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PRINCIPAL INVESTIGATOR: David R. Rowley, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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14. ABSTRACT The purpose of this project is to determine the role of FGF receptor 1 in reactive stroma during prostate tumorigenesis. We are using a novel approach to target transgene expression specifically to the reactive stroma of experimental prostate cancer. Using a modified approach, we are placing an inducible Cre recombinase behind the FAP gene promoter to target expression to reactive stroma. We will cross this mouse with Fgfr1flox mice (LoxP sites flanking FGF receptor 1 alleles). These mice will be crossed with TRAMP mice (prostate cancer model). Induced expression of Cre at sites of reactive stroma generated in the cancer foci will function to excise the FGF receptor 1 alleles and create a conditional knockout mouse. Progression of tumorigenesis in this line of knockout mice will be compared to heterozygous and wild type controls. Progress has been made in each Task. We have completed all cloning steps and have putative founder mice with the FAP-Cre sequence. The Fgfr1flox and have crossed it into the appropriate backgrounds and the TRAMP mice were crossed with the Fgfr1flox mice. This study will pinpoint the role of FGF receptor 1 in reactive stroma promotion of prostate.					
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FINAL REPORT: W81XWH-04-1-0189 "Role of Reactive Stroma in Prostate Cancer Progression" Submitted: 2/3/2008

Introduction:

A tumor microenvironment composed of a reactive stroma co-evolves with cancer foci during the initiation of prostate cancer. This reactive stroma is similar to a wound repair stroma and continues to co-evolve during prostate cancer progression. Our studies show that reactive stroma promotes the rate of tumorigenesis. The purpose of our study was to devise a mouse model to target gene expression to the reactive stroma compartment and to understand the specific regulators of reactive stroma. The overall and long-range goal is to develop a novel therapeutic approach to target the reactive stroma as a strategic method to control prostate cancer via targeting its niche. As this specific study ends, we now better understand the key regulators of reactive stroma and we have developed a putative mouse model. Our work is far from complete and we plan to continue toward our long-range goal.

Our previous reports and current data since the last Progress Report suggests that a putative stromal progenitor cell that lies just outside epithelial acini are activated to form the initial reactive stroma ([Figure 1](#)). These cells are vimentin positive ([Fig. 1A](#), arrow) and a subset of cells are CD34 positive ([Fig. 1B](#), arrow), suggesting a bone marrow origin of these putative mesenchymal progenitor cells. During the initiation of prostatic intraepithelial neoplasia (PIN), a

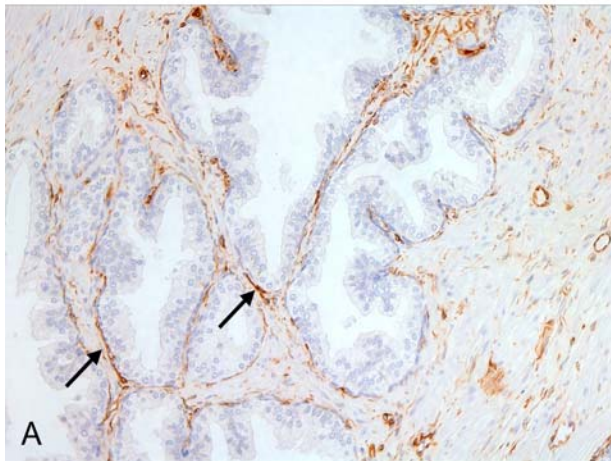


Fig 1A: Vimentin staining in human prostate

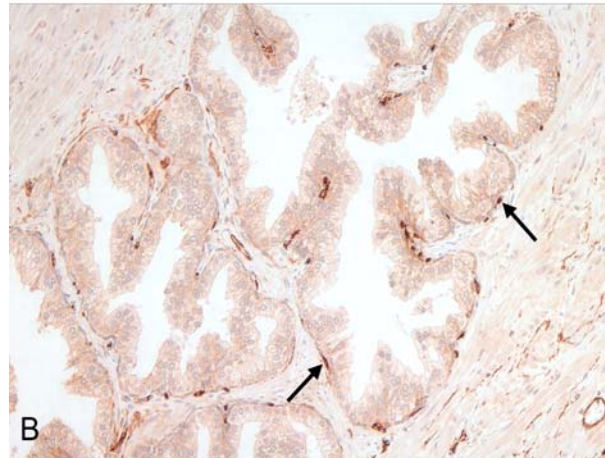


Fig 1B: CD34 staining in human prostate

pre-malignant lesion, these cells appear to give rise to a specific reactive stroma characterized by expression of vimentin, smooth muscle α -actin, tenascin-C, fibroblast activation protein (FAP) and pro-collagen I ([Figure 2, next page](#)). These cells are classified as myofibroblasts.

