-DD and pBABE:MEK2-DD vector constructs

1c) We determined that EGF and TGF β 1 treatment together was required for EMT of parental cell lines and that TGF β 1 treatment alone induced EMT only in cell variants transduced with active Ras, RasV12S25 and MEK2-DD.

1d) We determined that 1 ng/ml of EGF was sufficient to induce EMT in response to 10 ng/ml of TGF β 1, and that cells began to undergo EMT after 6 days of treatment, however, effects were maximal by treating cells with 10 ng/ml of both TGF- β 1 and EGF over a period of nine days.

1e) We determined that there was no difference between cell lines in receptor expression. We could not observe expression of TGF- β RIII.

1f) We determined that in all conditions in which EMT was induced as determined by induction of vimentin expression that cells exhibited a more invasive phenotype both in a modified boyden chamber assay and in a 3D culture model

1g) We stably transduced PC3-ML cells with a c-myc shRNA construct and scrambled construct and found that c-myc expression was required for expression of EMT genes that PC3-ML cells constitutively express and that knockdown of c-myc correlated with a less invasive phenotype in these cells. We also found that these cells over-express ras compared to our primary cell lines, however, they do not have elevated expression of erk activity as a result of this over-expression.

1h) We found that despite the fact that ras activity could induce EMT and an invasive phenotype in our cells, levels of erk activation, which are regulated by ras, did not correlate with conditions where EMT was induced. We transduced IBC-10a and -20a cells with pLNCX:Erk2 WT, pBABE:shErk2, and pLNCX:Erk2 D319N and determined that erk2 expression was required for TGF β 1 induced EMT and that expression of an Erk2 mutant that preferentially locates to the nucleus was sufficient for TGF-beta induced EMT.

1i) This data was prepared into a manuscript submitted to Carcinogenesis, we responded to all reviewer comments it was accepted for publication in Carcinogenesis in June of 2012.

In regards to TASK2 of our statement of work we were able to successfully complete 5 out of the 9 tasks in the SOW. We wrote and submitted a manuscript on the data obtained, however, due to technical difficulties and time constraints from my program as a result of my graduation we were unable to respond to reviewer comments and have the manuscript published. Please see attached submitted manuscript in the appendix for data regarding this task.

2a) We attempted to knockdown HMGA2 in IBC-10a cells, however, our shRNA construct was ineffective in reducing HMGA2 levels in response to combined treatment with EGF and TGF β 1. Due to time constraints we were unable to test other constructs or obtain a pBABE:HMGA2 cDNA construct.

2b) Because we could not successfully knockdown or over-express HMGA2 as outlined in 2a we could not adequately determine the effects of EGF and TGF β 1 on these cell variants.

2c) Because we could not successfully knockdown or over-express HMGA2 as outlined in 2a we could not adequately determine growth and invasive characteristics of these cell variants.

2d) We were able to carry out HMGA2 immunolabeling on prostate cancer tissue arrays and observed that HMGA2 appears to be expressed in more metastatic and invasive carcinomas.

2e) We were able to measure the levels of let-7a expression and lin-28b in our cells and found that in conditions where EMT was induced we observed an increase in lin-28b expression and decrease in let-7a expression.

2f) We were able to stably transduce IBC-10a and PCa-20a cells with a let-7a over-expression construct and an shRNA to lin-28b and observed that over-expression of let-7a inhibited EMT induction in our cells, however, knockdown of lin-28b was insufficient to inhibit EMT.

2g) We were able to observed that over-expression of let-7a and knockdown of lin-28b both inhibited the ability of cells to become invasive in response to EGF and TGF β 1 treatment.

2h) We also successfully transduced PC3-ML cells with a let-7a over-expression construct and lin-28b shRNA and found that transduction with these constructs resulted in a decrease in expression of EMT genes and invasive ability of cells.

2i) We prepared a manuscript with the data related to TASK2 and submitted it to the Prostate journal in April of 2012. Given time constraints as a result of my graduation we were unable to respond to reviewers comments and perform additional experiments.

As part of training related activities I was able to successfully attend and contribute to all weekly seminar and journal club meetings. I also sat on the committee that invited and hosted outside speakers to come to Drexel University. These included Dr. Harold Moses from Vanderbilt, an expert on TGF-beta signaling, Dr. Joshua Mendell from John Hopkins, an expert on the role of lin-28 and let-7 in cancer, and Dr. Bruce Zetter, an expert on prostate cancer. Furthermore, I successfully organized all thesis committee meetings as outlined in the statement of work, and presented my formal thesis proposal earlier than expected at the beginning of 2012. My committee felt I had done sufficient work to begin writing my thesis and I successfully wrote and defended my thesis on April 20th of 2012 and subsequently graduated from the program in May of that year.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterized PCa-20a and PCa-30a cell lines in terms of ability of TGF-beta to induce EMT and express pro-metastatic genes.
- Determined that neither expression with constitutively active Ras nor treatment with EGF is sufficient to abrogate TGF-beta's negative effect on cell proliferation
- Created IBC-10a and PCa-20a cells that over-express c-myc and determined c-myc over-expression is not sufficient for TGF-beta to induce EMT.
- Created IBC-10a and PCa-20a and PC3-ML cells that have knockdown of c-myc and determined c-myc over-expression is required for TGF-beta to induce EMT.
- Also observed c-myc expression is required for the increased invasive capacity observed during EMT.
- Created IBC-10a and PCa-20a cell lines that expressed constitutively active Ras and three Ras effector mutants (S35, G37 and C40) and determined that Ras activation of Raf is sufficient for TGF-beta to induce EMT in IBC-10a and PCa-20a cell lines.
- Created IBC-10a and PCa-20a cell lines that over-express constitutively active MEK1 and MEK2, downstream effectors of Ras activation of Raf, and determined that MEK1, but not MEK2, activation was sufficient for TGF- β 1 induction of EMT and invasion.
- Created IBC-10a cell lines and PCa-20a cell lines that express Erk2 WT, shRNA targeting Erk2, and an Erk2 D319N mutant that preferentially locates to the nucleus and determined that erk2 expression was required for TGF β 1 induced EMT and also that MEK1-induced Erk2 nuclear localization was required for TGF-beta induced EMT and invasion in prostate cancer.
- We determined that HMGA2 expression is induced in malignant and metastatic human prostate cancers when compared to normal and benign tissue.
- We determined that IBC-10a cells do not express LIN28A but strongly express Lin28B with EGF and EGF and TGF-beta in combination treatment.
- Determined by time course that increases in vimentin expression positively correlate with Lin28B expression and negatively with let-7a expression
- Created Lin-28B knockdown cells and determined that Lin-28B knock-down inhibits EMT and the invasive capacity of cells
- Created IBC-10a and PCa-20a and PC3-ML cells that over-express let-7a and show that let-7a is down-regulation is required for TGF-beta to induce EMT.
- Determined that over-expression of let-7a also inhibits invasive capacity of cells.
- Determined that let-7a expression is down-regulated in malignant and invasive prostate cancer when compared to normal tissue.

REPORTABLE OUTCOMES:

1. Research presented at 2010 and 2011 Discovery Day at Drexel University College of Medicine. 2012 presentation won second place prize for best presentation
2. Presented research poster at 2011 IMPaCT Conference in Orlando, FL.
3. Received Office of Research Award for Biomedical Graduate Student based on research conducted for this grant at Drexel University Research Day, 2011.
4. Created cell lines isolated from Gleason score 6 and 7 prostate cancer patients that over-express c-myc, have c-myc knockdown, express constitutively active Ras, express three Ras effector mutants, express both constitutively active MEK1 and MEK2, have Erk2 knock down, express Erk D319N mutant, over-express pre-let7a miRNA and have Lin28B knockdown.
5. Published Data as part of Task1 in the SOW in *Carcinogenesis*. Submitted data as part of Task 2 in SOW to *Prostate*.
6. Successfully wrote and defended thesis on April 20th, 2012 and graduated in May with PhD.
7. Obtained a Post-Doctoral position studying cancer epigenetics at the Wistar Institute in Philadelphia in October of 2012.

CONCLUSIONS:

We made significant progress in the research proposed by this grant. We were able to successfully complete 80% of the research related tasks in our SOW and 100% of my training related tasks. As a result of this progress I was able to finish my degree earlier than expected and successfully defended my thesis April 20th of 2012. Consequently, funding for this grant was ended early in July of 2012 because of my graduation from the program.

As part of aim 1 we were able to transfect cells with an shRNA targeting c-myc and found that c-myc expression was critical for our cells to undergo EMT and exhibit an invasive phenotype. We also transduced cells with a c-myc over-expression construct, however, were unable to induce EMT with TGF-beta alone as hypothesized in aim 1. We therefore set out to understand the role of ras signaling as proposed as an alternative set of experiments in aim 1 and which is the basis for a manuscript we were able to publish in *Carcinogenesis*. We created cells that over-express constitutively active ras, 3 ras effector mutants and both a constitutively active MEK1 and MEK2 construct. With these cells we were able to elucidate a novel mechanism by which EGF promotion of MEK1, but not MEK2, activity can regulate TGF-beta's ability to induce invasion and EMT in early stage prostate cancer cells. This appeared to result from the ability of MEK1, but not MEK2, to promote nuclear accumulation of the transcription factor Erk2. As part of aim 1 we also set out to understand whether EGF stimulation or Ras activity regulated TGF-beta's ability to slow proliferation and found that neither EGF or Ras abrogated the anti-proliferative effects of TGF-beta. TGF-beta also had an anti-proliferative effect on highly metastatic PC3-ML cells and increased expression of p21 in all cell lines. We concluded from this data that signaling pathways that promote TGF-beta's ability to induce invasion and metastasis likely do not coordinately regulate TGF-beta's anti-proliferative effects.

In regards to Aim 3, because of this data above we felt that further examination of Growth characteristics and growth genes were less important than understating the role that let-7 expression might have on regulating TGF-beta responses that we had observed in our preliminary data. We therefore cloned a pre-let7 sequence into a pBABE vector and induce over-expression of let-7 in our cells and found that let-7 inhibited TGF-beta's ability to induce EMT and invasion in our early stage prostate cancer cells lines as well as in highly metastatic PC3-ML cells. Since then we have also been able to observe a correlation with increased Lin28B expression and decreases in let-7 expression in our model and have seen that knockdown of lin28B in inhibits E + T induce invasion and EMT in our cells as outlined in Aim 3. Additionally, as part of Aim 2 we have observed increases in HMGA2 expression in malignant and metastatic prostate cancer when compared to benign and normal prostate tissue and we were able to observe a coordinate decrease of let-7a expression in metastatic prostate cancer vs normal prostate. These results were submitted to the journal *Prostate*, but were not published because of time constraints governed by my graduation.

Thus, our studies have provided significant insights into the specific mechanisms regulating TGF-beta's ability to induce EMT and invasion in prostate cancer and assigns novel functions to c-myc and the tumor suppressor microRNA let-7a. Specifically, we have been able to show that c-myc expression is required for EMT and that EGF and/or Raf activation induced down-regulation of microRNA let-7a is required for TGF-beta to induce EMT. Furthermore, that Ras induced Erk2 nuclear localization is sufficient to induce EMT and an invasive phenotype in primary prostate cancer cells. Taken together, this implies that targeting the EGF-Ras-Raf-MAPK-Lin28B-Let-7a pathway could inhibit invasion and metastasis and might restore the tumor suppressive effects of TGF-beta. Moreover, our data suggests that HMGA2 expression might serve as a diagnostic marker for the metastatic potential of prostate cancer.

REFERENCES:

- Amatangelo, M.D., Goodyear, S., Varma, D., and Stearns, M.E. (2012). c-Myc expression and MEK1-induced Erk2 nuclear localization are required for TGF-beta induced epithelial-mesenchymal transition and invasion in prostate cancer. *Carcinogenesis* 33, 1965-1975.
- Compere, S.J., Baldacci, P., Sharpe, A.H., Thompson, T., Land, H., and Jaenisch, R. (1989). The ras and myc oncogenes cooperate in tumor induction in many tissues when introduced into midgestation mouse embryos by retroviral vectors. *Proc Natl Acad Sci U S A* 86, 2224-2228.



c-myc expression and MEK1 induced Erk2 nuclear localization are required for TGF-beta induced epithelial-mesenchymal transition and invasion in prostate cancer

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3 **beta induced epithelial-mesenchymal transition and invasion in prostate cancer.**
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8 **Running Title:** EMT requires MEK1 activation of Erk2 and c-myc
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EMT requires MEK1 activation of Erk2 and c-myc

Abstract

Understanding the initial mechanisms by which epithelial cells transform to an invasive phenotype is critical to the development of diagnostics which can identify the metastatic potential of cancers as well as therapeutic agents which can prevent metastases. Changes in cellular response to the TGF-beta cytokine are known to promote epithelial cell invasion and metastasis in part through induction of epithelial to mesenchymal transitions (EMT). In this report we demonstrate that non-metastatic human prostate cancer cell lines of increasing Gleason Score can be induced to undergo EMT when treated with TGF-beta in combination with EGF. Mechanistic studies revealed that in cells stably transfected with activated Ras, TGF-beta alone induced EMT and that a Ras-Raf-MEK1, but not MEK2, signaling cascade is necessary and sufficient for Erk2 nuclear localization which works in concert with TGF-beta to promote EMT. Furthermore, we show for the first time that expression of the transcription factor c-myc, which is phosphorylated by Erk2, is required for EMT. Characteristically, EMT involved adoption of a spindle-shaped morphology, loss of E-cadherin and increased expression of Vimentin, Fibronectin and Fibroblast Specific Protein-1 (S100A4). Prostate cells undergoing EMT became invasive and expressed several genes associated with metastasis including MT-MMP1, MMP-2/9, the MMP-9 homodimer, Slug and Twist2. In sum, we demonstrate a novel mechanism by which non-invasive primary prostate tumor cells transition to an invasive phenotype characteristic of malignant tumor cells in response to TGF-beta signaling.

Introduction

Epithelial-mesenchymal transition (EMT) is mostly described as part of germ layer reorganization and tissue remodeling during embryonic development. However, it has become increasingly clear that a reactivation of the EMT developmental program primes malignant epithelial cells for the dissemination, and invasion required for metastatic spread of solid tumors, the foremost cause of mortality in prostate cancer patients(1). During EMT, tumor cells lose cell-cell contacts and the cobblestone networks characteristic of epithelial tissues and adopt a spindle shaped morphology and migratory phenotype typical of fibroblasts(2). Additionally, E-cadherin and β -catenin expression at cell-cell junctions is lost as cells express mesenchymal-

1 EMT requires MEK1 activation of Erk2 and c-myc
2 associated genes such as Vimentin, Fibronectin and Fibroblast Specific Protein-1 (FSP-1; also known as
3 S100A4)(3). Importantly, these changes in gene expression are correlated with an increasingly invasive and
4 aggressive tumor cell phenotype that is associated with a poorer patient prognosis(4-6). Silencing of Vimentin
5 or re-expression of E-cadherin in invasive cells also decreases their invasive phenotype; emphasizing that these
6 genes play a major role in controlling the metastatic behavior of tumor cells(7-9). Therefore, understanding the
7 initial molecular mechanisms regulating the EMT phenotype in prostate cancer will aid in identification of new
8 tumor biomarkers or therapeutics to target cells with a higher metastatic potential. Currently little is known on
9 what the key regulators of metastatic potential are in prostate cancer.

10 EMT is induced by various growth factors, specifically, transforming growth factor-beta (TGF- β)
11 appears to be the most ubiquitous instigator of EMT during development and cancer(3, 10, 11). In canonical
12 TGF- β signaling, TGF- β ligands activate TGF- β transmembrane receptors which phosphorylate latent Smad
13 proteins that form transcription factor complexes which regulate the expression of TGF- β responsive genes(12).
14 In addition, TGF- β activates a variety of non-canonical pathways, including, the AKT, mitogen activated
15 protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and NF-kappaB pathways(13-16). Interestingly, both
16 TGF- β induced Smad signaling and non-canonical Ras-MAPK activation are required for EMT, however, many
17 cancer cell lines exhibiting proficient TGF- β signal transduction, do not undergo TGF- β mediated EMT(17-20).
18 These findings suggest that TGF- β may require significant cross talk with other pathways to coordinate EMT.
19 For example, in some instances TGF- β induced EMT and metastasis is dependent on sustained elevated levels
20 of active Ras-MAPK signaling resulting from Ras over-expression or hyperactivity(21-23). Thus, while the
21 importance of Ras signaling in promoting EMT is well documented, why non-conical TGF- β activation of the
22 Ras-MAPK pathway is not sufficient to induce EMT alone in these models remains unresolved.

23 In studies of the prostate cancer ArCAP model using transformed cells, simultaneous treatment with
24 epidermal growth factor (EGF) and TGF- β induces both EMT and increased metastatic potential(22). One
25 plausible explanation is that EGF activates signaling events controlling Ras signaling dynamics that work in
26 concert with TGF-beta to help induce EMT in earlier stages of cancer. Utilizing non-transformed and hTERT
27

EMT requires MEK1 activation of Erk2 and c-myc immortalized primary prostate cells isolated from human prostates of increased Gleason score (GS 6 to 8), we report that TGF- β combined with EGF or Ras over-expression drives EMT and invasion in earlier cancer stages. Specifically, we found that MEK1 signaling downstream of Ras was necessary and sufficient for TGF- β induced EMT and that EGF and MEK1 signaling was sufficient to induce nuclear accumulation of the MEK1/2 effector molecule, Erk2, which correlated with EMT. Notably, TGF- β treatment alone was unable to induce Erk2 nuclear accumulation despite inducing its phosphorylation. Furthermore, we demonstrate that a mutant Erk2 construct which accumulates in the nucleus is sufficient to drive TGF- β induced EMT in early grade prostate cancer cells, and that this relies on expression of the c-myc transcription factor. In sum, we demonstrate a novel mechanism by which MEK1 signaling promotes the transition of primary non-invasive tumor cells to an invasive phenotype characteristic of malignant tumor cells in response to TGF- β .

Materials and Methods

Cells

IBC-10a, PCa-20a and PCa-30a cells were isolated from the right peripheral zone of a Gleason score 6, 7 and 8 prostate tumors respectively, as previously described(24). IBC-10a cells were immortalized by stable transfection with the pLXSN-hTERT retroviral plasmid (courtesy of John Rhim, USUHS, Bethesda, MD) and identified as intermediate basal cells. They possess minimal gross chromosomal abnormalities and express CK5, CK18, p63, PSA and PTEN(24). PCa-20a and PCa-30a cells expressed CK18, PTEN and PSA but not CK7 or p63. Cells were maintained in serum free complete keratinocyte media (cKm) containing EGF, bovine pituitary extract (Invitrogen Inc., Carlsbad, CA) and 50ug/ml penicillin/streptomycin (Mediatech, Manassas, VA). PC3-ML cells were isolated from PC3 prostate cancer cells (ATCC, Bethesda, MD) based on their ability to metastasize to the lumbar vertebrae(25). PC3-ML cells were maintained in DMEM with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 50 ug/ml penicillin/streptomycin (Mediatech, Manassas, VA).

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RasV12, Ras V12S35, RasV12C40 and RasV12G37 were stably over-expressed in both IBC-10a and PCa-20a cells using the pBABE-puro retroviral vector (a kind gift from Dr. Christian Sell, Drexel University College of Medicine). MEK1-DD and MEK2-DD were also over-expressed in cells using the pBABE-puro retroviral vector (a kind gift of Dr. Mauricio Reginato, Drexel University College of Medicine). HA-Erk2 WT and HA-Erk2 D319N were expressed in cells using the pLNCX retroviral vector (a kind gift from Dr. Claudio Torres, Drexel University College of Medicine). Scrambled shRNA constructs and shRNA constructs targeting c-myc (TRCN0000039641) were purchased from Sigma (St. Louis, MO). shRNA constructs targeting Erk2 were a kind gift from the lab of Dr. John Blenis (Harvard Medical School) and Dr. Peter Lelkes (Drexel University)(26). Retroviral and lentiviral production and maintenance of transfected cells was carried out according to methods previously described(27).

Antibodies

Western Blot and Immunoflourescenece was carried out according to previously described methods(28). For western analysis primary antibodies targeting Vimentin and Fibronectin were purchased from Sigma-Aldrich, St. Louis, MO (V6389 and F3648 respectively); E-cadherin, Tubulin, phosphorylated-Erk1/2, phosphorylated-Smad3, phosphorylated-Akt, c-myc and Slug were purchased from Cell Signaling Technology, Beverly, MA (2148, 4065, 9106, 9520, 4060, 9402 and 9585 respectively); FSP-1 and Twist2 were purchased from Abcam, Cambridge, MA (ab27597 and ab57997 respectively); and phosphorylated-c-myc was purchased from Millipore, Bellerica, MA (04-217). For IF, primary antibodies targeting Vimentin were purchased from Sigma-Aldrich, St. Louis, MO (V6389); β-catenin was purchased from Cell Signaling Technology, Beverly, MA (9582); and Erk2 were purchased from Santa Cruz biotechnology, Santa Cruz, CA (sc-1647).

EMT Induction

Unless otherwise stated, for *in vitro* induction of EMT, cells were trypsinized and plated in cKm at a low density (2 x 10⁴ cells/ml). The next day (day 0) cells were washed once in Km without supplements (i.e.

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pituitary extract and EGF) and media was replaced with Km supplemented with TGF- β 1 (10 *ng/ml* ; Peprotech,
Rocky Hill, NJ) and/or EGF (10 *ng/ml*; Invitrogen, Carlsbad, CA). Media in all experiments was changed on
days 3, 6 and 9, and cells were analyzed on day 10.

Quantitative Real-Time PCR

Total RNA was isolated using Qiagen's RNAeasy isolation kit per manufacturer instructions. Target
genes were amplified using the 1-step Brilliant SYBR Green qRT-PCR kit (Stratagene, La Jolla, CA) and the
MX3000P thermocycler (Stratagene, La Jolla, CA) per manufacturer instructions. Primers used were as follows:

Cyclophilin A: Fwd-GTGACTTCACACGCCTATATG, Rev-ACAAGATGCCAGGACCGTA;

Snail: Fwd-GCTGCCAATGCTCATCTGGGACT, Rev-CAGGGAGGTCAGCTCTGCCA;

Slug: Fwd-TCAGCTCAGGAGCATACAGC, Rev-GACTCCACTCGCCCCAAAGA;

Twist1: Fwd-GTCCGCAGTCTTACGAGGAG, Rev-CCAGCTTGAGGGTCTGAATC;

Twist2: Fwd-AGCAAGAAGTCGAGCGAAGA, Rev-CAGCTTGAGCGTCTGGATCT;

Zeb1: Fwd-TATGAATGCCCAAACCTGCAA, Rev-TGGTGATGCTGAAAGAGACG;

Zeb2: Fwd-CGCTTGACATCAATGAAGGA, Rev-CTTGCCACACTCTGTGCATT;

Vimentin: Fwd-CCCTCACCTGTGAAGTGGAT, Rev-TCCAGCAGCTTCCTGTAGGT;

MT-MMP1: Fwd-ACATTGGAGGAGACACCCAC Rev-TAGGCAGTGTTGATGGACGC;

MMP-2: Fwd-CAAAAACAAGAAGACATACATCTT. Rev-GCTTCCAACCTTCACGCT;

MMP-9- Fwd: CCCTGGAGACCTGAGAACCA, Rev: CCCGAGTGTAACCATAGCGG.

Utilizing the 2^{-ddCt} method, empty-vector or parent cells grown in minimal media were used to normalize gene
expression across treatments. Relative internal mRNA expression of target genes was normalized to
Cyclophilin-A expression in each sample. Each sample for each experiment was run in duplicate and averages
are representative of three independent experiments. Statistical significance was determined using Welch's
unpaired t-test.

EMT requires MEK1 activation of Erk2 and c-myc
Zymography

Evaluation of enzymatic activity of MMPs was assed using gelatin zymography as previously described(29). Media conditioned for 24 hrs on day 10 was collected and protein in conditioned media was concentrated 10 fold using Amicon Ultra-15 centrifugation filter devices according to manufacturer instructions (Millipore, Bellerica, MA). Between 0.1 and 1 μ g of protein depending on cell type was loaded and run on a 10% polyacrylamide gel containing 2 mg/ml of Gelatin A.

Invasion Assays

Following experimental treatments cells were trypsinized and seeded onto Matrigel coated invasion inserts with 0.8 μ m porous membranes (BD Biosciences, Bedford, MA) at a density of 5×10^4 cells per well in cKm and allowed to attach for 2 hrs. Media on the top chamber was then changed to experimental condition and bottom chamber was filled with cKm containing 5% FBS (Atlanta Biologicals, Lawrenceville, GA). Transwells were placed at 37°C for 48 hrs. Cells in top compartment were scraped off and cells which migrated to bottom were either fixed with 4% paraformaldehyde and stained with 0.1% crystal violet or trypsinized and counted using a hemocytometer. Data were averaged from three independent experiments. Prostashperes were produced as previously described and topped with Km containing experimental condition, 0.2% FBS (Atlanta Biologicals, Lawrenceville, GA) and 5% Matrigel(30). Media was changed every 3 days with experimental condition and 5% Matrigel. Prostasphere acini were analyzed after 12 days of culture.

Results

EGF and TGF- β function synergistically to induce EMT in primary non-invasive epithelial cells isolated from prostate cancer. We previously isolated 3 different human prostate epithelial cell lines (termed IBC-10a, PCa-20a and PCa-30a) from tumors of increasing Gleason Scores (GS6, GS7 and GS8, respectively)(24, 31). Previous studies have shown that TGF- β alone or in conjunction with other growth factors can induce EMT in transformed cells, but whether these ligands might normally induce EMT in non-immortalized primary cells has

EMT requires MEK1 activation of Erk2 and c-myc yet to be shown. Therefore, we treated each cell line with either minimal media (Km) as a control, EGF, TGF- β 1 (TGF) or both EGF and TGF- β 1 in combination (E + T) (as described in Materials and Methods) and analyzed the expression of mesenchymal and epithelial-associated proteins. Treatment of all three cell lines with Km or EGF failed to induce expression of several EMT associated genes, including Fibronectin and Vimentin (Figure 1A). In all cell lines TGF- β alone was sufficient to induce Fibronectin; however, a significant loss in E-cadherin expression, and induction of Vimentin and Fibroblast Specific Protein-1 (FSP-1) only occurred in more malignant PCa-30a cells (Figure 1A). In contrast, co-treatments of all three cell lines with E + T induced a robust EMT response as characterized by expression of Vimentin and FSP-1, loss of E-cadherin, disruption of epithelial cell-cell contacts, cytoplasmic accumulation of β -catenin and adoption of a spindle shaped morphology (Figure 1A, 1B and Supplemental Figure 1). We also found that the metastatic PC-3ML cell line constitutively expressed Fibronectin, Vimentin and FSP-1 and lacked E-cadherin expression (Figure 1A and Supplemental Figure 1).

Notably, a stable EMT phenotype was maintained as indicated by continued expression of Vimentin in cells cultured for an additional 4 days following discontinuation of the EMT inducing treatments (Figure 1C). To ensure that E + T induced EMT was not an artifact associated with cell lines, cell passage or continued growth in EGF containing media, we treated freshly established organ cultures from a GS 6 prostate cancer specimen with the different ligands. These organ cultures developed outgrowths of prostate epithelial cells and we observed that E + T, but not TGF- β alone, induced significant morphological changes reminiscent of EMT and promoted Vimentin expression after ~6 days of treatment (Figure 1D). Taken together these results suggest that signaling pathways activated by both EGF and TGF- β function synergistically to induce EMT in epithelial cells derived from low grade prostate tumors. Furthermore, they imply that induction of EMT by TGF- β does not require transformation of primary cell lines, rather, TGF- β induction of EMT may be a characteristic of epithelial cells isolated from higher grade tumors.

EMT requires MEK1 activation of Erk2 and c-myc
EGF signaling modulates cellular responses to TGF- β to induce the up-regulation of pro-metastatic genes and an invasive phenotype. Several transcription factors, including those of the Snail (Snail and Slug), Twist (Twist1 and Twist2) and Zeb (Zeb1 and Zeb2) families, have been identified as important regulators of EMT and are required for cell movement and metastatic spread in a variety of cancers(32-39). We observed that E + T treatment induced expression of Slug and Twist2 in IBC-10a cells (4 and 9-fold increase, respectively) and PCa-20a cells (3 and 8-fold increase respectively) (Figures 2A and 2B). Treatment of these cells with EGF or TGF- β alone failed to elicit significant changes in the expression of Slug. EGF alone induced Twist2 expression in both IBC-10a and PCa-20a cells, but less than that observed by E + T treatment (Figures 2A and 2B). In PC3-ML cells, TGF- β alone was sufficient to up-regulate Slug and Twist2 mRNA 2.5 and 3-fold, respectively (Figure 2A). EGF alone had no effect on the expression of these genes, and E + T treatment was as efficacious as TGF- β treatment alone (Figure 2A). In contrast, the expression of Snail, Twist1 and Zeb1/2 was not induced by these ligands in any of our primary cell lines (Supplemental Figure 2A). However, PC3-ML cells expressed a high basal level of Zeb1 and Twist2 (Supplemental Figure 2B). As expected, PC3-ML cells constitutively expressed high levels of Vimentin in minimal media regardless of treatment (Figure 2A and Supplemental Figure 2B).

The up-regulation of matrix metalloproteinases (MMPs), including MMP-2, MMP-9 and MT-MMP1, are also associated with acquisition of an EMT phenotype, and are important to breakdown stromal barriers during invasion and metastasis(40). In IBC-10a and PC-20a cells, treatment with E + T induced a robust increased in MMP-2, MMP-9 and MT-MMP-1 gene expression and accumulation of catalytically active MMP-2, MMP-9 and MMP-9 homodimer in conditioned media (Figure 2A and 2C). In contrast, treatment of PC3-ML cells with TGF- β alone was sufficient to promote the enzymatic activity of MMP-2, MMP-9 and the MMP-9 homodimer in conditioned media and EGF had no additive effect when combined with TGF- β (Figure 2C).

To functionally demonstrate the invasive capacity of cells undergoing EMT, we tested the affect of EGF, TGF- β and E + T on IBC-10a cells' ability to migrate through a matrigel-coated modified boyden chamber. While Km, EGF and TGF- β alone induced little to no invasion, IBC-10a cells treated with E + T

EMT requires MEK1 activation of Erk2 and c-myc exhibited significant increases in cell invasion and migration (Figure 2D). Furthermore, utilizing a 3-dimensional Matrigel model that recapitulates *in vivo* glandular organization(30), we observed that IBC-10a cells formed tight acinar-like structures (termed prostaspheres) in the presence of Km, EGF or TGF- β alone, however, in the presence of E + T, prostaspheres were disrupted and treatment promoted cell to emigration from the acini and their invasion through the surrounding Matrigel (Figure 2E). Notably, the invading IBC-10a cells were spindle shaped and expressed Vimentin, suggestive of EMT (Figure 2E).

Ras activation of Raf promotes TGF- β induced EMT. Ras is a major effector molecule of EGF signaling and has previously been implicated in promoting TGF- β mediated EMT(23). To determine the role of Ras in modulating TGF- β responses in IBC-10a and PCa-20a cells we stably transfected these cells with either a constitutively active Ras construct (pBABE:RasV12) or empty vector control (pBABE) and treated with Km, EGF, TGF- β , or E + T. In response to TGF- β or E + T treatments, Ras transfected cells showed a reduction in both 'cell-cell' junctions and E-cadherin expression, along with concomitant up-regulation of Vimentin (Figure 3A and Supplemental Figure 3A). Activated Ras is known to mediate its signaling through several downstream pathways, we therefore transfected IBC-10a and PCa-30a cells with specific Ras effector mutants including: RasV12-C40, which binds PI3-kinase to activate AKT signaling, RasV12-G37, which binds RalGDS to activate phospholipase D signaling, and RasV12-S35, which binds c-Raf to activate MAPK signaling(41). While all cells increased expression of Vimentin and FSP-1 in response to treatment with E + T, only cells transfected with RasV12-S35 also did so in the presence of TGF- β alone (Figure 3B and 3C). In response to TGF- β treatment RasV12-S35 transfected cells also expressed increased activity of MMP-2, MMP-9 and the MMP-9 homodimer and demonstrated enhanced cell motility and invasion exhibiting a >3-fold increase in migration and invasion in modified Boyden chamber assays when compared to controls (Figures 3D and 3E). Moreover, TGF- β treatment of IBC-10a or PC-20a cells transfected with either RasV12 or RasV12-S35 significantly increased expression of Vimentin, Slug, Twist2, MMP-2 and MMP-9 mRNA (Figure 3F and Supplemental Figure 3F). In contrast, IBC-10a and PCa-20a cells transfected with Empty vector, RasV12-C40 or RasV12-

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G37 failed to elicit any increase in expression of these genes in response to TGF- β (Figure 3F and Supplemental
Figure 3F). Taken together, these results indicate that EGF signaling through the Ras-Raf-MAPK cascade
potentiates TGF- β induction of EMT in non-invasive prostate epithelial cells.

MEK1, but not MEK2, activity is necessary and sufficient for TGF- β induced EMT. MEK1/2 activation
of Erk1/2 is the most well characterized downstream effect of Ras/Raf signaling and is critical for Ras induced
transformation(42). To better understand the signaling dynamics regulating EMT, IBC-10a cells were treated
with increasing concentrations of either a MEK 1/2 inhibitor (PD098059), a PI3K inhibitor (LY290042), or a
SMAD3 inhibitor (SIS3). As indicated by Vimentin and FSP-1 expression, we observed that the EMT response
was dramatically inhibited in a dose dependent manner by both PD098059 and SIS3 in IBC-10a cells (Figure
4A and Supplemental Figure 4A) suggesting that signaling through MAPK and Smad3 are both necessary for E
+ T induced EMT.

We also stably transfected IBC-10a cells with a constitutively active MEK1 or MEK2 construct
(pBABE: MEK1-DD, pBABE:MEK2-DD) and empty vector (pBABE), as a control. In response to TGF- β ,
MEK1-DD transfected cells demonstrated a decrease in E-cadherin expression and induction of Vimentin
(Figure 4B). In contrast, MEK2-DD transfected cells showed a partial reduction in E-cadherin expression, but
showed no induction of Vimentin (Figure 4B). Immunofluorescence imaging further demonstrated that
Vimentin expression was ubiquitously induced by TGF- β in MEK1-DD, but not in MEK2-DD transfected IBC-
10a cells (Supplemental Figure 4B). MEK1-DD and MEK2-DD transfected IBC-10a overexpressed MEK-1 and
MEK-2 respectively with no change in expression to the other MEK protein (Supplemental Figure 4C). MEK1-
DD and MEK2-DD transfected cells also both significantly increased phosphorylation of Erk 1/2 compared
with the empty vector cells (Figure 4C). We also observed that phosphorylation of Erk1/2 was elevated in IBC-
10a, PCa-20a and PCa-30a cells when treated with TGF- β alone and levels of activated Erk 1/2 were equal in
IBC-10a cells treated with either EGF, TGF- β or E + T (Figure 4D and 4E). Surprisingly, metastatic PC3-ML
cells exhibited decreased levels of Erk1/2 phosphorylation when compared to IBC-10a, PCa-20a and PCa-30a

EMT requires MEK1 activation of Erk2 and c-myc cells despite expressing significantly more Ras (Figure 4D). Functional Erk2, but not Erk1, is previously shown to be essential for EMT, and given the conflicting results above, we wanted to determine if Erk2 expression was required for EMT in our model(43). We transfected PCa-20a and PCa-30a cells with a scrambled shRNA or shRNA vector targeting Erk2 and observed that treatment with E + T or TGF- β in PCa-20a and PCa-30a cells with Erk2 knockdown failed to induce Vimentin and FSP-1 or down-regulate E-cadherin (Figure 4F). Taken together, these findings suggests that while MEK1 signaling specifically regulates EMT and Erk2 expression is required for EMT, differential levels of Erk2 phosphorylation are not regulating EMT.

Erk2 nuclear accumulation promotes and c-myc expression is required for TGF- β induced EMT.

MEK1 and MEK2 are often considered to be redundant in function, although MEK1 and MEK2 are shown to have differential effects on cellular localization of Erk2(44). Consistent with this observation, Erk2 accumulated in the nucleus of MEK1 transfected IBC-10a cells but not in MEK2 or empty vector transfected IBC-10a cells cultured in Km (Figure 5A). Additionally, we observed that TGF- β alone was insufficient to induce nuclear accumulation of Erk2 in IBC-10a cells, whereas E + T induced a dramatic increase in Erk2 nuclear staining (Figure 5B). Significantly, both TGF- β and E + T treatments induced sustained Erk2 accumulation in the nucleus of PCa-30a cells which undergo EMT with TGF- β treatment alone (Figure 5B). To further investigate the role of Erk2 nuclear accumulation, PCa-20a cells were transfected with a phosphatase resistant Erk2 mutant (D319N) which accumulates in the nucleus of cells and WT Erk2 as a control (Supplemental Figure 5D)(45). TGF- β treatment alone was sufficient to induce Vimentin and FSP-1 expression and promote EMT in cells transfected with mutant Erk2 but not WT Erk2 (Figure 5C). It is well established that nuclear Erk2 induces c-myc phosphorylation, as a functional consequence of Erk2 nuclear accumulation and we also observed an increase in phosphorylation of c-myc at serine 62 (Figure 5C)(46, 47). Moreover, transfection with MEK1 induced c-myc phosphorylation whereas knockdown of Erk2 decreased c-myc phosphorylation in response to E + T treatments in PCa-20a cells and treatment of TGF- β alone in PCa-30a cells further indicating that Erk2 nuclear accumulation is phosphorylating c-myc during EMT(Supplemental Figure 4E and 4F). These

EMT requires MEK1 activation of Erk2 and c-myc observations prompted us to discern the role of c-myc in promoting TGF- β induced EMT. We transfected IBC-10a cells with a c-myc over-expression construct and a c-myc targeting shRNA and treated them with TGF- β and E +T. We observed that c-myc over-expression was insufficient to promote TGF- β induced EMT, however, c-myc expression was required for induction of EMT in both IBC-10a and PCa-20a cells in response to E +T (Figure 5D and 5E). Knockdown of c-myc also significantly inhibited the invasive potential of IBC-10a cells in response to E+T (Figure 5F). Furthermore, knockdown of c-myc or Erk2 in PC3-ML cells decreased expression of Vimentin and FSP-1 (Figure 5G).

To test the enhanced metastatic potential associated with EMT, PC3-ML cells containing either Erk2 or c-myc shRNA constructs were injected intercardiacally into male NOD-SCID mice. Previous studies have demonstrated that PC3-ML cells readily metastasize in mice to distant organ sites by 4 wk post injection (PI)(48). We found that at 5 wks PI, 66% of mice injected with PC3-ML cells carrying a control scrambled shRNA construct exhibited liver and adrenal metastasis and 33% of these mice exhibited a brain metastasis (n=3) (Supplemental Figure 5 and Table 1). In contrast, shRNA-mediated knockdown of c-myc failed to produce distant metastasis in mice (n=6) and shRNA-mediated knockdown Erk2 produced only one distant metastasis (n=6) (Supplemental Figure 5A and 5B and Table 1). Knockdown of c-myc and Erk2 also inhibited the invasive phenotype typically observed in PC3-ML cells (Supplemental Figure 5C). Taken together these results suggest that nuclear accumulation of Erk2, which is stimulated by MEK1, but not MEK2, is a key regulator of TGF- β induced EMT and invasion. Moreover, these results indicate that c-myc expression, a target of activated Erk2 in the nucleus, is required for EMT and that inhibition of this pathway results in an overall decreased metastatic potential of highly invasive prostate cancer cells.

Discussion

To our knowledge, this is the first report to show that downstream of EGF, Ras and Raf signaling, active MEK1, but not MEK2, is necessary and sufficient for TGF- β induced EMT in a variety of normally non-invasive primary cells. These findings imply that activation of MEK1 and MEK2 has differential effects on

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TGF- β signaling and that their role in growth factor signaling is not interchangeable. While MEK1 and MEK2 share extensive homology, it is shown that MEK1-activated Erk2 preferentially accumulates in the nucleus(44). In agreement with a previous report(49), our findings indicate that over-expression of a mutant of Erk2 which accumulates in the nucleus, given its resistance to MAPK phosphatases, is sufficient for TGF- β alone to induce an EMT phenotype. This data strongly indicates that EGF signaling plays an important role in modulating TGF- β responses in prostate epithelial cells by inducing differential Erk2 shuttling to the nucleus which is critical for EMT. This data also suggests that there may be a role for MAPK phosphatases, which reside in the nucleus, in regulating EMT and TGF- β responses.

One of the functions of nuclear Erk2 is phosphorylation and stabilization of the transcription factor c-myc(46). Although *in vivo* breast cancer modeling suggests that over-expression of c-myc can elicit an EMT phenotype and that over-expression of c-myc alone can induce EMT in mammary epithelial cells, there is a lack of studies directly indicating whether c-myc expression is required for EMT in regards to TGF- β induced invasion(50-52). In this report, we demonstrate that expression of c-myc is critical for the EMT program and for TGF- β induced invasion. Interestingly, in normal epithelia TGF- β acts as a tumor suppressor in part by repressing c-myc, therefore it is conceivable that inhibition of c-myc down-regulation by TGF- β through the Ras-MAPK pathway is critical for the tumor promoting activities of TGF- β (53). Furthermore, our findings suggest that over-expression of c-myc is not sufficient for EMT; suggesting that post-translational phosphorylation of c-myc may have a larger functional role in tumor progression than simply stabilization of the c-myc protein. This finding is in agreement with a recent report showing that in mammary epithelial cells expressing a mutant myc protein possessing elevated levels of phosphorylated serine 62 results in invasive mammary carcinoma(54). In addition, c-myc is a driver of the pluripotent phenotype, regulating stem cell self-renewal and differentiation, and is shown to be required for growth of tumor initiating prostate cancer cells(24, 55, 56). Interestingly, EMT in human mammary epithelial cells also includes induction of classical stem cell markers and cells undergoing EMT exhibit some level of cellular plasticity(57, 58). Therefore, c-myc activity

1 EMT requires MEK1 activation of Erk2 and c-myc
2 might play a critical role in regulating EMT, the cellular plasticity associated with EMT and the tumor initiating
3 characteristics of cells undergoing EMT.
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6 Reportedly, Ras and Raf mutations, and/or amplification, are a rare event during the prostate and breast
7 cancer progression and has lead pathological studies to doubt the clinical contribution of Ras alone to cancer
8 metastasis and EMT(59, 60). However, alternative molecular processes may transiently up-regulate Ras and Raf
9 activity, including increased expression of Ras GEFs and reduced expression of Ras GAPs. For example,
10 enhancer of zeste homolog 2 (EZH2), a member of the Polycomb Repressive Complex 2, is shown to silence
11 disabled homolog 2-interacting protein (DAB2IP), a Ras GAP, thereby inducing hyperactive Ras and promoting
12 increased prostate cancer metastasis(61). Since EZH2 expression is greatly increased in metastatic prostate
13 cancer cells compared to localized prostate cancers, it is possible that a transient up-regulation of Ras activity
14 may contribute to EMT invasion and metastatic progression of human prostate cancer(62).
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18 While non-canonical MAPK activation by TGF- β appears to be required for TGF- β mediated EMT, it is
19 also apparent that constitutive activation of Ras along with TGF- β can act cooperatively to promote EMT when
20 TGF- β alone cannot(63, 64). Our findings suggest that the ability for EGF and MEK1 to differentially direct
21 Erk2 cellular localization may serve as a functional mechanism for the synergistic signaling between Ras and
22 TGF- β to induce EMT. From our findings, we propose a model by which Erk2 must be activated and shuttled
23 to the nucleus where it can phosphorylate c-myc and, in cooperation with TGF- β signaling, induce EMT.
24 Therefore, in circumstances where TGF- β alone cannot induce EMT, Erk2 may not have sufficiently
25 accumulated in the nucleus, or c-myc may not be adequately expressed. In this case, auxiliary pathways, such as
26 EGF activation of Ras, may be required for TGF- β mediated EMT. In agreement with this hypothesis, other
27 studies have shown that sustained MAPK signaling directed by Ras, Raf, EGF or Erb2 over-expression is often
28 necessary to promote robust and sustainable EMT in response to TGF- β treatment(21-23).
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32 Recent studies have suggested that EMT and metastatic dissemination may be an early event in
33 tumorigenesis(65, 66). Our results support this concept and suggest that early stage prostate cancer cells possess
34 the genetic repertoire necessary for EMT and invasion. In early stage tumors, it is feasible that increased TGF- β
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EMT requires MEK1 activation of Erk2 and c-myc and EGF levels may arise from chronic inflammation or the reactive stroma associated with early tumors to induce EMT and invasion(67, 68). Future studies examining the nuclear localization of Erk2 in cancer cells at the leading edges of tumors may aid identification of early stage cancers that are poised to metastasize, and identify patients with a poorer prognosis who may require more aggressive therapeutic intervention.