Our published data supports the hypothesis that transforming growth factor beta one (TGF- β 1) that is overexpressed in PIN and carcinoma cells, functions as a key regulator of the formation and biology of reactive stroma (1-3). Our work has also addressed other growth factors in reactive stroma are downstream of TGF- β 1 regulation. Among these are connective tissue growth factor (CTGF) and fibroblast growth factor-2 (FGF-2). Our published studies show these genes are TGF- β regulated and are responsible for many of the effects attributed to overexpression of TGF- β (3, 4). Attenuation of TGF- β signaling in carcinoma-associated stroma resulted in decreased angiogenesis and rate of tumor growth (3).

Initiation of Reactive Stroma in PIN:

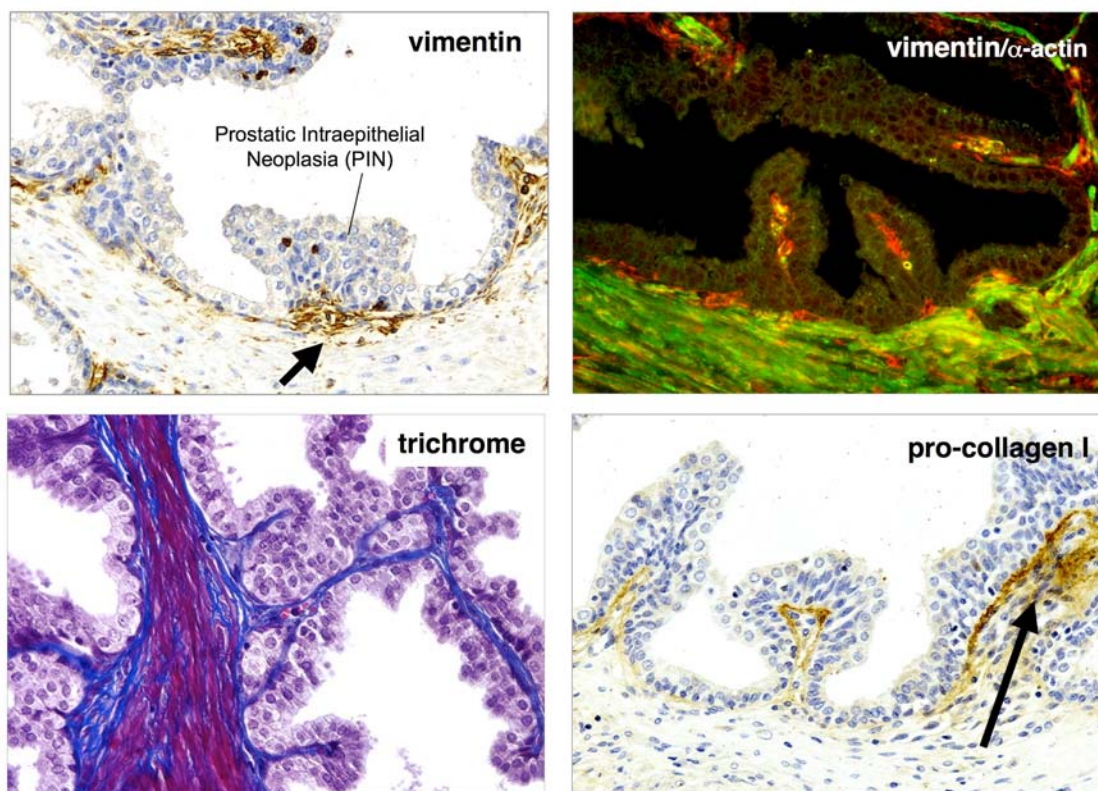


Figure 2: Initiation of reactive stroma (arrows) during PIN

The focus of this application is to provide unique mouse models with which to probe mechanisms of FGF-2 action in the tumor microenvironment. Little is understood about signaling and the downstream mediators of FGF-2 action. To address this, we showed that FGF-2 message was downregulated in prostate stromal cells that were engineered for knockout of the TGF β receptor type II (3). Moreover, our most recent study shows that TGF- β 1 not only regulates protein expression but also stimulates release of the 18 kDa isoform of FGF-2 from prostate stromal cells (3). Engineered expression of FGF-2 in the stromal cells with attenuated TGF- β signaling resulted in recovered angiogenesis and rate of tumor growth. These studies show that FGF-2 is a key factor in mediating TGF- β mechanisms of action in prostate cancer associated reactive stroma.