Supplementary Material

Supplementary Figures can be found at <http://carcin.oxfordjournals.org/>

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References

1. Xue C, et al. The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Res.* 2003 Jun 15;63(12):3386-94.
2. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nature reviews.* 2002 Jun;2(6):442-54.
3. Thiery JP, et al. Epithelial-mesenchymal transitions in development and disease. *Cell.* 2009 Nov 25;139(5):871-90.
4. Christofori G, et al. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci.* 1999 Feb;24(2):73-6.
5. Domagala W, et al. Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas. *Am J Pathol.* 1990 Dec;137(6):1299-304.
6. Rudland PS, et al. Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. *Cancer Res.* 2000 Mar 15;60(6):1595-603.
7. Singh S, et al. Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. *Cancer research.* 2003 May 1;63(9):2306-11.

EMT requires MEK1 activation of Erk2 and c-myc

8. Gilles C, et al. Vimentin contributes to human mammary epithelial cell migration. *Journal of cell science*. 1999 Dec;112 (Pt 24):4615-25.
9. Gottardi CJ, et al. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *J Cell Biol*. 2001 May 28;153(5):1049-60.
10. Giampieri S, et al. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol*. 2009 Nov;11(11):1287-96.
11. Massague J, et al. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J*. 2000 Apr 17;19(8):1745-54.
12. Brown KA, et al. A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. *J Cell Biochem*. 2007 May 1;101(1):9-33.
13. Gingery A, et al. TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. *Exp Cell Res*. 2008 Sep 10;314(15):2725-38.
14. Kato M, et al. TGF-beta activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN. *Nature cell biology*. 2009 Jul;11(7):881-9.
15. Lee MK, et al. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J*. 2007 Sep 5;26(17):3957-67.
16. Perlman R, et al. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nature cell biology*. 2001 Aug;3(8):708-14.
17. Brown KA, et al. Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro. *Breast Cancer Res*. 2004;6(3):R215-31.
18. Deckers M, et al. The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer research*. 2006 Feb 15;66(4):2202-9.
19. Do TV, et al. Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. *Mol Cancer Res*. 2008 May;6(5):695-705.
20. Xie L, et al. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia*. 2004 Sep-Oct;6(5):603-10.
21. Seton-Rogers SE, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci U S A*. 2004 Feb 3;101(5):1257-62.
22. Zhau HE, et al. Epithelial to mesenchymal transition (EMT) in human prostate cancer: lessons learned from ARCaP model. *Clin Exp Metastasis*. 2008;25(6):601-10.
23. Janda E, et al. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol*. 2002 Jan 21;156(2):299-313.
24. Goodyear SM, et al. Dysplasia of human prostate CD133(hi) sub-population in NOD-SCIDS is blocked by c-myc anti-sense. *Prostate*. 2009 May 15;69(7):689-98.
25. Wang M, et al. Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice. *Differentiation; research in biological diversity*. 1991;48(2):115-40.
26. Dimitri C, et al. Spatially separate docking sites on ERK2 regulate distinct signaling events in vivo. *Current biology : CB*. 2005;15(14):1319-43.
27. Michiels F, et al. Expression of Rho GTPases using retroviral vectors. *Methods Enzymol*. 2000;325:295-302.
28. Amatangelo M, et al. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. *The American journal of pathology*. 2005;167(2):475-563.
29. Stearns ME. Alendronate blocks TGF-beta1 stimulated collagen 1 degradation by human prostate PC-3 ML cells. *Clinical & experimental metastasis*. 1998 May;16(4):332-9.
30. Debnath J, et al. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods (San Diego, Calif)*. 2003;30(3):256-324.

EMT requires MEK1 activation of Erk2 and c-myc

31. Goodyear SM, et al. Role of the VEGFR3/VEGFD receptor axis in TGFbeta1 activation of primary prostate cell lines. *Prostate*. 2009 Jun 15;69(9):982-90.
32. Peinado H, et al. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*. 2007 Jun;7(6):415-28.
33. Barrallo-Gimeno A, et al. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development (Cambridge, England)*. 2005 Jul;132(14):3151-61.
34. Cano A, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature cell biology*. 2000 Feb;2(2):76-83.
35. Bolos V, et al. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *Journal of cell science*. 2003 Feb 1;116(Pt 3):499-511.
36. Comijn J, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell*. 2001 Jun;7(6):1267-78.
37. Eger A, et al. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene*. 2005 Mar 31;24(14):2375-85.
38. Ansieau S, et al. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell*. 2008 Jul 8;14(1):79-89.
39. Yang J, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004 Jun 25;117(7):927-39.
40. Pulyaeva H, et al. MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. *Clin Exp Metastasis*. 1997 Mar;15(2):111-20.
41. White MA, et al. Multiple Ras functions can contribute to mammalian cell transformation. *Cell*. 1995 Feb 24;80(4):533-41.
42. Troppmair J, et al. Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. *J Biol Chem*. 1994 Mar 4;269(9):7030-5.
43. Shin S, et al. ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Molecular cell*. 2010;38(1):114-41.
44. Skarpen E, et al. MEK1 and MEK2 regulate distinct functions by sorting ERK2 to different intracellular compartments. *FASEB J*. 2008 Feb;22(2):466-76.
45. Tresini M, et al. Modulation of replicative senescence of diploid human cells by nuclear ERK signaling. *J Biol Chem*. 2007 Feb 9;282(6):4136-51.
46. Chuang CF, et al. Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors. *FEBS Lett*. 1994 Jun 13;346(2-3):229-34.
47. Alvarez E, et al. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-myc and c-jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J Biol Chem*. 1991 Aug 15;266(23):15277-85.
48. Wang M, et al. Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice. *Differentiation*. 1991 Nov;48(2):115-25.
49. Shin S, et al. ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Mol Cell*. 2010 Apr 9;38(1):114-27.
50. Smith AP, et al. A positive role for Myc in TGFbeta-induced Snail transcription and epithelial-to-mesenchymal transition. *Oncogene*. 2009 Jan 22;28(3):422-30.
51. Trimboli AJ, et al. Direct evidence for epithelial-mesenchymal transitions in breast cancer. *Cancer Res*. 2008 Feb 1;68(3):937-45.
52. Cho KB, et al. Overexpression of c-myc induces epithelial mesenchymal transition in mammary epithelial cells. *Cancer Lett*. 2010 Jul 28;293(2):230-9.
53. Bierie B, et al. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nature reviews*. 2006 Jul;6(7):506-20.

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54. Wang X, et al. Phosphorylation regulates c-Myc's oncogenic activity in the mammary gland. *Cancer Res.* 2011 Feb 1;71(3):925-36.

55. Park IH, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature.* 2008 Jan 10;451(7175):141-6.

56. Wilson A, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes & development.* 2004 Nov 15;18(22):2747-63.

57. Mani SA, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell.* 2008 May 16;133(4):704-15.

58. Morel AP, et al. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One.* 2008;3(8):e2888.

59. Carter BS, et al. ras gene mutations in human prostate cancer. *Cancer research.* 1990 Nov 1;50(21):6830-2.

60. Rochlitz CF, et al. Incidence of activating ras oncogene mutations associated with primary and metastatic human breast cancer. *Cancer research.* 1989 Jan 15;49(2):357-60.

61. Min J, et al. An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. *Nat Med.* 2010 Mar;16(3):286-94.

62. Varambally S, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature.* 2002 Oct 10;419(6907):624-9.

63. Kim ES, et al. Transforming growth factor (TGF)-beta in conjunction with H-ras activation promotes malignant progression of MCF10A breast epithelial cells. *Cytokine.* 2005 Jan 21;29(2):84-91.

64. Horiguchi K, et al. Role of Ras signaling in the induction of snail by transforming growth factor-beta. *J Biol Chem.* 2009 Jan 2;284(1):245-53.

65. Weinberg RA. Leaving home early: reexamination of the canonical models of tumor progression. *Cancer cell.* 2008 Oct 7;14(4):283-4.

66. Klein CA. Cancer. The metastasis cascade. *Science.* 2008 Sep 26;321(5897):1785-7.

67. Wyckoff J, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* 2004 Oct 1;64(19):7022-9.

68. Eastham JA, et al. Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. *Lab Invest.* 1995 Nov;73(5):628-35.

Tables and Figure Legends

Table 1. Summary of PC3-ML tumor development in NOD-SCID. Male NOD-SCID mice were injected intercardiacally with 5 x 10⁴ cells of PC3-ML cells expressing GFP and transfected with a scrambled shRNA vector (n=3), an Erk2 targeted shRNA construct (n=6) or a c-myc targeted shRNA construct (n=6). Tumor development in thoracic cavity, abdominal cavity, adrenal gland, liver and brain was assessed 5 wk after injection. Totals represent number of tumors identified at given location per total number of mice injected. Tumors from the thoracic cavity were harvested weighed and averaged showing standard deviation (n = 9, 12 and 12 for tumors harvested from sh Scram, sh Erk2 and sh c-myc respectively),.

EMT requires MEK1 activation of Erk2 and c-myc

Figure 1. EGF and TGF- β act synergistically to induce EMT in primary human prostate epithelial cells.

(A) Western Blots of crude cell extracts from cells treated with minimal media (Km), or Km containing 10 ng/ml EGF (EGF), 10 ng/ml TGF- β (TGF) or 10 ng/ml of EGF + TGF- β (E + T) as described in Materials and Methods. IBC-10a cells, PCa-20a cells and PCa-30a cells were isolated from Gleason Score (GS) 6, 7 and 8 prostate cancers, respectively. Blots were probed with antibodies specific for Fibronectin, E-cadherin, Vimentin, Fibroblast Specific Protein-1 (FSP-1) and Tubulin (loading control). (B) Images of IBC-10a cells cultured in Km, EGF, TGF- β or E + T. Phase contrast images show a reduction in cell-cell contacts in E + T treated IBC-10a and acquisition of a spindle-shaped morphology (see red arrows) (Top Panel). Immunofluorescent images of cells labeled with antibodies specific for β -catenin (Middle Panel; green) and Vimentin (Bottom Panel; green). Cell nuclei (red) were labeled with propidium iodide. (Top/Middle Panels: 400x magnification) (Bottom Panel: 200x magnification). (C) Western Blot analysis of E-cadherin, Vimentin and Actin (loading control) expression in IBC-10a cells treated with E + T at day 0 through 9, and 4 days post treatment, where cells were cultured in cKm. (D) Induction of EMT in prostate organ cultures. Phase Contrast and immunofluorescent imaging show prostate epithelial cells derived from GS6 prostate cancer tissue, emigrating out of the tissue and strongly expressing vimentin (red) in response to concomitant E + T treatment, but not TGF- β alone, Nuclei labeled with DAPI (200x magnification).

Figure 2. EGF signaling modulates TGF- β signaling to up-regulate genes associated with an invasive phenotype in primary prostate epithelial cells. (A) Expression of Vimentin, Slug, Twist2, MT-MMP-1, MMP-2 and MMP-9 genes in IBC-10a (left), PCa-20a (middle) and PC3-ML cells (right). Effects of EGF (10 ng/ml) (light gray), TGF- β (10 ng/ml)(dark gray), and EGF + TGF- β (10 ng/ml each) (E + T) (black) treatments on gene expression was determined by qRT-PCR. Values were normalized to cyclophilin-A and represent the fold increase in expression relative to cells treated with Km (white). Data represent the mean $\pm$ S.D. from three independent experiments. Statistical significance comparing TGF- β treatment alone to E + T was determined

EMT requires MEK1 activation of Erk2 and c-myc using Welch's unpaired t-test, where * = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001; # = no significant difference (p-value > 0.1). (B) Western Blot analysis of Slug and Twist2 in IBC-10a cells treated 9 days with Km, EGF, TGF- β or E + T. Tubulin served as the loading control. (C) Representative gelatin zymographs of conditioned media from IBC-10a, PCa-20a and PC3-ML cells treated with EGF, TGF- β or E + T. Equal amounts of protein from conditioned medium from each treatment was resolved by 10% PAGE containing gelatin-A (2mg/ml). Coomassie Blue staining reveals relative levels of enzymatically active MMP-9 homodimer (210 kDa), MMP-9 (92kDa) and MMP-2 (72kDa) in media. (D) Modified Boyden Chamber Invasion assays showing cell migration following treatment with Km, EGF, TGF- β or E + T for 48 hr. Representative image of cells on bottom of chamber stained with brilliant blue dye. (E) Prostatesphere culture showing three-dimensional acinar structures formed by IBC-10a cells grown as single cell suspensions in Matrigel for 12 days in the presence of different growth factors. (Top Panel) Phase contrast images shows cells grew as rounded spheres in the presence of Km, EGF and TGF- β . In contrast, cells grown in the presence of E + T were irregularly shaped and cells invaded the surrounding Matrigel (see red arrows). (Lower Panel) Immunofluorescent labeling of Prostatespheres with Vimentin antibodies (green). Nuclei labeled with propidium iodide (200x magnification).

Figure 3. Ras activation of Raf induces TGF- β 's pro-invasive response. (A) Western Blot analysis for E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a (left) and PCa-20a (right) cells stably transfected with pBABE:RasV12, and treated with minimal media (Km), EGF (10 ng/ml), TGF- β (10 ng/ml), or EGF + TGF- β in combination (E + T). (B) Western Blot analysis for E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a and PCa-20a cells stably transfected with pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 vectors. Cells were treated with Km, EGF, TGF- β , or E + T. (C) Western Blot analyses of FSP-1 and Tubulin (loading control) expression in IBC-10a cells stably transfected with Empty vector (Empty), pBABE:RasV12-C40, pBABE:RasV12-G37, or pBABE:RasV12-S35 and treated with TGF- β . (D) Quantification of Modified Boyden Chamber invasion assays showing the invasive

EMT requires MEK1 activation of Erk2 and c-myc activity of IBC-10a cells stably transfected with Empty vector (Empty), pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 variants and treated with TGF- β . Values are representative of three independent replicates. Mean $\pm$ SD, where ** = p-value < 0.01, compared to Empty vector control. (E) Gelatin zymographs of conditioned media 24 hours after last treatment from pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 and pBABE:Empty variants of IBC-10a cells treated with TGF- β . (F) Relative expression of Slug, Twist-2, MMP-2 and MMP-9 mRNA in IBC-10a cells transfected with pBABE:RasV12-C40 (light gray bars); pBABE:RasV12-G37 (dark gray bars); pBABE:RasV12-S35 (black bars); and pBABE:Empty (white bars) variants treated with TGF- β by qRT-PCR. Values normalized against cyclophilin-A and represent the fold increase in expression compared to pBABE:Empty cells. Mean $\pm$ 1 S.D. from 3 independent experiments. Statistical significance was derived using Welch's unpaired t-test, where * = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001 when comparing RasV12 and RasV12-S35 cells to pBABE:Empty cells.

Figure 4. MEK1, but not MEK2, activity is necessary and sufficient for TGF- β induced EMT.

(A) Western Blot analysis of phosphorylated-Erk 1/2, Vimentin, FSP-1 and Tubulin (loading control) expression in IBC-10a cells cultured in either Km with 0.5% DMSO (Km + DMSO), Km with 10 *ng/ml* of EGF + TGF- β with 0.5% DMSO (E + T + DMSO), or Km with 10 *ng/ml* of EGF + TGF- β with increasing concentrations of the MEK1/2 inhibitor PD09859 (+PD098). (B) Western Blot analysis of E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD), and Empty vector (pBABE:Empty) and treated with TGF- β (10 *ng/ml*). (C) IBC-10a cells from (B) were cultured in minimal media the expression of phosphorylated-Erk1/2, total-Erk 1/2 and Tubulin (loading control) examined by Western Blot. (D) Western Blot analysis of phosphorylated-Erk1/2, total-Erk1/2, Ras and Tubulin (loading control) expression in IBC-10a, PCa-20a, PCa-30a and PC3-ML cells following treatment with to TGF- β . (E) Western Blot analysis of phosphorylated-Erk1/2, total-Erk1/2 and Tubulin (loading control) expression in IBC-10a cells treated with minimal media (Km), EGF

EMT requires MEK1 activation of Erk2 and c-myc (10 ng/ml), TGF- β (10 ng/ml), or EGF + TGF- β (10 ng/ml) (E + T). (F) Western Blot analysis of E-cadherin, Vimentin, FSP-1, total Erk1/2 and Tubulin (loading control) expression in PCa-20a and PCa-30a cells stably transfected with a scrambled shRNA construct or a shRNA construct targeting Erk2 after treatment with TGF- β , EGF, or E + T.

Figure 5. Erk2 nuclear localization and c-myc expression promote TGF- β induced EMT. (A) Immunofluorescent imaging of Erk2 localization (red) in IBC-10a cells transfected with pBABE:Empty, pBABE:MEK1-DD, or pBABE:MEK2-DD. Cells were grown in minimal media and probed for Erk2 localization (400x magnification). (B) Immunofluorescent images of PCa-20a and PCa-30a cells treated with TGF- β (10 ng/ml) (TGF) or EGF + TGF- β (10 ng/ml each) (E + T) (400x magnification). (C) Western Blot analysis of E-cadherin, Vimentin, serine 62 phosphorylated c-myc (c-myc pS62), FSP-1, and Tubulin (loading control) expression in PCa-20a cells stably transfected with Erk2 WT construct (pLNCX:HA-Erk2), a phosphatase resistant mutant Erk2 (pLNCX:HA-Erk2 D319N) or empty vector (pBABE:Empty) as control and treated with TGF- β . (D) Western Blot analysis of E-cadherin, Vimentin, c-myc, FSP-1 and Tubulin (loading control) expression in IBC-10a cells stably transfected with an empty vector, c-myc overexpression, scrambled shRNA or a shRNA targeting c-myc construct. Cells were treated with TGF- β alone or concomitant EGF + TGF- β (E + T). (E) Western Blot analysis of E-cadherin, Vimentin and Tubulin (loading control) expression in PCa-20a cells stably transfected with a scrambled shRNA or a shRNA targeting c-myc construct and treated with concomitant EGF + TGF- β (E + T). (F) Quantification of Modified Boyden Chamber invasion assays showing the invasive activity of IBC-10a cells stably transfected with a scrambled shRNA or a shRNA targeting c-myc and treated with E + T. Values are representative of three independent replicates. Mean $\pm$ SD, where ** = p-value < 0.01, compared to Empty vector control. (G) Western Blot analysis of Vimentin, c-myc, total-Erk 1/2, FSP-1 and Tubulin (loading control) expression in PC3-ML cells stably transfected with a scrambled shRNA, a shRNA targeting Erk2 or a shRNA targeting c-myc construct.

EMT requires MEK1 activation of Erk2 and c-myc

Supplemental Figure 1. Immunofluorescent images of PCa-20a and PCa-30a cells grown in minimal media (Km), EGF (10 *ng/ml*), TGF- β 1 (10 *ng/ml*) (TGF) or EGF + TGF- β 1 (10 *ng/ml* each) (E + T). Similarly, PC3-ML cells were grown in DMEM supplemented with EGF, TGF- β or E + T. Cells were labeled with antibodies for β -catenin (top row; green, 400x magnification) and Vimentin (bottom row; green, 200x magnification). Cell nuclei (red) were labeled with propidium iodide.

Supplemental Figure 2. (A) qRT-PCR measurements of gene expression in IBC-10a (left), PCa-20a (middle) and PC3-ML cells (right). Cells were treated with in Km (white), 10 *ng/ml* of EGF (light gray), 10 *ng/ml* TGF- β 1 (dark gray) or 10 *ng/ml* EGF + TGF- β 1 (black). Primers were specific for Snail, Twist1, Zeb1 and Zeb2. Values normalized for cyclophilin-A and represented as the fold expressed relative to cells treated with Km (set at 1). Values represent the mean $\pm$ 1 S.D. from 3 experiments. (B) qRT-PCR analysis of gene expression in IBC-10a (white), PCa-20a (gray) and PC3-ML cells (black) cells grown in Km for 10 days. Shows basal levels of Snail, Slug, Twist1, Twist2, Zeb1, Zeb2 and Vimentin mRNA normalized for cyclophilin-A mRNA and represented as fold expressed relative to IBC-10a cells.

Supplemental Figure 3. (A-C) Western Blots of crude cell extracts from IBC-10a cells stably transfected with Empty vector (pBABE:Empty), pBABE:RasV12 or Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; and pBABE:RasV12-S35). Variants were grown in minimal media (Km). Blotted with antibodies specific for E-cadherin, Vimentin, Ras, pAKT, total AKT, pErk1/2, total Erk 1/2 and Tubulin (loading control). (D) qRT-PCR analysis of Vimentin mRNA in IBC-10a (left) and PCa-20a (right) cells stably transfected with a constitutively active Ras construct (pBABE:RasV12) or Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) and Empty vector (pBABE:Empty) and treated with TGF- β (10 *ng/ml*). Values normalized for cyclophilin-A and represented as fold expressed relative to empty vector. Values represent the mean $\pm$ 1 S.D. from 3 experiments. (E) Immunofluorescent images of IBC-10a and PCa-20a cells stably transfected with pBABE-S35 and grown in minimal media (Km), or Km

EMT requires MEK1 activation of Erk2 and c-myc plus 10 *ng/ml* EGF (EGF), TGF- β 1 (TGF) or EGF + TGF- β 1 (E + T). Cells labeled with Vimentin (green, 200x magnification). Cell nuclei (blue) labeled with DAPI. (F) qRT-PCR analysis of Slug, Twist-2, MMP-2 and MMP-9 mRNA levels in IBC-20a cells stably transfected with pBABE:RasV12-C40 (light gray); pBABE:RasV12-G37 (dark gray); pBABE:RasV12-S35 (black); and pBABE:Empty (white) and treated with TGF- β . Values normalized for cyclophilin-A and represented as fold expressed relative to empty vector cells. Values represent the mean +/- 1 S.D. from 3 experiments.

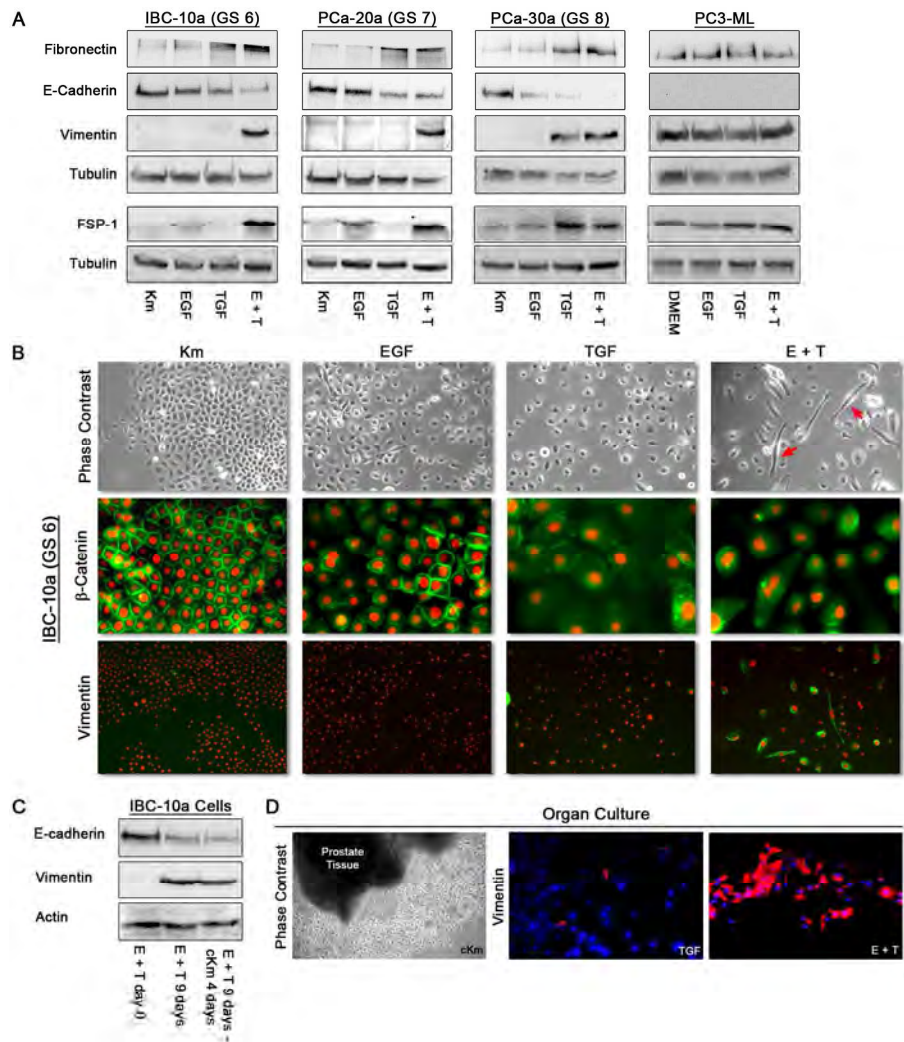
Supplemental Figure 4. (A) Western Blots of crude cell extracts from IBC-10a cells grown in either Km with 0.5% DMSO (Km + DMSO), or Km supplemented with 10 *ng/ml* of EGF + TGF- β 1 with 0.5% DMSO (E + T + DMSO); or Km supplemented with 10 *ng/ml* of EGF + TGF- β 1 with increasing concentrations of either SIS3 (left)(a SMAD3 inhibitor) or LY290042 (right) (a PI3-kinase inhibitor). Blotted with antibodies specific for phosphorylated SMAD 3 (pSMAD3) (left) or phosphorylated AKT (pAKT) (right), or with Vimentin, Fibroblast Specific Protein-1 (FSP-1) and Tubulin (loading control) antibodies. (B) Western Blots of crude cell extracts from IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD) constitutively active MEK2 (pBABE:MEK2-DD) and Empty vector (pBABE:Empty) and blotted with antibodies for MEK1, MEK2 and Tubulin (loading control). (C) Immunofluorescent images of IBC-10a cells transfected with activated MEK constructs (pBABE:MEK1-DD and pBABE:MEK2-DD), treated with TGF- β and labeled for Vimentin (green; 400x magnification). Nuclei (blue) stained with DAPI. (D) Immunofluorescent images of PCa-20a cells transfected with empty vector (pBABE:Empty), a WT Erk2 construct (pLNCX:HA-Erk2) or a phosphatase resistant Erk2 construct (pLNCX:HA-Erk2 D319N). Cells were plated in growth media and grown in minimal media for 24 hrs and probed for Erk2 localization (red, 400x magnification.). (E) Western Blot analysis of crude cell extracts from IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD), and Empty vector (pBABE:Empty) grown in minimal media and blotted for serine 62 phosphorylated c-myc (c-myc pS62) and Tubulin (loading control). (F) Western Blot analysis of crude cell extracts from PCa-20a and PCa-30a cells stably transfected with a

EMT requires MEK1 activation of Erk2 and c-myc scrambled shRNA construct or an shRNA construct targeting Erk2 after treatment with 10 *ng/ml* EGF and TGF- β in combination (E + T) or TGF- β alone (TGF), respectively. Blotted with antibodies to serine 62 phosphorylated c-myc (c-myc pS62), total c-myc and Tubulin (loading control).

Supplemental Figure 5. (A) Representative light and green fluorescent images of tumors within the thoracic cavity of mice injected intercardiacally with PC3-ML cells stably transfected with a scrambled shRNA construct, a shRNA targeting Erk2 construct or a shRNA targeting c-myc construct 5 weeks post injection. (B) Representative light and green fluorescent images of tumors found within the abdominal cavity, adrenal gland, liver and brain of mice injected intercardiacally with PC3-ML cells stably transfected with a scrambled shRNA construct 5 weeks post injection. (C) Phase contrast imaging of three-dimensional acinar structures formed by PC3-ML cells stably transfected with a scrambled shRNA, a shRNA targeting Erk2 or a shRNA targeting c-myc construct and grown as a single cell suspensions in Matrigel for 12 days.

Table 1. Summary of PC3-ML tumor development in NOD-SCID.

Cell Line	Thoracic Cavity	Tumor mass (mg)	Abdominal Cavity Met	Adrenal Metastasis	Liver Metastasis	Brain Metastasis
PC3-ML sh Scram	3/3	16.5 $\pm$ 11	2/3	2/3	2/3	1/3
PC3-ML sh Erk2	6/6	7.2 $\pm$ 3.0	1/6	1/6	1/6	0/6
PC3-ML sh c-myc	5/6	6.3 $\pm$ 3.1	0/6	0/6	0/6	0/6

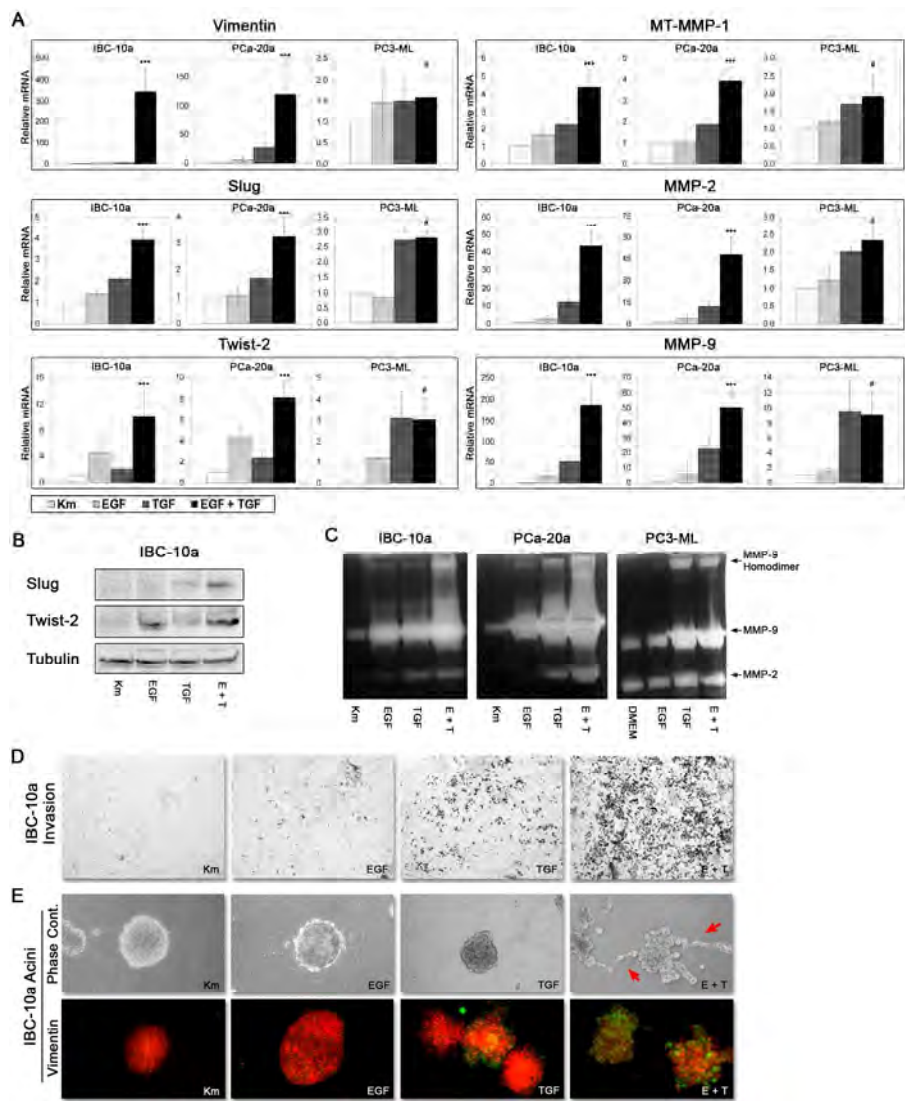


(A) Western Blots of crude cell extracts from cells treated with minimal media (Km), or Km containing 10 ng/ml EGF (EGF), 10 ng/ml TGF-beta (TGF) or 10 ng/ml of EGF + TGF-beta (E + T) as described in Materials and Methods. IBC-10a cells, PCa-20a cells and PCa-30a cells were isolated from Gleason Score (GS) 6, 7 and 8 prostate cancers, respectively. Blots were probed with antibodies specific for Fibronectin, E-cadherin, Vimentin, Fibroblast Specific Protein-1 (FSP-1) and Tubulin (loading control). (B) Images of IBC-10a cells cultured in Km, EGF, TGF-beta or E + T. Phase contrast images show a reduction in cell-cell contacts in E + T treated IBC-10a and acquisition of a spindle-shaped morphology (see red arrows) (Top Panel). Immunofluorescent images of cells labeled with antibodies specific for beta-catenin (Middle Panel; green) and Vimentin (Bottom Panel; green). Cell nuclei (red) were labeled with propidium iodide. (Top/Middle Panels: 400x magnification) (Bottom Panel: 200x magnification). (C) Western Blot analysis of E-cadherin, Vimentin and Actin (loading control) expression in IBC-10a cells treated with E + T at day 0 through 9, and 4 days post treatment, where cells were cultured in cKm. (D) Induction of EMT in prostate organ cultures. Phase Contrast and immunofluorescent imaging show prostate epithelial cells derived from GS6 prostate

cancer tissue, emigrating out of the tissue and strongly expressing vimentin (red) in response to concomitant E + T treatment, but not TGF-beta alone, Nuclei labeled with DAPI (200x magnification).

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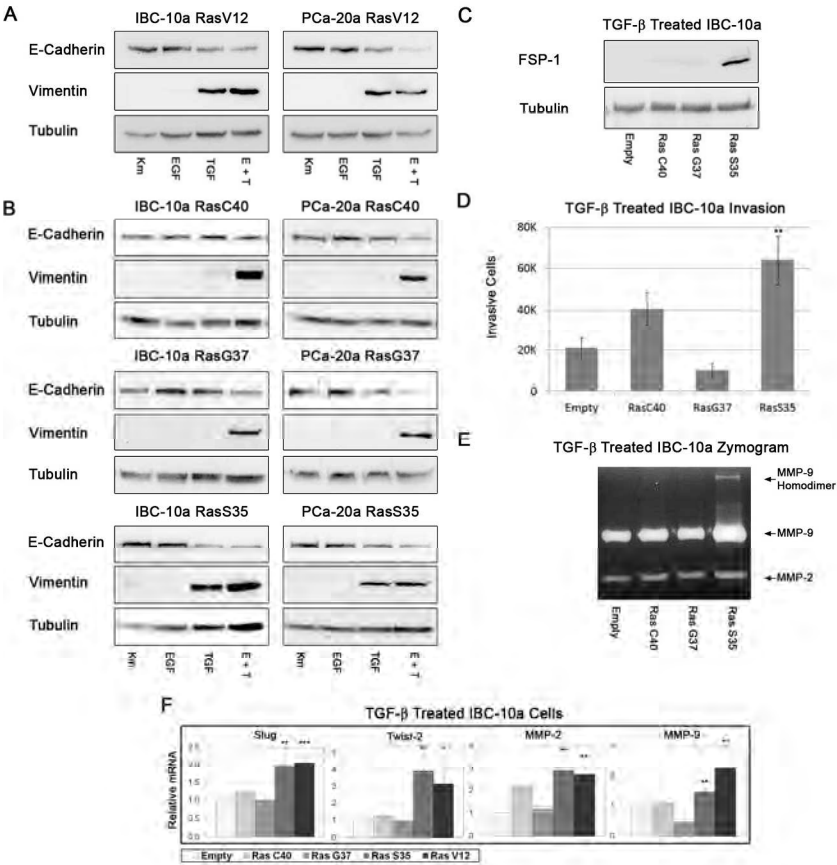
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(A) Expression of Vimentin, Slug, Twist2, MT-MMP-1, MMP-2 and MMP-9 genes in IBC-10a (left), PCa-20a (middle) and PC3-ML cells (right). Effects of EGF (10 ng/ml) (light gray), TGF-beta (10 ng/ml)(dark gray), and EGF + TGF-beta (10 ng/ml each) (E + T) (black) treatments on gene expression was determined by qRT-PCR. Values were normalized to cyclophilin-A and represent the fold increase in expression relative to cells treated with Km (white). Data represent the mean +/- S.D. from three independent experiments. Statistical significance comparing TGF-beta treatment alone to E + T was determined using Welch's unpaired t-test, where * = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001; # = no significant difference (p-value > 0.1). (B) Western Blot analysis of Slug and Twist2 in IBC-10a cells treated 9 days with Km, EGF, TGF-beta or E + T. Tubulin served as the loading control. (C) Representative gelatin zymographs of conditioned media from IBC-10a, PCa-20a and PC3-ML cells treated with EGF, TGF-beta or E + T. Equal amounts of protein from conditioned medium from each treatment was resolved by 10% PAGE containing gelatin-A (2mg/ml). Coomassie Blue staining reveals relative levels of enzymatically active MMP-9 homodimer (210 kDa), MMP-9 (92kDa) and MMP-2 (72kDa) in media. (D) Modified Boyden Chamber

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3 Invasion assays showing cell migration following treatment with Km, EGF, TGF-beta or E + T for 48 hr.
4 Representative image of cells on bottom of chamber stained with brilliant blue dye. (E) Prostatesphere culture
5 showing three-dimensional acinar structures formed by IBC-10a cells grown as single cell suspensions in
6 Matrigel for 12 days in the presence of different growth factors. (Top Panel) Phase contrast images shows
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9 arrows). (Lower Panel) Immunofluorescent labeling of Prostatespheres with Vimentin antibodies
10 (green). Nuclei labeled with propidium iodide (200x magnification).
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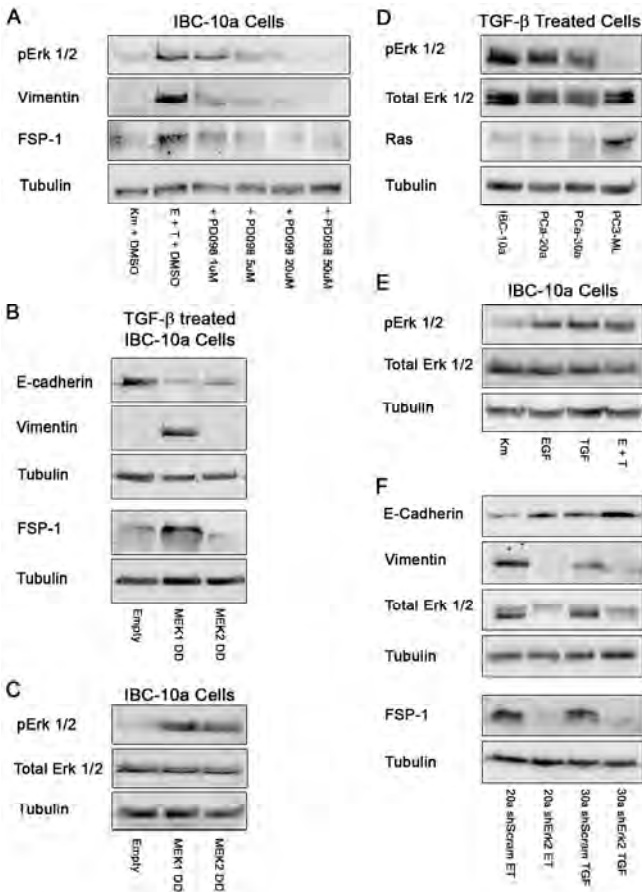


(A) Western Blot analysis for E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a (left) and PCa-20a (right) cells stably transfected with pBABE:RasV12, and treated with minimal media (Km), EGF (10 ng/ml), TGF- β (10 ng/ml), or EGF + TGF-beta in combination (E + T). (B) Western Blot analysis for E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a and PCa-20a cells stably transfected with pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 vectors. Cells were treated with Km, EGF, TGF- β , or E + T. (C) Western Blot analyses of FSP-1 and Tubulin (loading control) expression in IBC-10a cells stably transfected with Empty vector (Empty), pBABE:RasV12-C40, pBABE:RasV12-G37, or pBABE:RasV12-S35 and treated with TGF-beta. (D) Quantification of Modified Boyden Chamber invasion assays showing the invasive activity of IBC-10a cells stably transfected with Empty vector (Empty), pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 variants and treated with TGF-beta. Values are representative of three independent replicates. Mean $\pm$ SD, where ** = p-value < 0.01, compared to Empty vector control. (E) Gelatin zymographs of conditioned media 24 hours after last treatment from pBABE:RasV12-C40, pBABE:RasV12-G37, and pBABE:RasV12-S35 and pBABE:Empty

variants of IBC-10a cells treated with TGF-beta. (F) Relative expression of Slug, Twist-2, MMP-2 and MMP-9 mRNA in IBC-10a cells transfected with pBABE:RasV12-C40 (light gray bars); pBABE:RasV12-G37 (dark gray bars); pBABE:RasV12-S35 (black bars); and pBABE.Empty (white bars) variants treated with TGF- β by qRT-PCR. Values normalized against cyclophilin-A and represent the fold increase in expression compared to pBABE:Empty cells. Mean $\pm$ 1 S.D. from 3 independent experiments. Statistical significance was derived using Welch's unpaired t-test, where * = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001 when comparing RasV12 and RasV12-S35 cells to pBABE:Empty cells.