Not only does FGF-2 regulate the tumor-associated stroma, but also there is emerging evidence that suggests that FGF-2 may mediate epithelial to mesenchymal transition of carcinoma cells during tumor progression to metastasis. Accordingly, completion of our proposed study will provide new models and new data on the specific responses and downstream pathways mediated by FGF-2 in tumor stroma.

Our project is focused on understanding FGF-2 biology via the generation of a novel mouse model to knock out FGF-2 signaling in the reactive stromal compartment. This project was proposed to address specific mechanisms and pinpoint those biologies specifically regulated by FGF-2 signaling. Fibroblast activation protein (FAP) is expressed in reactive

stroma of prostate cancer (1). FAP has also been shown to have restricted expression specifically in reactive stroma in adult stromal cells (5, 6). In our study, we have used the upstream promoter of the murine FAP gene to specifically target the expression of an inducible Cre recombinase to cancer associated reactive stroma. Three Specific Aims and Tasks were proposed that will generate a conditional knock out of the FGF receptor 1 gene (cognate receptor for FGF-2) in the reactive stroma tumor microenvironment of the TRAMP mouse model for prostate cancer. The use of the FAP-inducible Cre mouse will also benefit researchers studying other cancers, as FAP is generally expressed in reactive stroma of many different carcinomas.

Body:

Task 1 will knock-in DNA encoding the Mifepristone (RU 486) inducible Cre recombinase (CrePR1) into the fibroblast activation protein (FAP) locus.

The purpose of Task 1 (Aim 1) is to target an inducible Cre recombinase to the reactive stroma via knock-in to FAP gene locus in a transgenic mouse. Our initial intent was to insert a Mifepristone-regulated Cre recombinase (CrePR1). However, several studies have shown that the Mifepristone inducible system may be too leaky for our needs. FAP is expressed in the mesenchyme during development; hence, leaky activation of the inducible Cre could generate an embryonic phenotype that would be undesirable. Accordingly, we decided to use the tamoxifen inducible Cre recombinase and we disclosed this in previous Progress Reports. The tamoxifen inducible system typically exhibits less leaky activation and is the current method of choice for these types of gene switch systems. This will allow for expression and activation of Cre recombinase (nuclear transport) in specifically in prostate cancer reactive stroma, once this mouse has been crossed with the TRAMP mice and tamoxifen delivered (Task 3).

The initial approach proposed in the original application was to knock-in the inducible Cre directly into the endogenous FAP locus. As discussed extensively in the previous Progress Report, this approach was thought to be too risky, as it had been shown that disruption of the endogenous FAP gene could itself produce a phenotype that would complicate analysis. As disclosed previously, we switched to using bacterial artificial clones (BAC) that contain the murine FAP gene for recombination. The strategy was to then use this construct to generate a transgenic mouse. In this manner the endogenous mouse FAP gene would not be disrupted.

During the initial and second progress period, we followed the steps as outlined in the original application. All steps up to the actual recombination steps were successful. However, as disclosed in the Previous Progress Report, we experienced extraordinary difficulty in producing a recombined BAC clone using this strategy. Every possible approach was attempted. The strategic knock-in of constructs into some genes is sometimes not possible, likely due to some structure of the gene that is not permissive. We do not understand the specific reason why this was unsuccessful with the FAP gene-containing BAC clone. We used multiple different 5' and 3' homologous DNA sequences of different lengths, under different conditions of recombination with no success. We have considerable experience with homologous recombination having generated a mouse that is null for the *wfdc1/ps20* gene in the past. Hence, we were confident in our procedures.