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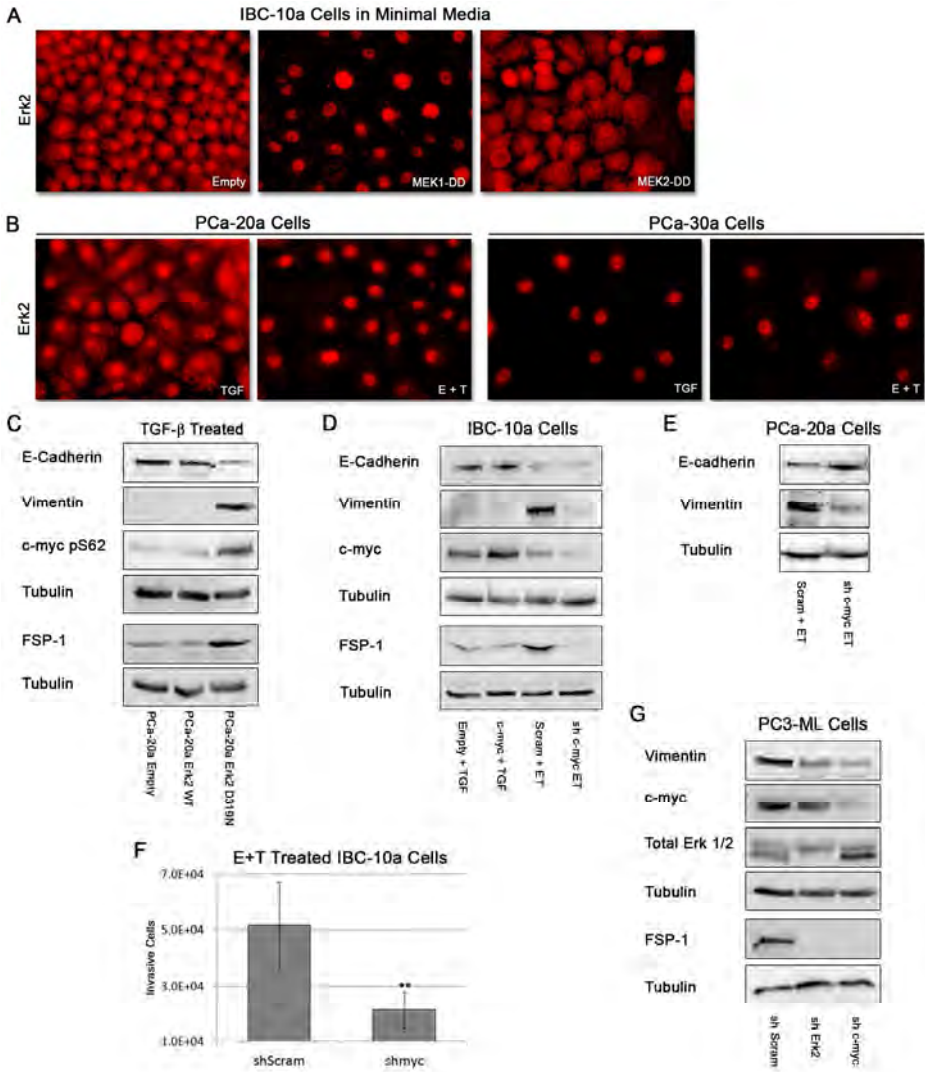
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(A) Western Blot analysis of phosphorylated-Erk 1/2, Vimentin, FSP-1 and Tubulin (loading control) expression in IBC-10a cells cultured in either Km with 0.5% DMSO (Km + DMSO), Km with 10 ng/ml of EGF + TGF-beta with 0.5% DMSO (E + T + DMSO), or Km with 10 ng/ml of EGF + TGF-beta with increasing concentrations of the MEK1/2 inhibitor PD09859 (+PD098). (B) Western Blot analysis of E-cadherin, Vimentin and Tubulin (loading control) expression in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD), and Empty vector (pBABE:Empty) and treated with TGF-beta (10 ng/ml). (C) IBC-10a cells from (B) were cultured in minimal media the expression of phosphorylated-Erk1/2, total-Erk 1/2 and Tubulin (loading control) examined by Western Blot. (D) Western Blot analysis of phosphorylated-Erk1/2, total-Erk1/2, Ras and Tubulin (loading control) expression in IBC-10a, PCa-20a, PCa-30a and PC3-ML cells following treatment with to TGF-beta. (E) Western Blot analysis of phosphorylated-Erk1/2, total-Erk1/2 and Tubulin (loading control) expression in IBC-10a cells treated with minimal media (Km), EGF (10 ng/ml), TGF-beta (10 ng/ml), or EGF + TGF-beta (10 ng/ml) (E + T). (F) Western Blot analysis of E-cadherin, Vimentin, FSP-1, total Erk1/2 and Tubulin (loading control) expression

in PCa-20a and PCa-30a cells stably transfected with a scrambled shRNA construct or a shRNA construct targeting Erk2 after treatment with TGF-beta, EGF, or E + T.
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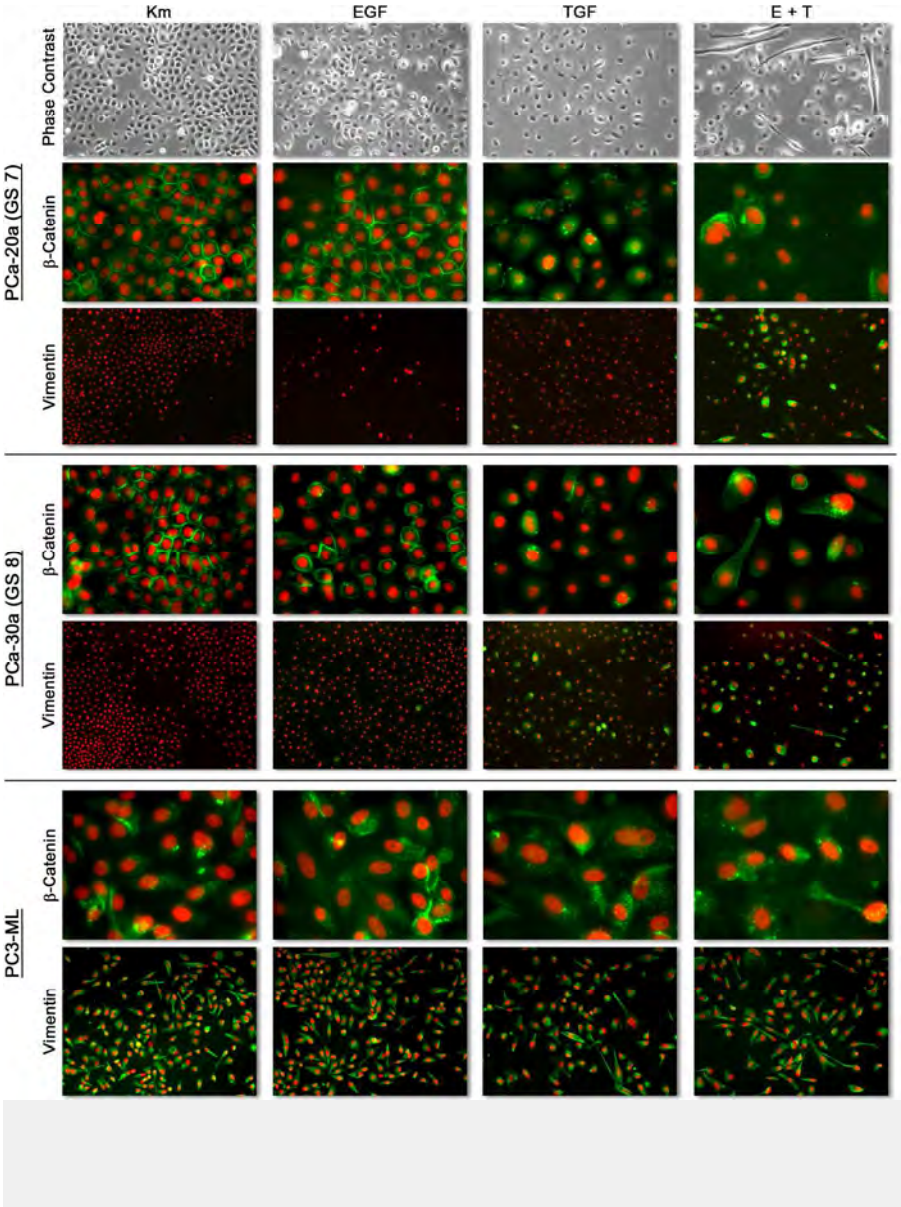
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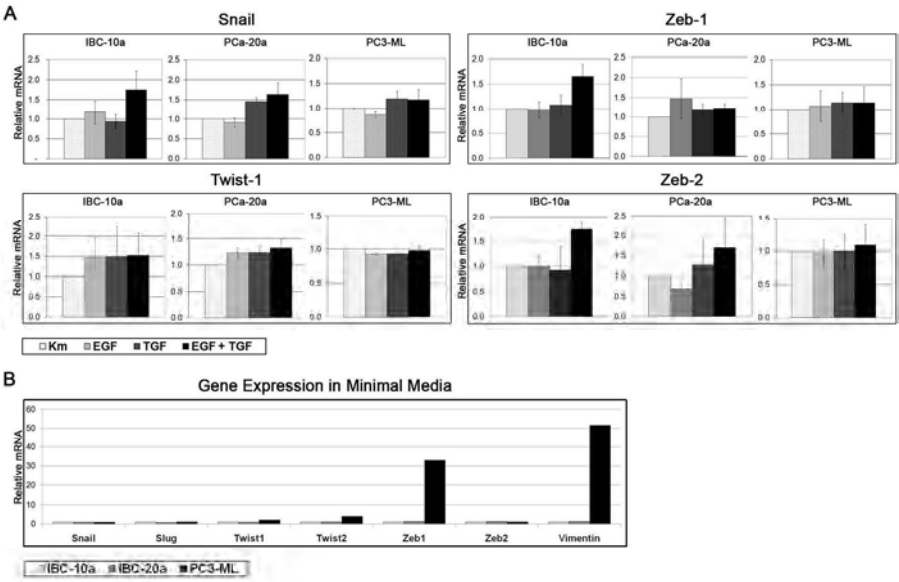
(A) Immunofluorescent imaging of Erk2 localization (red) in IBC-10a cells transfected with pBABE:Empty, pBABE:MEK1-DD, or pBABE:MEK2-DD. Cells were grown in minimal media and probed for Erk2 localization (400x magnification). (B) Immunofluorescent images of PCa-20a and PCa-30a cells treated with TGF-beta (10 ng/ml) (TGF) or EGF + TGF-beta (10 ng/ml each) (E + T) (400x magnification). (C) Western Blot analysis of E-cadherin, Vimentin, serine 62 phosphorylated c-myc (c-myc pS62), FSP-1, and Tubulin (loading control) expression in PCa-20a cells stably transfected with Erk2 WT construct (pLNCX:HA-Erk2), a phosphatase resistant mutant Erk2 (pLNCX:HA-Erk2 D319N) or empty vector (pBABE:Empty) as control and treated with TGF-beta. (D) Western Blot analysis of E-cadherin, Vimentin, c-myc, FSP-1 and Tubulin (loading control) expression in IBC-10a cells stably transfected with an empty vector, c-myc overexpression, scrambled shRNA or a shRNA targeting c-myc construct. Cells were treated with TGF-beta alone or concomitant EGF + TGF-beta (E + T). (E) Western Blot analysis of Vimentin, c-myc, total-Erk 1/2, FSP-1 and Tubulin (loading control) expression in PC3-ML cells stably transfected with a scrambled shRNA, a shRNA targeting Erk2 or a shRNA targeting c-myc construct. (E) Phase contrast imaging of three-

dimensional acinar structures formed by PC3-ML cells stably transfected with a scrambled shRNA, a shRNA targeting Erk2 or a shRNA targeting c-myc construct and grown as a single cell suspensions in Matrigel for 12 days.
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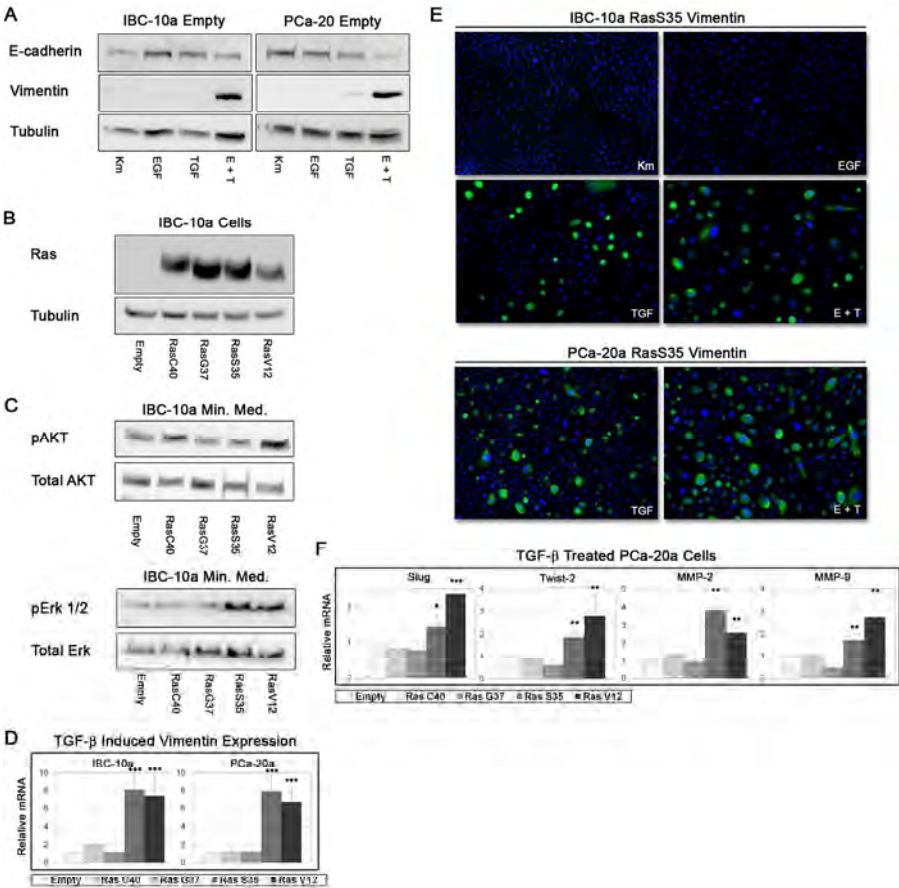


Immunofluorescent images of PCa-20a and PCa-30a cells grown in minimal media (Km), EGF (10 ng/ml), TGF-β1 (10 ng/ml) (TGF) or EGF + TGF-β1 (10 ng/ml each) (E + T). Similarly, PC3-ML cells were grown in DMEM supplemented with EGF, TGF-β or E + T. Cells were labeled with antibodies for β-catenin (top row; green, 400x magnification) and Vimentin (bottom row; green, 200x magnification). Cell nuclei (red) were labeled with propidium iodide. 175x235mm (300 x 300 DPI)



(A) qRT-PCR measurements of gene expression in IBC-10a (left), PCa-20a (middle) and PC3-ML cells (right). Cells were treated with in Km (white), 10 ng/ml of EGF (light gray), 10 ng/ml TGF- β 1 (dark gray) or 10 ng/ml EGF + TGF- β 1 (black). Primers were specific for Snail, Twist1, Zeb1 and Zeb2. Values normalized for cyclophilin-A and represented as the fold expressed relative to cells treated with Km (set at 1). Values represent the mean $\pm$ 1 S.D. from 3 experiments. (B) qRT-PCR analysis of gene expression in IBC-10a (white), PCa-20a (gray) and PC3-ML cells (black) cells grown in Km for 10 days. Shows basal levels of Snail, Slug, Twist1, Twist2, Zeb1, Zeb2 and Vimentin mRNA normalized for cyclophilin-A mRNA and represented as fold expressed relative to IBC-10a cells.

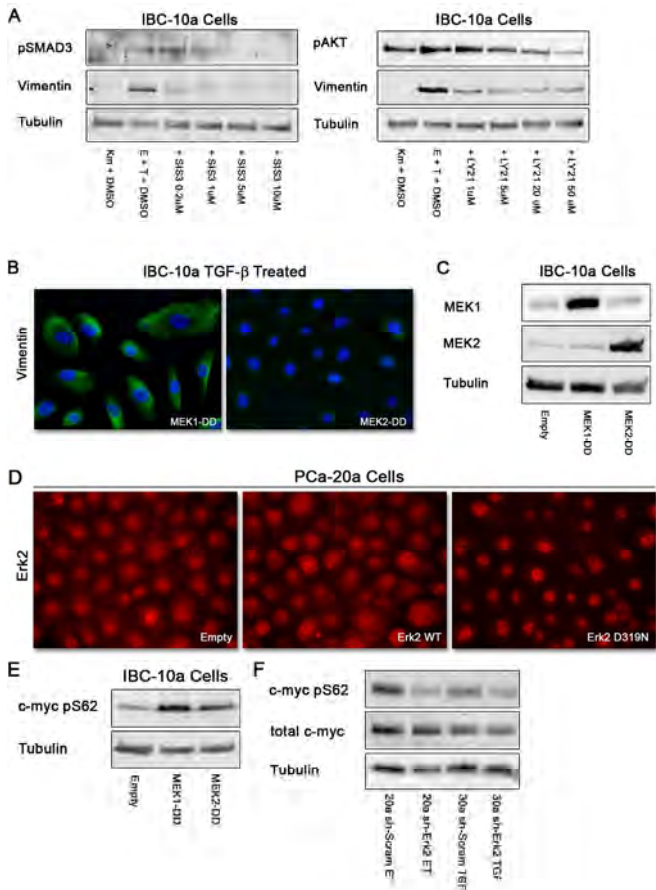
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(A-C) Western Blots of crude cell extracts from IBC-10a cells stably transfected with Empty vector (pBABE:Empty), pBABE:RasV12 or Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; and pBABE:RasV12-S35). Variants were grown in minimal media (Km). Blotted with antibodies specific for E-cadherin, Vimentin, Ras, pAKT, total AKT, pErk1/2, total Erk 1/2 and Tubulin (loading control). (D) qRT-PCR analysis of Vimentin mRNA in IBC-10a (left) and PCa-20a (right) cells stably transfected with a constitutively active Ras construct (pBABE:RasV12) or Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) and Empty vector (pBABE:Empty) and treated with TGF- β (10 ng/ml). Values normalized for cyclophilin-A and represented as fold expressed relative to empty vector. Values represent the mean $\pm$ 1 S.D. from 3 experiments. (E) Immunofluorescent images of IBC-10a and PCa-20a cells stably transfected with pBABE-S35 and grown in minimal media (Km), or Km plus 10 ng/ml EGF (EGF), TGF- β 1 (TGF) or EGF + TGF- β 1 (E + T). Cells labeled with Vimentin (green, 200x magnification). Cell nuclei (blue) labeled with DAPI. (F) qRT-PCR analysis of Slug, Twist-2, MMP-2 and MMP-9 mRNA levels in IBC-20a cells stably transfected with pBABE:RasV12-C40 (light gray); pBABE:RasV12-G37 (dark gray);

pBABE:RasV12-S35 (black); and pBABE:Empty (white) and treated with TGF- β . Values normalized for cyclophilin-A and represented as fold expressed relative to empty vector cells. Values represent the mean $\pm$ 1 S.D. from 3 experiments.
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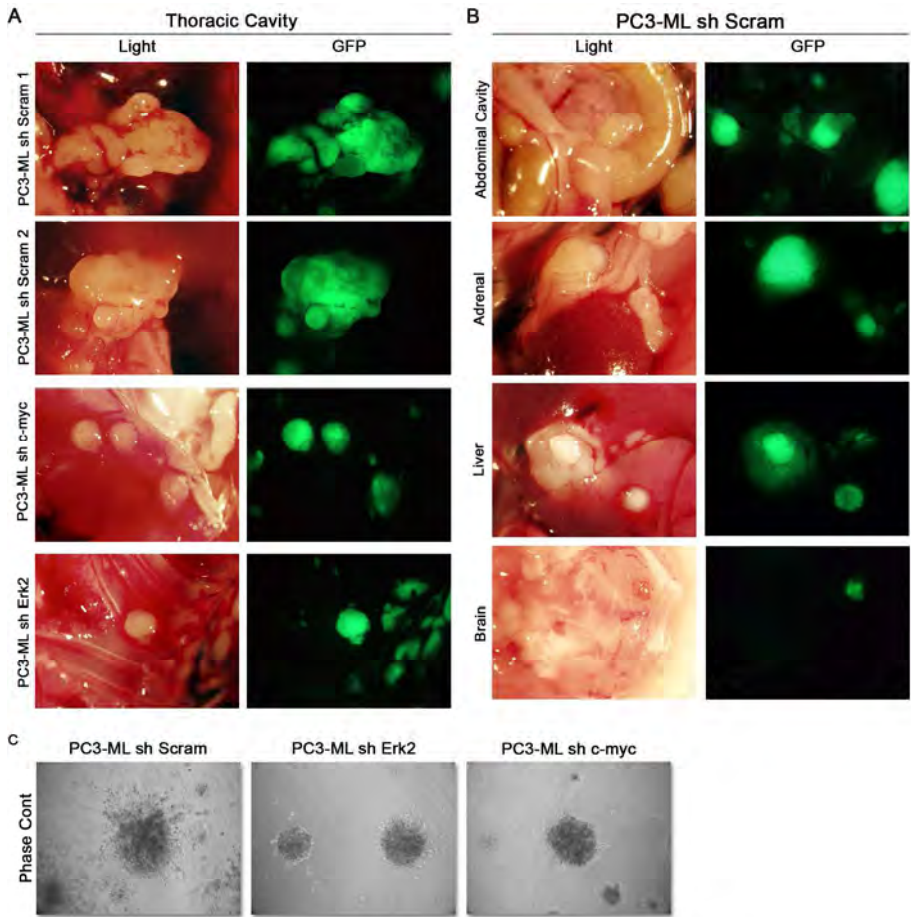
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(A) Western Blots of crude cell extracts from IBC-10a cells grown in either Km with 0.5% DMSO (Km + DMSO), or Km supplemented with 10 ng/ml of EGF + TGF-beta1 with 0.5% DMSO (E + T + DMSO); or Km supplemented with 10 ng/ml of EGF + TGF-beta1 with increasing concentrations of either SIS3 (left)(a SMAD3 inhibitor) or LY290042 (right) (a PI3-kinase inhibitor). Blotted with antibodies specific for phosphorylated SMAD 3 (pSMAD3) (left) or phosphorylated AKT (pAKT) (right), or with Vimentin, Fibroblast Specific Protein-1 (FSP-1) and Tubulin (loading control) antibodies. (B) Western Blots of crude cell extracts from IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD) constitutively active MEK2 (pBABE:MEK2-DD) and Empty vector (pBABE:Empty) and blotted with antibodies for MEK1, MEK2 and Tubulin (loading control). (C) Immunofluorescent images of IBC-10a cells transfected with activated MEK constructs (pBABE:MEK1-DD and pBABE:MEK2-DD), treated with TGF-beta and labeled for Vimentin (green; 400x magnification). Nuclei (blue) stained with DAPI. (D) Immunofluorescent images of PCa-20a cells transfected with empty vector (pBABE:Empty), a WT Erk2 construct (pLNCX:HA-Erk2) or a phosphatase resistant Erk2 construct (pLNCX:HA-Erk2 D319N). Cells were plated in growth media and grown in minimal

media for 24 hrs and probed for Erk2 localization (red, 400x magnification.). (E) Western Blot analysis of crude cell extracts from IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD), and Empty vector (pBABE:Empty) grown in minimal media and blotted for serine 62 phosphorylated c-myc (c-myc pS62) and Tubulin (loading control). (F) Western Blot analysis of crude cell extracts from PCa-20a and PCa-30a cells stably transfected with a scrambled shRNA construct or an shRNA construct targeting Erk2 after treatment with 10 ng/ml EGF and TGF-beta in combination (E + T) or TGF-beta alone (TGF), respectively. Blotted with antibodies to serine 62 phosphorylated c-myc (c-myc pS62), total c-myc and Tubulin (loading control).
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Representative light and green fluorescent images of tumors within the thoracic cavity of mice injected intercardiacally with PC3-ML cells stably transfected with a scrambled shRNA construct, a shRNA targeting Erk2 construct or a shRNA targeting c-myc construct 5 weeks post injection. (B) Representative light and green fluorescent images of tumors found within the abdominal cavity, adrenal gland, liver and brain of mice injected intercardiacally with PC3-ML cells stably transfected with a scrambled shRNA construct 5 weeks post injection. (C) Phase contrast imaging of three-dimensional acinar structures formed by PC3-ML cells stably transfected with a scrambled shRNA, a shRNA targeting Erk2 or a shRNA targeting c-myc construct and grown as a single cell suspensions in Matrigel for 12 days.

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Table 1. Summary of PC3-ML tumor development in NOD-SCID.

Cell Line	Thoracic Cavity	Tumor mass (mg)	Abdominal Cavity Met	Adrenal Metastasis	Liver Metastasis	Brain Metastasis
PC3-ML sh Scram	3/3	16.5 ± 11	2/3	2/3	2/3	1/3
PC3-ML sh Erk2	6/6	7.2 ± 3.0	1/6	1/6	1/6	0/6
PC3-ML sh c-myc	5/6	6.3 ± 3.1	0/6	0/6	0/6	0/6



Let-7 miRNA down regulation via EGF-Raf promotes TGF-beta induced epithelial-mesenchymal transition in prostate cancer cells

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Key Words:	TGF-beta, EMT, Cancer Invasion, Let-7, HMGA2

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Title: Let-7 miRNA down regulation via EGF-Raf promotes TGF-beta induced epithelial-mesenchymal transition in prostate cancer cells

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Running Head: Let-7a Down-regulation is required for EMT.

Abstract:

Background

Identifying mechanisms by which prostate epithelial cells transform to an invasive phenotype is critical to the development of diagnostics and therapeutics which can identify the metastatic potential of cancers and possibly prevent their metastases. We have previously observed that EGF regulates TGF-beta induced invasion and epithelial to mesenchymal transition (EMT) in prostate epithelial cells.

Methods

We isolated primary prostate epithelial cell lines from lower Gleason Score human prostate cancers and utilized a variety of molecular biology techniques to examine how EGF regulates TGF-beta induced invasion and EMT in these cells.

Results

We identify that EGF contributes to EMT by down regulation of the tumor suppressor microRNA let-7a which targets Ras and c-myc, both important promoters of the EMT program. We show that let-7a down-regulation is critical for TGF-beta's tumor promoting activities as over-expression of let-7a, inhibits the ability of these cells to undergo EMT and inhibits cellular invasion in metastatic cells. Furthermore, we observe decreases in let-7a expression in human prostate cancer samples when compared to normal tissues and increases in expression of HMGA2, a let-7a target that is associated with invasive and metastatic disease.

Conclusions

We conclude that early stage prostate cancer cells require let-7a down-regulation to undergo TGF-beta induced EMT and transformation to an invasive phenotype, and that HMGA2 might serve as a diagnostic marker for the invasive and metastatic potential of prostate cancer.

Introduction

During development cells of epithelial and mesenchymal origin convert between the two phenotypes in what has been described as Epithelial-Mesenchymal Transition (EMT) and Mesenchymal-Epithelial Transition (MET). Studies in cancer models have identified an analogous plasticity of some epithelial cancer cells which acquire mesenchymal features as a means to escape the primary tumor mass and prime themselves for metastasis [1]. EMT can be phenotypically characterized as a loss of cell-cell contacts along with the cobblestone network characteristic of epithelial tissues, and adoption of a spindle-shaped morphology and migratory phenotype. This process is associated with changes in gene expression that include the down-regulation of E-cadherin and up-regulation of vimentin [1, 2]. Importantly, these changes correlate with increasingly invasive and aggressive tumors associated with poorer patient prognosis [3-5]. Silencing of vimentin or re-expression of E-cadherin in invasive cells decreases their invasive phenotype; emphasizing that EMT associated changes in gene expression play a major role in controlling the invasive behavior of tumor cells [6-8]. Therefore, understanding the molecular mechanisms regulating EMT in prostate cancer will aid in identification of new tumor biomarkers or therapeutics to target cells with a higher metastatic potential.

EMT is induced by various growth factors, with transforming growth factor-beta (TGF-β) appearing to be the most ubiquitous instigator of EMT during development and cancer [1, 9, 10]; however, many cancer cell lines exhibiting proficient TGF-β signal transduction, do not undergo TGF-β mediated EMT. Rather, EMT is dependent on additional factors including sustained elevated levels of active Ras-MAPK signaling, which may result from Ras over-expression or hyperactivity [11-17]. We have recently found that in conjunction with TGF-β signaling, oncogenic pathways activated by EGF, such as MEK1 activation of Erk2 and c-myc expression, are also required for efficient EMT in prostate epithelial cells[18]. Interestingly, in normal adult epithelial cells TGF-β functions as a tumor suppressor inducing G1 growth arrest, however as malignancies progress TGF-β signaling promotes tumor invasion and metastasis in part through EMT [19]. Therefore, we hypothesize that tumor suppressive pathways exist that prevent TGF-β induced EMT in normal and early stage cancer cells which EGF signaling can inhibit.

MicroRNAs (miRNAs) have been shown to be key players in a broad range of developmental and physiological processes and their deregulation has been closely linked to human diseases including cancer. miRNAs are a diverse family of 18–24 nucleotide non-coding RNAs that post-transcriptionally regulate the stability and translational efficiency of target mRNAs through imperfect complimentary base pairing with specific sequences in their 3' untranslated regions (UTRs). One of the more intriguing miRNAs that might serve as a master regulator of EMT and whose role in EMT has not been adequately addressed is the let-7 family of miRNAs. Let-7 was one of the first mammalian miRNAs to be identified and is ubiquitously expressed in normal adult tissues [20, 21]. However, in a plethora of human malignancies, including lung, ovarian, gastric, prostate cancer and melanoma, down-regulation of let-7 family members occurs during tumor development [22–26]. The human let-7 family of miRNAs possesses potent tumor suppressor activity by targeting multiple oncogenes, including those involved in EMT. Specifically, let-7 has been shown to directly target Ras, negatively correlate with Ras expression, target the proliferative transcription factor c-myc and inhibit the growth of human cancer cells [27–29]. In addition, the let-7 family of miRNAs are also strong negative regulators of high mobility group A2 protein (HMGA2, also known as HMGI-C), binding to multiple target sites in its 3' UTR [30]. HMGA2 is a chromatin associated protein that is widely expressed during early embryogenesis and cancer, however, it is almost completely absent in differentiated cells and normal adult tissues [31]. Interestingly, HMGA2 expression has been described as a critical factor necessary for TGF- β induced EMT in mouse primary mammary epithelial and human pancreatic cancer cells [32, 33]. Recently it has also been shown that over-expression of Lin-28B, which negatively regulates let-7 and targets it for degradation, is sufficient to induce EMT and invasion in hepatocellular carcinoma cell lines [34, 35]. Moreover, miRNA expression analysis of cancer cell lines suggests that let-7 is a marker for less advanced cancers and negatively correlates with reactivation of embryonic mesenchymal genes [36]. However, the significance of Lin-28 effects on let-7a expression in promoting EMT and tumor progression remain unclear.

We have found that the addition of EGF or transfection with Ras mutants that specifically activate Raf to TGF- β treatment induces EMT in primary prostate epithelial cell lines isolated from early stage human prostate cancers. In this report our data shows that EGF induces down-regulation of let-7a through a Ras-Raf

1 signaling cascade which correlates with EMT. Mechanistic studies revealed that let-7a expression controls
2 cellular response to TGF-beta by inhibiting their ability to undergo EMT and become invasive. Finally we
3 show that expression of let-7a is lost in advanced and invasive human prostate cancer and that HMGA2, a target
4 of let-7a, is induced in invasive and metastatic cancer. Taken together, these results suggest that decreases in
5 let-7a expression are required for TGF-β induced EMT and invasive phenotypes and that HMGA2 might be a
6 marker for the invasive and metastatic potential of prostate cancer.
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17 **Materials and Methods**

18 *Cells*

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20 IBC-10a and PCa-20a cells were isolated from the right peripheral zone of a Gleason score 6 and 7
21 prostate tumors respectively, as previously described[37]. IBC-10a cells were immortalized by stable
22 transfection with the pLXSN-hTERT retroviral plasmid (courtesy of John Rhim, USUHS, Bethesda, MD) and
23 identified as intermediate basal cells. They possess minimal gross chromosomal abnormalities and express
24 CK5, CK18, p63, PSA and PTEN[37]. PCa-20a cells expressed CK18, PTEN and PSA but not CK7 or p63.
25 Cells were maintained in serum free complete keratinocyte media (cKm) containing EGF, bovine pituitary
26 extract (Invitrogen Inc., Carlsbad, CA) and 50ug/ml penicillin/streptomycin (Mediatech, Manassas, VA). PC3-
27 ML cells were isolated from PC3 prostate cancer cells (ATCC, Bethesda, MD) based on their ability to
28 metastasize to the lumbar vertebrae[38]. PC3-ML cells were maintained in DMEM with 10% fetal bovine
29 serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 50 ug/ml penicillin/streptomycin (Mediatech,
30 Manassas, VA).
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47 Ras V12S35, RasV12C40 and RasV12G37 were stably over-expressed in both IBC-10a and PCa-20a
48 cells using the pBABE-puro retroviral vector (a kind gift from Dr. Christian Sell, Drexel University College of
49 Medicine). MEK1-DD and MEK2-DD were also over-expressed in cells using the pBABE-puro retroviral
50 vector (a kind gift of Dr. Mauricio Reginato, Drexel University College of Medicine). Scrambled shRNA
51 constructs and shRNA constructs targeting Lin-28B (TRCN0000140870) were purchased from Sigma (St.
52 Louis, MO). The Pre-let-7a-1 oligonucleotide was obtained from Promega and Age1/EcoR1 sites were inserted
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by PCR. Pre-let-7a-1 oligonucleotide was then cloned in the pBABE vector according to Sambrook et al. [39]. Retroviral and lentiviral production and maintenance of transfected cells was carried out according to methods previously described[40].

Reagents and Antibodies

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Primary antibodies targeting Vimentin were purchased from Sigma-Aldrich, St. Louis, MO (V6389); E-cadherin, Tubulin, Slug, Lin28A and Lin-28B were purchased from Cell Signaling Technology, Beverly, MA (2148, 4065, 9585, 3978 and 4196 respectively); Twist2 was purchased from Abcam, Cambridge, MA (ab57997) and HMGA2 was purchased from BioCheck, Foster City, CA (59170AP).

Epithelial-Mesenchymal Transition (EMT) Induction

Unless otherwise stated, for *in vitro* induction of EMT, cells were trypsinized and plated in cKm at a low density (2×10^4 cells/ml). The next day (day 0) cells were washed once in Km without supplements (i.e. pituitary extract and EGF) and media was replaced with Km supplemented with TGF- β 1 (10 *ng/ml*; Peprotech, Rocky Hill, NJ) and/or EGF (10 *ng/ml*; Invitrogen, Carlsbad, CA). Media in all experiments was changed on days 3, 6 and 9, and cells were analyzed on day 10.

Western Blotting

Western blots were performed according to standard procedures previously described[41]. Briefly, denatured protein lysates in Laemmli buffer were resolved on an SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Bellerica, MA) using a semi-dry transfer cell (Bio-Rad Laboratories, Inc, Hercules, CA). Membranes were blocked in 5% non-fat dry milk and incubated overnight at 4°C with primary antibodies in 3% BSA.. Secondary antibody HRP reactions were visualized and documented using Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL) and the ChemiDoc™ XRS Gel Documentation system (Bio-Rad Laboratories, Inc., Hercules, CA).

Northern blotting

Total RNA from samples was isolated using the SV Total RNA Isolation Kit (Promega, Madison, WI). Approximately 20 µg per lane was used for Northern blotting as described previously [42]. Antisense, 32P-ATP end-labeled DNA oligonucleotides [20 nucleotides (nt); Dupont Nucleotides, Inc.], which recognize let-7 mature miRNA, and 5S RNA, were used for Northern blots. Blots were visualized using HyBlot CL autoradiography film (Denville Scientific, Inc, Metuchen, NJ) and developed on a Kodak X-OMAT 1000A Processor (Kodak, Rochester, NY).

Quantitative Real-Time PCR

Total small RNA was isolated using the Absolutely RNA miRNA isolation kit (Agilent, La Jolla, CA) per manufacturer instructions. miRNA's were polyadenylated and cDNA was synthesized using the miRNA 1st strand cDNA synthesis kit (Agilent, La Jolla, CA) per manufacturer instructions. cDNA corresponding to target RNAs were amplified using the Brilliant SYBR Green qPCR kit (Aligent, La Jolla, CA) and the MX3000P thermocycler (Agilent, La Jolla, CA) per manufacturer instructions. A universal reverse primer and forward primer specific for let-7a (GAGGTAGTAGGTTGTATA) and U6 (Purchased from Aligent, La Jolla, CA) were used. Quantification was performed utilizing the 2^{-ddCt} method, where empty-vector or parent cells were used to normalize gene expression. Relative internal miRNA expression of target genes was normalized to U6 RNA expression in each sample. Each sample for each experiment was run in duplicate and averages are representative of three independent experiments. Statistical significance was determined using Welch's unpaired t-test.

Invasion Assays

Following experimental treatments cells were trypsinized and seeded onto Matrigel coated invasion inserts with 0.8 µm porous membranes (BD Biosciences, Bedford, MA) at a density of 6x10⁴ cells per well in cKm (for IBC-10a cell variants) or at a density of 3 x10⁴ cells per well in DMEM (for PC3-ML cell variants)

and allowed to attach for 2 hrs. Media on the top chamber was then changed to experimental condition and bottom chamber was filled with media containing 5% FBS (Atlanta Biologicals, Lawrenceville, GA). Transwells were placed at 37°C for 48 hrs. Cells in top compartment were scraped off and cells which migrated to bottom were fixed with 4% paraformaldehyde, stained with propidium iodide and counted. Data were averaged from three independent experiments. Statistical significance was determined using Welch's unpaired t-test.

Prostasphere Assay

Cells were Typsinized and plated at a density of 1×10^3 cells per well on a growth factor reduced Matrigel (BD Biosciences, Bedford, MA) coated 8 well chamber slides (BD Biosciences, Bedford, MA) and topped with DMEM containing 0.2% FBS (Atlanta Biologicals, Lawrenceville, GA) and 5% Matrigel. Media was changed every 3 days with experimental condition and 5% Matrigel. Prostasphere acini were analyzed after 12 days of culture.

In situ hybridization

Tissue sections for *in situ* hybridization were purchased from Usbiomax (Cat# T191, Rockville, MD). Biotin labeled let-7a LNA probes were purchased from Exiqon (Möln, MA) and hybridization was done modified from manufacturer instructions. Briefly, tissue samples were rehydrated in sequential washes with xylene followed by decreasing concentrations of EtOH in Depec treated PBS. Tissue was then incubated for 15 min in 5 ug/ml proteinase k in Depec-H₂O followed by 20 min in 0.2N HCl and 10 min in 0.25% acetic anhydride in 0.1M triethanolamine to acetylate remaining proteins. Sample was then incubated for 2 hr in Pre-hybridization mix at 45°C followed by an overnight incubation with LNA probe (5 nM final concentration) in Hybridization mix (HM) at 50°C. A series of stringency washes were performed to wash unhybridized probe. Sequentially samples were washed in 100% HM at hybridization temperature, 75% HM/25% 2x SCC at hybridization temperature, 50% HM/50% 2x SCC at hybridization temperature, 25% HM/75% 2x SCC at hybridization temperature, 100% 2x SCC at hybridization temperature, 0.2x SCC at hybridization temperature,

0.2x SCC/25% PBS + 0.05% Tween-20 (PBST) at room temperature, 50% 0.2x SCC/50% PBST at room temperature, 25% 0.2x SCC/75% PBST at room temperature, 100% PBST. Samples were then blocked using Peroxidase quenching solution and 50% Blocking buffer/50% PBST (Cat# 1859346, Thermo Scientific, Waltham, MA). Samples were then incubated with Streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) in 50% Blocking buffer/50% PBST at room temperature for 2 hrs. HRP activity was visualized after a 60 min incubation with DAB substrate (Cat# 1859346, Thermo Scientific, Waltham, MA). Slides were finally counterstained, washed, dehydrated and mounted.

Immunohistochemistry

Tissue sections for immunohistochemistry were purchased from Usbiomax (Cat# PR484, Rockville, MD). Briefly, tissue samples were rehydrated in sequential washes with xylene followed by decreasing concentrations of EtOH in PBS. Antigen retrieval was done by incubating slides in Citrate buffer for 20 min at 95°C and allowed to cool to room temp. Samples were then blocked using Peroxidase quenching solution and Blocking buffer (Cat# 1859346, Thermo Scientific, Waltham, MA) followed by an overnight incubation at 4°C with primary HMGA2 antibody (59170AP, BioCheck, Foster City, CA) in Blocking buffer. Samples were then incubated with biotinylated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) in Blocking buffer at room temperature for 1 hr followed by an incubation in Streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) in Blocking buffer at room temperature for 1 hr. HRP activity was visualized after a roughly 15 min incubation with DAB substrate (Cat# 1859346, Thermo Scientific, Waltham, MA). Slides were finally counterstained, washed, dehydrated and mounted.

Results

We have previously isolated primary prostate epithelial cells (termed IBC-10a and PCa-20a) from prostate cancer samples of Gleason Score 6 and 7, respectively. These cells grow as monolayers, forming uniform confluent ‘cobblestone’ patterns. We found that co-treatments of cells with both EGF (10 ng/ml) and

1 TGF- β (10 ng/ml) (E + T) for 10 days was required for the down-regulation of E-cadherin, induction of
2 vimentin expression and acquisition of a spindle-shaped morphology and invasive phenotype characteristic of
3 EMT[18]. Furthermore, we found that EMT was dependent on signaling through Ras-Raf-MEK1 and
4 expression of c-myc. Interestingly, both Ras and c-myc are known targets of the miRNA let-7a which has
5 surfaced as a potent tumor suppressor in a variety of cancers [28, 29]. Therefore, we sought to elucidate any
6 significance to let-7a expression in modulating TGF- β responses in our primary prostate epithelial cells lines.
7 We found that treatment of IBC-10a cells with EGF or with E + T resulted in a significant down-regulation of
8 the let-7a miRNA (Figure 1A). Additionally, the expression of the let-7a target gene, HMGA2, which is
9 associated with EMT, was induced with both EGF and E + T treatments in both IBC-10a and PCa-20a cells
10 (Figure 1B). Time course analysis of EMT indicated that vimentin expression began on day 6 and was robust by
11 day 9 (Figure 1C). Accordingly, let-7a expression was reduced on day 6 and strongly decreased by day 9
12 (Figure 1C).

13 Ras is known to be activated by EGF and is previously implicated in promoting TGF- β mediated EMT;
14 therefore, we sought to further understand the role of downstream Ras signaling in modulating let-7a
15 expression. We stably transfected IBC-10a cells with the Ras effector mutants; RasV12-C40, which specifically
16 binds to PI3-kinase and activates Akt signaling, RasV12-G37, which specifically binds RalGDS and activates
17 phospholipase D signaling, and RasV12-S35, which specifically binds cRaf and activates MAPK signaling
18 [43]. An empty vector (pBABE) was used as a control and the effect of TGF- β treatment alone was examined.
19 Only cells expressing the RasS35 mutant demonstrated a down-regulation of let-7a expression in response to
20 TGF- β treatment when compared to the other cell variants (Figure 1D). Moreover, only TGF- β treatment of
21 cells transfected with the RasS35 mutant induced expression of HMGA2 when compared to other cell variants
22 (Figure 1E). This data suggests that let-7a expression is being regulated by EGF activation of the Ras-Raf-
23 MAPK pathway, the same pathway that regulates EMT in these cells.

24 Mature miRNA mimetics can be excised from transcripts containing miRNA precursor sequences[44].
25 Thus, to directly assess the role of let-7a expression in EMT we transfected IBC-10a and PCa-20a cells with a
26 pBABE.pre-let-7a-1 construct to induce over expression of let-7a. Utilizing qRT-PCR we were able to detect a

roughly 2.5 fold increase in pre- let-7a in pBABE:pre- let-7a transfected cells treated with E + T compared to cells transfected with a pBABE empty vector (Figure 2A). While over-expressing let-7a did not induce discernible differences in cells grown in minimal media, we did observed that over-expression of let-7a in both IBC-10a and PCa-20a cells inhibited EMT as characterized by decreases in expression of the mesenchymal markers vimetin and Fibroblast Specific Protein-1 (FSP-1) (Figure 2B). Over-expression of pre-let7a was also associated with a decrease in the let-7a target gene HMGA2 (Figure 2B). Several transcription factors reported to be important regulators of EMT, including those of the Twist and Snail family, are essential for metastasis in a variety of cancers [45], and we observed that EMT in our cells correlated with an increase in Twist2 and Slug (Snail2) expression (Figure 2C). Consistent with the above results, over-expression of pre-let-7a inhibited expression of these transcription factors in response to E + T treatments (Figure 2C).

We extended our studies to PC-3ML cells which express EMT markers, are highly invasive and readily metastasize in mice to distant organ sites [46]. Furthermore, PC3-ML cells also over-express Ras when compared to less tumorigenic cell lines. Transfection of PC-3ML cells with the pre-let-7a expression construct decreased basal expression of Ras, HMGA2 as well as vimentin (Figure 2D). Importantly, transfection with the pre-let-7a expression construct also reduced their invasive capacity by roughly 50% as assessed by reduced migration through matrigel towards a serum gradient in a modified boyden chamber assay (Figure 2E). Taken together, over-expression of let-7a appears to inhibit the expression of mesenchymal genes and reduce the invasive capacity of cells.