As disclosed in the last Progress Report we followed alternative approaches to correct this problem. These steps are shown in [Figure 3](#) (following page). During the last Progress Period, we cloned out approximately 10 kb of the immediate upstream promoter in the murine FAP gene. We used this to construct a plasmid that contains this piece upstream of a tamoxifen

inducible Cre. We used this to generate a transgenic mouse. To date, we have a potential founder; we have mated this founder and are in the process of screening litters to identify mice with germ line transmission. We are also in the cue in our Genetically Engineered Mouse (GEM) Core facility for injecting more embryos to generate a second line of mice. We plan to

Task 1:

- Subcloning of 10 kb of FAP immediate upstream promoter from BAC clone containing the FAP gene.
- Acquisition of the tamoxifen inducible Cre recombinase.
- Gel purification of fragments.
- Ligation and cloning of construct.
- Verification of proper orientation and sequence fidelity.
- Provide verified construct to the GEM core.
- Characterization of founder mice and breeding to verify germ line transmission.
- Cross mice with ROSA reporter mice to verify induced expression in wound repair studies and leakiness in plus or minus tamoxifen conditions.
- Use mice for Task 2 and 3 experiments.

develop at least two separate lines, possibly three. This approach was proposed as an "Alternative Approach" and we requested these changes in the last Progress Report in order to accomplish this Task. Hence, Task 1 is nearing completion. Although DOD funding for this project has officially ended, it is our plan to continue to make these mice and report our results. We are committed to completion of this project even though we are outside the formal project window. We will forward all future publications regarding these mice to the DOD.

Figure 3: Alternative approaches in Task 1

Task 2, is to cross these mice with a EF-1 α / lox stop cassette / FGFR1 Δ (dominant negative FGF receptor type I-myc tag) to create a FAP(CrePR1) / lox(stop) FGFR1 Δ bigenic animal. **Modification approved in the previous Progress Report:** The *Fgfr1*^{flox} mouse will be used instead of the FGFR1 Δ mouse for Task 2 in order to produce a conditional knockout.

Task 2 is designed to attenuate expression of FGF receptor I at sites of reactive stroma. To accomplish this, we generated an *Fgfr1*^{flox} mouse that has loxP sites flanking elements in the FGF receptor I locus. As disclosed in the Previous Progress Report, we took this approach Instead of expressing the lox(stop) FGFR1 Δ dominant negative as this approach would be more likely to attenuate (knockdown) native gene FGFR1 expression as compared with a dominant negative approach. Hence, we decided to use a mouse that contains floxed alleles of the FGF receptor I (*Fgfr1*^{flox} mice) to knockout signaling. This is a much more straightforward approach to assure a conditional knockout of FGFR1 instead of expressing a competing dominant negative receptor. As discussed in the previous Progress Report, the *Fgfr1*^{flox} line (provided to by Juha Partanen, University of Helsinki, Finland) (7) was in the ICR background. We first rederived this mouse via embryo transfer (to assure a pathogen-free line) and then backcrossed this mouse into the C57BL/6 and FVB genetic background. We have made over 10+ generations of crosses (0.39% ICR remaining). This background is optimal for crossing with the FAP-Cre mice creating the bigenic FAP(Cre) / *Fgfr1*^{flox} (FVB) mouse line (heterozygous floxed FGFR1 allele). This step will complete Task 2. Figure 4 (following page) shows the lines of mice and crosses to be made for each Task. In addition to the proposed experiments for Task 2, we have also generated a fibroblast cell line from the *Fgfr1*^{flox} mouse line peritoneum. Use of this cell line will aid us in extended studies to address specific signaling mechanisms. Hence Task 2 remains in progress at the end of this DOD funded project. Again, we remain committed to completion of this Task in the future even though our funding has ended. As soon as we have a mature male mouse from Aim 1, this will be crossed with multiple female *Fgfr1*^{flox} mice to generate the bigenic FAP(Cre) / *Fgfr1*^{flox} (FVB) lines as proposed.

Task 3, is to cross this bigenic animal with TRAMP mice. The TRAMP / FAP(CrePR1) / lox(stop) FGFR1Δ bigenic cross should exhibit RU 486 regulated expression of the dominant negative FGF receptor I transgene in TRAMP reactive stroma. **Modification approved in the previous Progress Report:** The resulting cross will produce the FAP(CrePR1) / *Fgfr1*^{lox} / TRAMP mouse that will result in RU 468 regulated FGF receptor I knockout in cancer associated reactive stroma (see Figure 3).