Members of the Lin-28 protein family have been shown to induce let-7a degradation by terminal uridylation [35]. We sought to investigate whether changes in Lin-28 expression might regulate let-7a expression during EMT. We observed that while Lin-28A was not expressed in IBC-10a cells under any conditions, Lin-28B expression was enhanced by treatment with either EGF or the combination treatment of EGF and TGF- β (E+T) (Figure 3A). Notably, these are the same conditions associated with reduced let-7a expression in Figure 1A. Time course analysis indicated that Lin-28B expression was gradually increased over the 9 days of E + T treatment (Figure 3A), and consistent with our above findings, IBC-10a cells transfected with RasS35 mutant showed a robust increase in Lin-28B expression when treated with TGF- β . Again, IBC-

1 10a cells transfected with RasG37, RasC40 or Empty vector constructs showed no apparent affect on EMT, thus
2 indicating that Lin-28B was being induced through the Ras-Raf-MAPK pathway (Figure 3B). Given these
3 results we transfected IBC-10a cells with a shRNA targeting Lin-28B, or a scrambled shRNA as a control, to
4 assess the function of Lin-28B in the induction of EMT in our cells. We observed an efficient decrease in Lin-
5 28B expression as well as a failure to induce HMGA2 expression, suggesting enhanced expression of let-7a, in
6 E + T treated IBC-10a cells transfected with the Lin-28B shRNA construct (Figure 3C). Knockdown of Lin-
7 28B also inhibited IBC-10a cells from undergoing EMT as characterized by a failure to induce vimentin
8 expression and decrease E-cadherin expression when compared to control cells treated with E + T (Figure 3C).
9 Importantly, and in agreement with other reports showing Lin-28B expression promotes invasion [34],
10 knockdown of Lin-28B reduced the invasive capacity of IBC-10a cells by roughly 50% in a modified Boyden
11 chamber assay when treated with E + T (Figure 3D).

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Activation of MEK1/2 is the most well-characterized downstream effect of Ras/Raf signaling and is
critical for Ras induced transformation and EMT induction [47]. To further assess the signaling pathways
regulating Lin-28B expression we stably transfected IBC-10a cells with a constitutively active MEK1 or MEK2
construct (pBABE: MEK1-DD, pBABE:MEK2-DD, respectively) and empty vector (pBABE), as a control. We
observed that in conjunction with TGF- β treatment, constitutive MEK1 activity, but not MEK2, induced EMT
in IBC-10a cells (Figure 3E). Surprisingly, in response to TGF- β , MEK1-DD transfected cells displayed no
demonstrable increase in Lin-28B expression, despite inducing HMGA2 expression; an event thought indicative
of let-7a down-regulation (Figure 3E). Rather, constitutive MEK1 activity appeared to down-regulate Lin28B
expression when compared to cells transfected with constitutively active MEK2 or empty vector control (Figure
3E). We also observed by qRT-PCR that expression of let-7a was down-regulated by both MEK1-DD and
MEK2-DD transfection, suggesting that regulation of let-7a and its effects on EMT are associated with more
convoluted signaling dynamics than those explored in this current study. It appears that Lin-28B expression is
might not necessary for let-7a down-regulation, and likewise that down-regulation of let-7a is not sufficient for
TGF- β induced EMT. However, taken together, our results do suggest that Lin-28B expression is critical for
TGF- β induced EMT and invasive responses.

1 Regulators of EMT have been identified in a variety of cancers to regulate the metastatic ability of cells,
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3 thus expression of such regulators might serve as possible biomarkers for the metastatic potential of certain
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5 cancers. Our results prompted us to determine whether changes in expression of let-7a or HMGA2 were
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7 associated with more invasive and metastatic human prostate cancers. Utilizing *in situ* hybridization and a HRP
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9 labeled let-7a LNA probe we observed that let-7a expression was down-regulated when comparing normal
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11 prostate tissue to malignant prostate characterized as poorly differentiated invasive carcinoma (Figure 4A).
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13 Likewise, we observed an increase in nuclear HMGA2 expression in poorly differentiated invasive carcinoma
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15 as well as a rectal metastasis sample when compared to normal prostate tissue or benign tumor (Figure 4B). We
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17 proceeded to count cell nuclei which stained positive for HMGA2 from three normal tissue samples available
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19 (two 28 y.o. males and one 43 y.o male), four malignant prostate cancer tissue samples (one 73 y.o male with
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21 adenocarcinoma with a known distance metastasis, one 70 y.o. male with adenocarcinoma and two 60 y.o.
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23 males with undifferentiated carcinoma) and two rectal metastasis tissue samples (two 62 y.o. males with
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25 metastatic adenocarcinoma spread to the rectum). We found that 7% (45/667) of cells in the normal samples
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27 expressed HMGA2, 23% (354/1566) of cells in the malignant samples expressed HMGA2 and 31% (250/803)
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29 of cells in the metastatic samples expressed HMGA2. These results indicated that HMGA2 expression is
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31 associated with malignant and metastatic prostate cancer and suggest that let-7a, a major regulator of HMGA2,
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33 might play a role in the metastatic potential of prostate cancers.
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43 **Conclusions and Discussion**

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45 Despite recent advances in prostate cancer diagnostics and treatment, the long term survival rate for men
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47 diagnosed with prostate cancer has not changed significantly. Understanding the steps involved in prostate
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49 cancer progression are critical for the development of effective therapeutics and diagnostics. Currently there are
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51 no available therapies to stop metastases or diagnostics to distinguish invasive cancers in the early stages of
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53 tumor progression, primarily because the processes broadly involved in prostate cancer cell invasion are poorly
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55 understood. This is the first report that we are aware of that indicates let-7a down-regulation is associated with
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57 TGF- β induced EMT and invasion in prostate cancer cell lines. Furthermore, we establish that let-7a expression
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is down-regulated in advanced human prostate cancer and is regulated through a Ras-Raf-MAPK signaling pathway.

Let-7 miRNAs are known to negatively regulates the broad architectural transcription factor, High Mobility Group A2 (HMGA2) [48]. HMGA2 is expressed primarily by mesenchymal cells during embryogenesis, but not normal adult tissues [49]. HMGA2 seems to be re-expressed by malignant tissues and its over-expression correlates with occurrence of metastasis and poor prognoses in several human cancers [50, 51]. It has also been associated with prostate cancer in dog models, however, its expression and role in human prostate cancer has not been explored. We have found an increase in HMGA2 expression is associated with more invasive and metastatic human prostate cancers. Interestingly, HMGA2 has also been described as a critical factor necessary for TGF- β induced EMT in mouse primary mammary epithelial cells where HMGA2 directly up-regulates Snail1 expression to induce EMT [33, 52]. We have found that HMGA2 is also expressed during TGF- β induced EMT in epithelial cells isolated from prostate cancer and correlates with expression of Slug (Snail2) and Twist2 expression in these cells.

Our work demonstrates that the let-7a negative regulator, Lin-28B, is expressed as cells undergo EMT and that Lin-28B is up-regulated through Ras-Raf-MAPK signaling. Knock-down of Lin-28B also inhibited our cells from undergoing EMT and becoming invasive. However, contradictory to this, constitutive MEK1 activity activated TGF- β induced EMT, let-7a down-regulation and HMGA2 expression while also down-regulating Lin-28B expression. This could imply that signaling through MEK1 might regulate let-7a expression at the transcriptional level. We have previously shown that MEK1 mediates EMT through Erk2 nuclear localization and requires expression of c-myc. Interestingly c-myc has been shown to bind upstream of the let-7a promoter and its over-expression results in down-regulation of let-7 [53]. However, it has also been shown that Lin-28B transactivation is necessary for myc-mediated let-7 repression [54]. Additionally, the efficiency of miRNA processing, including processing of let-7a, has also been shown to be regulated by both MEK and TGF- β associated signaling events [55, 56]. Further studies into the dynamic nature of Lin-28 and let-7a expression and its role in EMT will be required.

1 Our results and these observations could be reconciled if upon initial transfection of cells with activated
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3 MEK1 or treatment of transfected cells with TGF- β , Lin-28B was induced to degrade let-7a before it was down-
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5 regulated in an unknown negative feedback loop stemming from MEK1 signaling. Future studies including time
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7 course analysis of Lin-28B expression following transfection with MEK1-DD and with TGF- β treatment might
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9 shed light on to such possibilities. Lin-28B activity might also be regulated by an unknown post translational
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11 modification which could suggest that total Lin-28B levels might not accurately reflect Lin-28B activity. In this
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13 scenario Lin-28B activity might actually be induced in MEK1 transfected cells to allow for let-7a degradation
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15 and therefore decrease the efficiency of let-7a processing despite the decreased expression of Lin-28B.
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18 Regardless, MEK2 activity also decreased let-7a expression without promoting TGF- β induced EMT. This
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20 indicates that let-7a down-regulation is not sufficient for TGF- β induced EMT and thus, let-7a expression
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22 merely acts as break to inhibit pro-invasive responses to TGF- β , likely by reducing expression of Ras and c-
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24 myc. These dynamics are further complicated by recent reports showing that competitive endogenous RNAs
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26 exist (ceRNAs) which act as miRNA sponges by competitively inhibiting the efficiency of miRNAs in binding
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28 to target mRNAs[57]. Interestingly, the Kras pseudo- gene has been identified as such a ceRNA and likely also
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30 regulates let-7a function given the homology of its 3' UTR to the Kras gene[58]. Future studies exploring the
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32 role of this ceRNA in regulating TGF-beta induced EMT and invasion and how its expression is regulated could
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34 be enlightening.
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41 Many cells undergoing EMT adopt a cancer stem cell-like phenotype and are able to produce metastases
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43 in mice at very low titer [59, 60]. Thus, cells which exhibit the plasticity to undergo EMT might also be highly
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45 adaptable and more suited for forming distant metastases. The let-7 family of miRNAs was first discovered as
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47 factors regulating developmental timing and differentiation. Therefore, it is possible that let-7a down-
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49 regulation serves to promote a de-differentiated state that may be required as part of the EMT process, thus
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51 regulating TGF- β mediated EMT. Interestingly, recent findings have connected Lin-28 and myc expression
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53 with stem cell maintenance and the pluripotent state, where expression of myc and Lin-28 appear to be
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55 interchangeable [61, 62]. Although our results are conflicting, induced expression of Lin-28B may further serve
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57 to regulate let-7a expression and EMT, and suggests that regulation of let-7 expression by myc and Lin-28
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might pose as a connection between EMT, pluripotency, cancer stem cells and metastatic potential. The current findings along with our prior reports suggest that c-myc and Erk2 activity are required for EMT indicate that a signaling cascade involving Ras-Raf-MAPK-myc down regulation of let-7 plays a critical role in regulating invasion and EMT in cancer [63-65].

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Figure Legends

Figure 1. Down-regulation of let-7a correlates with EMT. (A) Expression of let-7a in IBC-10a cells treated with minimal media (Km), or Km containing 10 ng/ml EGF (EGF), 10 ng/ml TGF-beta1 (TGF) or 10 ng/ml of EGF + TGF-beta1 (E + T) as described in Materials and Methods and determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to cells treated with Km. Data represent the mean +/- S.D. from three independent experiments, where * = p-value < 0.05 and ** = p-value < 0.01, compared to expression in Km. **(B)** Western blot analysis of HMGA2 expression in crude cell extracts from IBC-10a and PCa-20a cells treated with minimal media (Km), EGF, TGF-beta or E + T. Tubulin served as the loading control. **(C)** Time course analysis after day 3, 6 and 9 of IBC-10a grown in 10 ng/ml of both EGF and TGF-beta1 as described in Materials and Methods. LEFT: Western blot analysis of vimentin expression, tubulin served as loading control. RIGHT: Northern blot analysis for mature let-7a expression, 5S RNA served as loading control (performed by Youji Hu). **(D)** Expression of let-7a in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) or empty vector as a control and treated with 10 ng/ml of TGF-beta1 as determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells. Data represent the mean +/- S.D. from three independent experiments where * = p-value < 0.05 compared to empty

vector control. **(E)** Western blot analysis of HMGA2 expression in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) and empty vector as a control and treated with 10 *ng/ml* of TGF-beta1. Tubulin served as loading control.

Figure 2. *Let-7a* down-regulation is required for EMT and an invasive phenotype. **(A)** Expression of *let-7a* in IBC-10a and PCa-20a cells stably transfected with pBABE:pre-*let-7a*-1 or empty vector as control and treated with EGF + TGF-beta1 (E + T) as described in Materials and Methods and determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells of same variant. **(B)** Western blot analysis of vimentin, Fibroblast Specific Protein-1 (FSP-1) and HMGA2 expression in crude cell extracts from IBC-10a and PCa-20a stably transfected with pBABE:pre-*let-7a*-1 or empty vector as control and treated with minimal media (Km) or Km containing or 10 *ng/ml* of EGF + TGF-beta1 (E + T). Tubulin served as the loading control. **(C)** Western blot analysis of Slug and Twist2 expression in crude cell extracts from IBC-10a cells stably transfected with pBABE:pre-*let-7a*-1 or empty vector as control and treated with minimal media (Km) or Km containing 10 *ng/ml* of EGF + TGF-beta1 (E + T). Tubulin served as the loading control. **(D)** Western blot analysis of vimentin, Ras and HMGA2 expression in crude cell extracts from PC3-ML Cells stably transfected with pBABE:pre-*let-7a*-1 or empty vector as control and grown in DMEM with 10% FBS. Tubulin served as the loading control. **(E)** Quantification of Modified Boyden Chamber invasion assays showing the invasive activity through Matrigel of PC3-ML cells stably transfected with pBABE:pre-*let-7a* or empty vector as control after 48 hrs. Values are representative of three independent replicates. Mean +/- SD, where * = p-value < 0.05, compared to empty vector control.

Figure 3. *Lin28B* expression correlates with EMT and its knock-down inhibits EMT and an invasive phenotype. **(A)** Western blot analysis of *lin-28A* and *lin-28B* expression in crude cell extracts from IBC-10a cells. LEFT: Cells treated with minimal media (Km), or Km containing 10 *ng/ml* EGF (EGF), 10 *ng/ml* TGF-beta1 (TGF) or 10 *ng/ml* of EGF + TGF-beta1 (E + T) as described in Materials and Methods. RIGHT: Time course analysis after day 3, 6 and 9 of IBC-10a grown in 10 *ng/ml* of each EGF and TGF-beta1, changing media every 3 days.

Tubulin served as the loading control. **(B)** Western blot analysis of lin-28B expression in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) or empty vector as a control and treated with 10 ng/ml of TGF-beta1. Tubulin served as loading control. **(C)** Western blot analysis of lin-28B, HMGA2, vimentin and E-cadherin expression in crude cell extracts from IBC-10a stably transfected with shlin-28B construct or scrambled shRNA construct as control and treated with 10 ng/ml of EGF + TGF-beta1 in combination (E + T). Tubulin served as the loading control. **(D)** Quantification of Modified Boyden Chamber invasion assays showing the invasive activity through Matrigel of IBC-10a cells stably transfected with shlin-28B construct or scrambled shRNA construct as control. Values are representative of three independent replicates. Mean +/- SD, where * = p-value < 0.05, compared to scrambled control. **(E)** Western blot analysis of lin-28B, HMGA2 and vimentin expression in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD) or empty vector as a control and treated with 10 ng/ml of TGF-beta1. Tubulin served as the loading control. **(F)** Expression of let-7a in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD) or empty vector as a control as determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells. Data represent the mean +/- S.D. from three independent experiments, where * = p-value < 0.05, compared to empty vector control.

Figure 4. *Let-7a down-regulation and HMGA2 expression are induced in malignant prostate cancer. (A)* *In situ* hybridization analysis of let-7a expression on prostate cancer tissue array using biotin labeled let-7a LNA probe and HRP conjugated streptavidin. Counter stained with Harris Modified Hematoxylin. LEFT: Normal prostate from a 28 y.o. Male. RIGHT: Undifferentiated carcinoma from 60 y.o. male. **(B)** Immunohistochemistry analysis of HMGA2 expression using HRP conjugated secondary antibodies on prostate cancer tissue array. Counterstained with Harris Modified Hematoxylin. TOP LEFT: Normal prostate from a 28 y.o. Male. TOP RIGHT: Chronic Prostatitis with hyperplasia from 71 y.o. male. BOTTOM LEFT: Undifferentiated carcinoma from 60 y.o. male. BOTTOM RIGHT: Metastatic adenocarcinoma from rectum from 62 y.o. male.

References

1. Thiery, J.P., H. Acloque, R.Y. Huang, and M.A. Nieto, *Epithelial-mesenchymal transitions in development and disease*. Cell, 2009. **139**(5): p. 871-90.

2. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression*. Nat Rev Cancer, 2002. **2**(6): p. 442-54.

3. Christofori, G. and H. Semb, *The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene*. Trends Biochem Sci, 1999. **24**(2): p. 73-6.

4. Domagala, W., J. Lasota, A. Dukowicz, M. Markiewski, G. Striker, K. Weber, and M. Osborn, *Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas*. Am J Pathol, 1990. **137**(6): p. 1299-304.

5. Rudland, P.S., A. Platt-Higgins, C. Renshaw, C.R. West, J.H. Winstanley, L. Robertson, and R. Barraclough, *Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer*. Cancer Res, 2000. **60**(6): p. 1595-603.

6. Singh, S., S. Sadacharan, S. Su, A. Beldegrun, S. Persad, and G. Singh, *Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer*. Cancer Res, 2003. **63**(9): p. 2306-11.

7. Gilles, C., M. Polette, J.M. Zahm, J.M. Tournier, L. Volders, J.M. Foidart, and P. Birembaut, *Vimentin contributes to human mammary epithelial cell migration*. J Cell Sci, 1999. **112 (Pt 24)**: p. 4615-25.

8. Gottardi, C.J., E. Wong, and B.M. Gumbiner, *E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner*. J Cell Biol, 2001. **153**(5): p. 1049-60.

9. Giampieri, S., C. Manning, S. Hooper, L. Jones, C.S. Hill, and E. Sahai, *Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility*. Nat Cell Biol, 2009. **11**(11): p. 1287-96.

10. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system*. EMBO J, 2000. **19**(8): p. 1745-54.

11. Seton-Rogers, S.E., Y. Lu, L.M. Hines, M. Koundinya, J. LaBaer, S.K. Muthuswamy, and J.S. Brugge, *Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells*. Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1257-62.

12. Zhau, H.E., V. Odero-Marah, H.W. Lue, T. Nomura, R. Wang, G. Chu, Z.R. Liu, B.P. Zhou, W.C. Huang, and L.W. Chung, *Epithelial to mesenchymal transition (EMT) in human prostate cancer: lessons learned from ARCaP model*. Clin Exp Metastasis, 2008. **25**(6): p. 601-10.

13. Janda, E., K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, and S. Grunert, *Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways*. J Cell Biol, 2002. **156**(2): p. 299-313.

14. Brown, K.A., M.E. Aakre, A.E. Gorska, J.O. Price, S.E. Eltom, J.A. Pietenpol, and H.L. Moses, *Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro*. Breast Cancer Res, 2004. **6**(3): p. R215-31.

15. Deckers, M., M. van Dinther, J. Buijs, I. Que, C. Lowik, G. van der Pluijm, and P. ten Dijke, *The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells*. Cancer Res, 2006. **66**(4): p. 2202-9.

16. Do, T.V., L.A. Kubba, H. Du, C.D. Sturgis, and T.K. Woodruff, *Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition*. Mol Cancer Res, 2008. **6**(5): p. 695-705.

17. Xie, L., B.K. Law, A.M. Chytil, K.A. Brown, M.E. Aakre, and H.L. Moses, *Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro*. Neoplasia, 2004. **6**(5): p. 603-10.

18. Amatangelo, M.D., Goodyear, S. M., Varma, D., Stearns, M. E., *c-Myc Expression and MEK1 induced Erk2 Nuclear Localization are Required for TGF-beta Induced EMT and Invasion in Prostate Cancer*. Carcinogenesis, In Press.
19. Bieri, B. and H.L. Moses, *Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer*. Nat Rev Cancer, 2006. **6**(7): p. 506-20.
20. Pasquinelli, A.E., B.J. Reinhart, F. Slack, M.Q. Martindale, M.I. Kuroda, B. Maller, D.C. Hayward, E.E. Ball, B. Degan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson, and G. Ruvkun, *Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA*. Nature, 2000. **408**(6808): p. 86-9.
21. Sempere, L.F., S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky, and V. Ambros, *Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation*. Genome Biol, 2004. **5**(3): p. R13.
22. Nam, E.J., H. Yoon, S.W. Kim, H. Kim, Y.T. Kim, J.H. Kim, J.W. Kim, and S. Kim, *MicroRNA expression profiles in serous ovarian carcinoma*. Clin Cancer Res, 2008. **14**(9): p. 2690-5.
23. Motoyama, K., H. Inoue, Y. Nakamura, H. Uetake, K. Sugihara, and M. Mori, *Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family*. Clin Cancer Res, 2008. **14**(8): p. 2334-40.
24. Yanaihara, N., N. Caplen, E. Bowman, M. Seike, K. Kumamoto, M. Yi, R.M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G.A. Calin, C.G. Liu, C.M. Croce, and C.C. Harris, *Unique microRNA molecular profiles in lung cancer diagnosis and prognosis*. Cancer Cell, 2006. **9**(3): p. 189-98.
25. Schultz, J., P. Lorenz, G. Gross, S. Ibrahim, and M. Kunz, *MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth*. Cell Res, 2008. **18**(5): p. 549-57.
26. Ozen, M., C.J. Creighton, M. Ozdemir, and M. Ittmann, *Widespread deregulation of microRNA expression in human prostate cancer*. Oncogene, 2008. **27**(12): p. 1788-93.
27. Kumar, M.S., S.J. Erkeland, R.E. Pester, C.Y. Chen, M.S. Ebert, P.A. Sharp, and T. Jacks, *Suppression of non-small cell lung tumor development by the let-7 microRNA family*. Proc Natl Acad Sci U S A, 2008. **105**(10): p. 3903-8.
28. Johnson, S.M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K.L. Reinert, D. Brown, and F.J. Slack, *RAS is regulated by the let-7 microRNA family*. Cell, 2005. **120**(5): p. 635-47.
29. Sampson, V.B., N.H. Rong, J. Han, Q. Yang, V. Aris, P. Soteropoulos, N.J. Petrelli, S.P. Dunn, and L.J. Krueger, *MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells*. Cancer Res, 2007. **67**(20): p. 9762-70.
30. Calin, G.A., C. Sevignani, C.D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini, and C.M. Croce, *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers*. Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2999-3004.
31. Rogalla, P., K. Drechsler, G. Frey, Y. Hennig, B. Helmke, U. Bonk, and J. Bullerdiek, *HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors*. Am J Pathol, 1996. **149**(3): p. 775-9.
32. Watanabe, S., Y. Ueda, S. Akaboshi, Y. Hino, Y. Sekita, and M. Nakao, *HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells*. Am J Pathol, 2009. **174**(3): p. 854-68.
33. Thuault, S., U. Valcourt, M. Petersen, G. Manfioletti, C.H. Heldin, and A. Moustakas, *Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition*. J Cell Biol, 2006. **174**(2): p. 175-83.
34. Wang, Y.C., Y.L. Chen, R.H. Yuan, H.W. Pan, W.C. Yang, H.C. Hsu, and Y.M. Jeng, *Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma*. Carcinogenesis, 2010. **31**(9): p. 1516-22.
35. Newman, M.A., J.M. Thomson, and S.M. Hammond, *Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing*. RNA, 2008. **14**(8): p. 1539-49.

36. Shell, S., S.M. Park, A.R. Radjabi, R. Schickel, E.O. Kistner, D.A. Jewell, C. Feig, E. Lengyel, and M.E. Peter, *Let-7 expression defines two differentiation stages of cancer*. Proc Natl Acad Sci U S A, 2007. **104**(27): p. 11400-5.

37. Goodyear, S.M., M.D. Amantangelo, and M.E. Stearns, *Dysplasia of human prostate CD133(hi) sub-population in NOD-SCIDS is blocked by c-myc anti-sense*. Prostate, 2009. **69**(7): p. 689-98.

38. Wang, M. and M. Stearns, *Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice*. Differentiation; research in biological diversity, 1991. **48**(2): p. 115-140.

39. Sambrook, J. and D.W. Russell, *Molecular cloning : a laboratory manual*. 3rd ed. 2001, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

40. Michiels, F., R.A. van der Kammen, L. Janssen, G. Nolan, and J.G. Collard, *Expression of Rho GTPases using retroviral vectors*. Methods Enzymol, 2000. **325**: p. 295-302.

41. Amantangelo, M.D., D.E. Bassi, A.J. Klein-Szanto, and E. Cukierman, *Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts*. Am J Pathol, 2005. **167**(2): p. 475-88.

42. Blevins, T., *Northern blotting techniques for small RNAs*. Methods Mol Biol, 2010. **631**: p. 87-107.

43. White, M.A., C. Nicolette, A. Minden, A. Polverino, L. Van Aelst, M. Karin, and M.H. Wigler, *Multiple Ras functions can contribute to mammalian cell transformation*. Cell, 1995. **80**(4): p. 533-41.

44. Zeng, Y., E.J. Wagner, and B.R. Cullen, *Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells*. Mol Cell, 2002. **9**(6): p. 1327-33.

45. Peinado, H., D. Olmeda, and A. Cano, *Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?* Nat Rev Cancer, 2007. **7**(6): p. 415-28.

46. Wang, M. and M.E. Stearns, *Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice*. Differentiation, 1991. **48**(2): p. 115-25.

47. Troppmair, J., J.T. Bruder, H. Munoz, P.A. Lloyd, J. Kyriakis, P. Banerjee, J. Avruch, and U.R. Rapp, *Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation*. J Biol Chem, 1994. **269**(9): p. 7030-5.

48. Lee, Y.S. and A. Dutta, *The tumor suppressor microRNA let-7 represses the HMGA2 oncogene*. Genes Dev, 2007. **21**(9): p. 1025-30.

49. Chiappetta, G., V. Avantiaggiato, R. Visconti, M. Fedele, S. Battista, F. Trapasso, B.M. Merciai, V. Fidanza, V. Giancotti, M. Santoro, A. Simeone, and A. Fusco, *High level expression of the HMGI (Y) gene during embryonic development*. Oncogene, 1996. **13**(11): p. 2439-46.

50. Abe, N., T. Watanabe, T. Masaki, T. Mori, M. Sugiyama, H. Uchimura, Y. Fujioka, G. Chiappetta, A. Fusco, and Y. Atomi, *Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins*. Cancer Res, 2000. **60**(12): p. 3117-22.

51. Di Cello, F., J. Hillion, A. Hristov, L.J. Wood, M. Mukherjee, A. Schuldenfrei, J. Kowalski, R. Bhattacharya, R. Ashfaq, and L.M. Resar, *HMGA2 participates in transformation in human lung cancer*. Mol Cancer Res, 2008. **6**(5): p. 743-50.

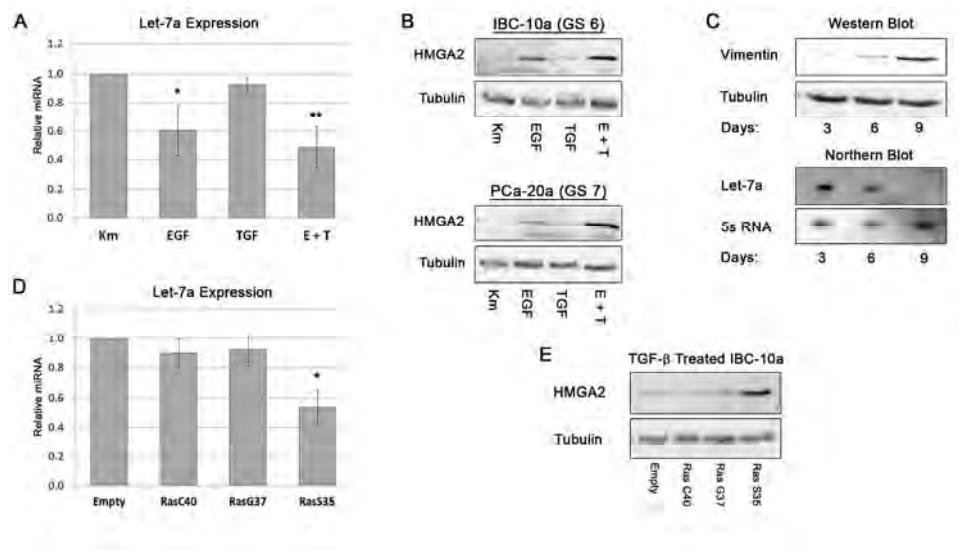
52. Thuault, S., E.J. Tan, H. Peinado, A. Cano, C.H. Heldin, and A. Moustakas, *HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition*. J Biol Chem, 2008. **283**(48): p. 33437-46.

53. Chang, T.C., D. Yu, Y.S. Lee, E.A. Wentzel, D.E. Arking, K.M. West, C.V. Dang, A. Thomas-Tikhonenko, and J.T. Mendell, *Widespread microRNA repression by Myc contributes to tumorigenesis*. Nat Genet, 2008. **40**(1): p. 43-50.

54. Chang, T.C., L.R. Zeitels, H.W. Hwang, R.R. Chivukula, E.A. Wentzel, M. Dews, J. Jung, P. Gao, C.V. Dang, M.A. Beer, A. Thomas-Tikhonenko, and J.T. Mendell, *Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation*. Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3384-9.

55. Davis, B.N., A.C. Hilyard, G. Lagna, and A. Hata, *SMAD proteins control DROSHA-mediated microRNA maturation*. Nature, 2008. **454**(7200): p. 56-61.

56. Paroo, Z., X. Ye, S. Chen, and Q. Liu, *Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling*. Cell, 2009. **139**(1): p. 112-22.
57. Karreth, F.A., Y. Tay, D. Perna, U. Ala, S.M. Tan, A.G. Rust, G. DeNicola, K.A. Webster, D. Weiss, P.A. Perez-Mancera, M. Krauthammer, R. Halaban, P. Provero, D.J. Adams, D.A. Tuveson, and P.P. Pandolfi, *In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma*. Cell, 2011. **147**(2): p. 382-95.
58. Poliseno, L., L. Salmena, J. Zhang, B. Carver, W.J. Haveman, and P.P. Pandolfi, *A coding-independent function of gene and pseudogene mRNAs regulates tumour biology*. Nature, 2010. **465**(7301): p. 1033-8.
59. 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, and R.A. Weinberg, *The epithelial-mesenchymal transition generates cells with properties of stem cells*. Cell, 2008. **133**(4): p. 704-15.
60. Kong, D., S. Banerjee, A. Ahmad, Y. Li, Z. Wang, S. Sethi, and F.H. Sarkar, *Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells*. PLoS One, 2010. **5**(8): p. e12445.
61. Nakagawa, M., M. Koyanagi, K. Tanabe, K. Takahashi, T. Ichisaka, T. Aoi, K. Okita, Y. Mochiduki, N. Takizawa, and S. Yamanaka, *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts*. Nat Biotechnol, 2008. **26**(1): p. 101-6.
62. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, *Induction of pluripotent stem cells from adult human fibroblasts by defined factors*. Cell, 2007. **131**(5): p. 861-72.
63. Chuang, C.F. and S.Y. Ng, *Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors*. FEBS Lett, 1994. **346**(2-3): p. 229-34.
64. Zhu, J., J. Blenis, and J. Yuan, *Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1*. Proc Natl Acad Sci U S A, 2008. **105**(18): p. 6584-9.
65. Dangi-Garimella, S., J. Yun, E.M. Eves, M. Newman, S.J. Erkeland, S.M. Hammond, A.J. Minn, and M.R. Rosner, *Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7*. EMBO J, 2009. **28**(4): p. 347-58.

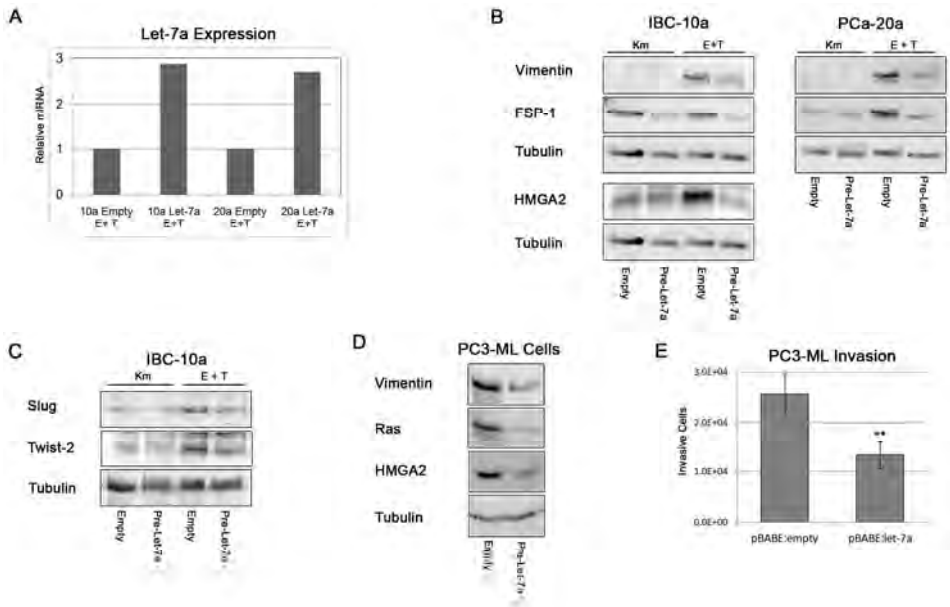


Down-regulation of let-7a correlates with EMT. (A) Expression of let-7a in IBC-10a cells treated with minimal media (Km), or Km containing 10 ng/ml EGF (EGF), 10 ng/ml TGF-beta1 (TGF) or 10 ng/ml of EGF + TGF-beta1 (E + T) as described in Materials and Methods and determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to cells treated with Km. Data represent the mean +/- S.D. from three independent experiments, where * = p-value <0.05 and ** = p-value < 0.01, compared to expression in Km. (B) Western blot analysis of HMGA2 expression in crude cell extracts from IBC-10a and PCa-20a cells treated with minimal media (Km), EGF, TGF-beta or E + T. Tubulin served as the loading control. (C) Time course analysis after day 3, 6 and 9 of IBC-10a grown in 10 ng/ml of both EGF and TGF-beta1 as described in Materials and Methods. LEFT: Western blot analysis of vimentin expression, tubulin served as loading control. RIGHT: Northern blot analysis for mature let-7a expression, 5S RNA served as loading control (performed by Youji Hu). (D) Expression of let-7a in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) or empty vector as a control and treated with 10 ng/ml of TGF-beta1 as determined by

qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells. Data represent the mean $\pm$ S.D. from three independent experiments where * = p-value < 0.05 compared to empty vector control. (E) Western blot analysis of HMGA2 expression in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) and empty vector as a control and treated with 10 ng/ml of TGF-beta1. Tubulin served as loading control.

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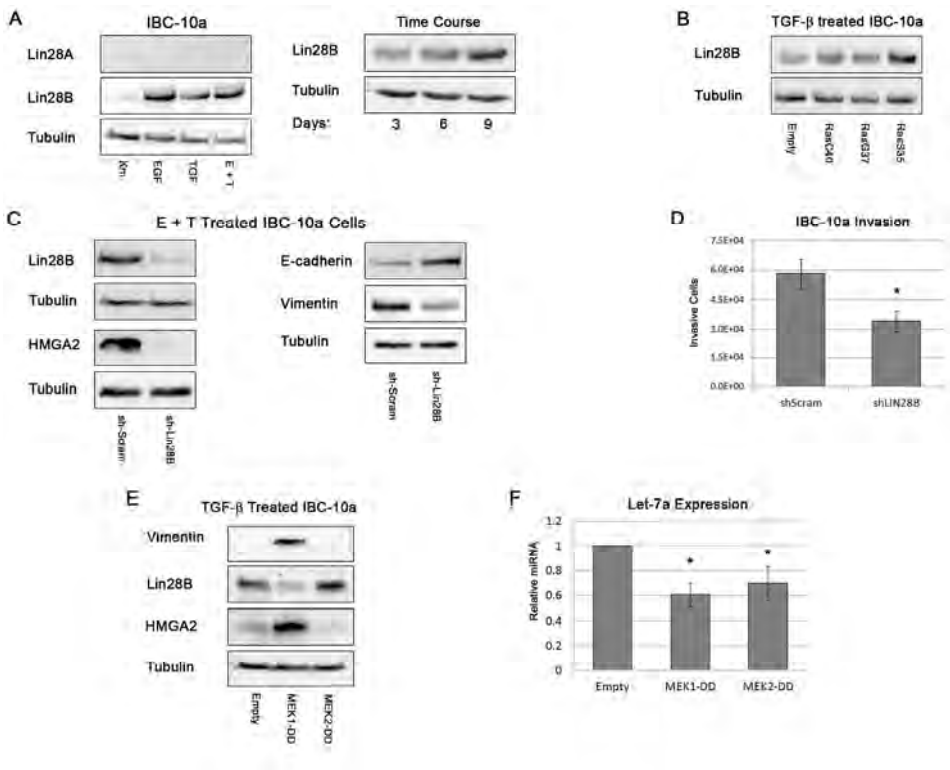


Let-7a down-regulation is required for EMT and an invasive phenotype. (A) Expression of let-7a in IBC-10a and PCa-20a cells stably transfected with pBABE:pre-let-7a-1 or empty vector as control and treated with EGF + TGF-beta1 (E + T) as described in Materials and Methods and determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells of same variant. (B) Western blot analysis of vimentin, Fibroblast Specific Protien-1 (FSP-1) and HMGA2 expression in crude cell extracts from IBC-10a and PCa-20a stably transfected with pBABE:pre-let-7a-1 or empty vector as control and treated with minimal media (Km) or Km containing or 10 ng/ml of EGF + TGF-beta1 (E + T). Tubulin served as the loading control. (C) Western blot analysis of Slug and Twist2 expression in crude cell extracts from IBC-10a cells stably transfected with pBABE:pre-let-7a-1 or empty vector as control and treated with minimal media (Km) or Km containing 10 ng/ml of EGF + TGF-beta1 (E + T). Tubulin served as the loading control. (D) Western blot analysis of vimentin, Ras and HMGA2 expression in crude cell extracts from PC3-ML Cells stably transfected with pBABE:pre-let-7a-1 or empty vector as control and grown in DMEM with 10% FBS. Tubulin served as the loading control. (E) Quantification of Modified Boyden Chamber

invasion assays showing the invasive activity through Matrigel of PC3-ML cells stably transfected with pBABE:pre-let-7a or empty vector as control after 48 hrs. Values are representative of three independent replicates. Mean +/- SD, where * = p-value < 0.05, compared to empty vector control.

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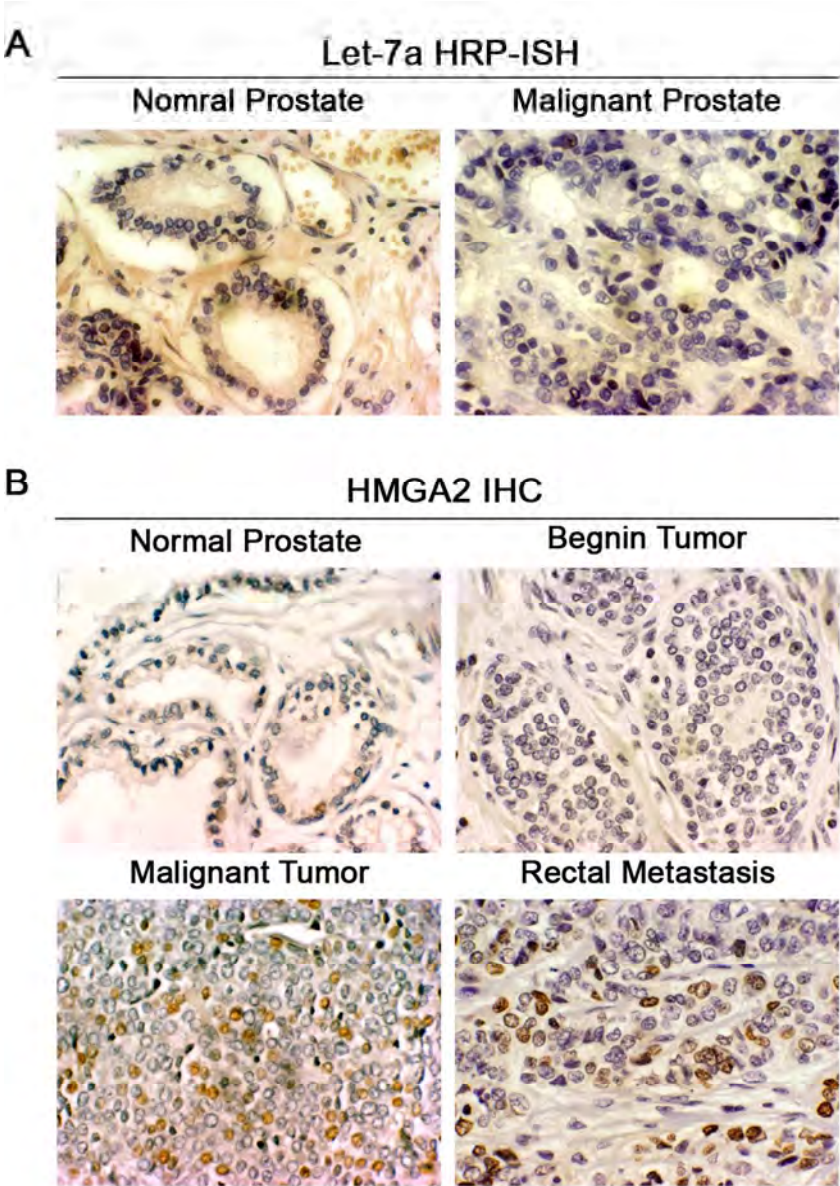
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Lin28B expression correlates with EMT and its knock-down inhibits EMT and an invasive phenotype. (A) Western blot analysis of lin-28A and lin-28B expression in crude cell extracts from IBC-10a cells. LEFT: Cells treated with minimal media (Km), or Km containing 10 ng/ml EGF (EGF), 10 ng/ml TGF-beta1 (TGF) or 10 ng/ml of EGF + TGF-beta1 (E + T) as described in Materials and Methods. RIGHT: Time course analysis after day 3, 6 and 9 of IBC-10a grown in 10 ng/ml of each EGF and TGF-beta1, changing media every 3 days. Tubulin served as the loading control. (B) Western blot analysis of lin-28B expression in IBC-10a cells stably transfected with constitutively active Ras effector mutants (pBABE:RasV12-C40; pBABE:RasV12-G37; pBABE:RasV12-S35) or empty vector as a control and treated with 10 ng/ml of TGF-beta1. Tubulin served as loading control. (C) Western blot analysis of lin-28B, HMGA2, vimentin and E-cadherin expression in crude cell extracts from IBC-10a stably transfected with shlin-28B construct or scrambled shRNA construct as control and treated with 10 ng/ml of EGF + TGF-beta1 in combination (E + T). Tubulin served as the loading control. (D) Quantification of Modified Boyden Chamber invasion assays showing the invasive activity through Matrigel of IBC-10a cells stably transfected with shlin-28B construct or scrambled shRNA

construct as control. Values are representative of three independent replicates. Mean $\pm$ SD, where * = p-value < 0.05, compared to scrambled control. (E) Western blot analysis of lin-28B, HMGA2 and vimentin expression in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD) or empty vector as a control and treated with 10 ng/ml of TGF-beta1. Tubulin served as the loading control. (F) Expression of let-7a in IBC-10a cells stably transfected with constitutively active MEK1 (pBABE:MEK1-DD), MEK2 (pBABE:MEK2-DD) or empty vector as a control as determined by qRT-PCR. Values were normalized to U6 RNA and represent the fold increase in expression relative to empty vector cells. Data represent the mean $\pm$ S.D. from three independent experiments, where * = p-value < 0.05, compared to empty vector control.

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Let-7a down-regulation and HMGA2 expression are induced in malignant prostate cancer. (A) In situ hybridization analysis of let-7a expression on prostate cancer tissue array using biotin labeled let-7a LNA probe and HRP conjugated streptavidin. Counter stained with Harris Modified Hematoxylin. LEFT: Normal prostate from a 28 y.o. Male. RIGHT: Undifferentiated carcinoma from 60 y.o. male. (B) Immunohistochemistry analysis of HMGA2 expression using HRP conjugated secondary antibodies on prostate cancer tissue array. Counterstained with Harris Modified Hematoxylin. TOP LEFT: Normal prostate from a 28 y.o. Male. TOP RIGHT: Chronic Prostatitis with hyperplasia from 71 y.o. male. BOTTOM LEFT: Undifferentiated carcinoma from 60 y.o. male. BOTTOM RIGHT: Metastatic adenocarcinoma from rectum from 62 y.o. male.

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