During the last Progress Period we have expanded our colony of TRAMP (transgenic mouse for prostate cancer) mice for this project. The TRAMP mouse uses the probasin minimal promoter to drive expression of SV40 large T antigen. Tumors with optimal progression rates

Figure 4: Mouse lines generated in each Task

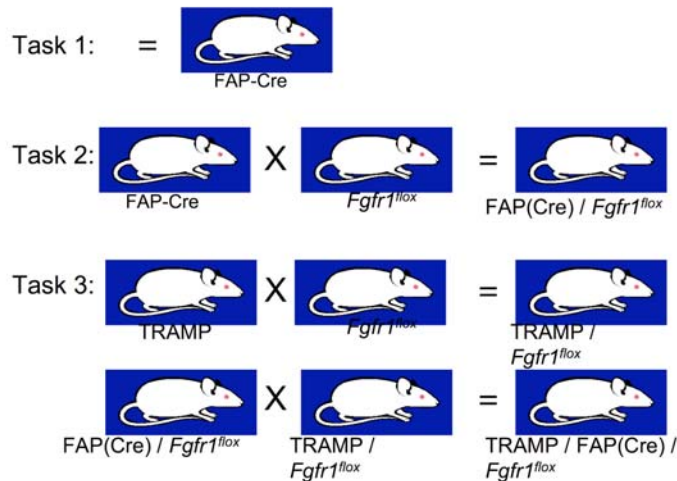


Figure 4: Mouse lines that will be developed

yield [TRAMP / *Fgfr1*^{lox} (C57BL/6)]/[FAP(Cre) / *Fgfr1*^{lox} (FVB)] mice (Figure 4). Administration of tamoxifen to mice will induce the conditional knock out of FGFR1 alleles at sites of reactive stroma via tamoxifen-induction of Cre recombinase activity and removal of floxed alleles in FAP positive carcinoma associated stromal cells. Control tumors will be from TRAMP mice having heterozygous or wild type FGFR1 alleles and hence, functioning FGFR1. When exposed to Cre, a single allele knockout in heterozygous mice for floxed FGFR1 alleles showed a wild type phenotype in the previous studies of Partanen, which focused on developing mid- and hindbrain (7). Completion of Task 3 will produce data that will directly address the central hypothesis. Once our tamoxifen inducible Cre mouse is created, we will be able to rapidly proceed to Task 3. We remain committed to the completion of this Aim even though the funding period of this project has ended. We will report any manuscripts generated from this work to the DOD.

Key Research Accomplishments:

Task 1:

- Acquisition of BAC (clone RP23-161B24) containing the mouse FAP gene. Verification.
- Acquisition and subcloning of Mifepristone inducible Cre recombinase (CrePR1). Verification of sequence.
- Acquisition and subcloning of an SV40 Neo selection cassette flanked with Flp recombinase sites. Sequence verification.
- Construction and subcloning of a downstream IRES element. Sequence verification.
- Construction and subcloning of a downstream CrePR1. Sequence verification.

- Addition of a poly A tail downstream of CrePR1. Sequence verification.
- Step-wise construction and subcloning of flanking upstream and downstream 55 bp FAP gene sequence in reverse orientation (homologous to Exon 2 region of FAP gene). Sequence verification at each step for proper reverse orientation.
- Acquisition of EL250 cells containing an arabinose inducible *flpe* gene and all recombineering reagents.
- Multiple experiments and conditions tested to recombine FAPupstream-IRES-CrePR1-FAPdownstream construct into BAC clones via homologous recombination.
- Acquisition of a tamoxifen-inducible Cre recombinase construct
- Isolation and cloning of an approximately 10 kb piece of the immediate upstream promoter of the murine FAP gene.
- Construction and cloning of a plasmid containing approximately 10 kb FAP promoter ligated upstream of the tamoxifen-inducible Cre recombinase sequence.
- Removal of plasmid sequences, purification and preparation of the FAP promoter / tamoxifen inducible Cre recombinase construct.
- Injection of these into mouse embryos for generation of a transgenic mouse.
- Identification of putative founder mouse (positive for transgene in tail clips) from the first transgenic litters.
- Breeding of founder male mouse with several females (litters are being screened).

Task 2:

- Acquisition of the *Fgfr1^{fllox}* mice (ICR background) and confirmation of floxed alleles.
- Rederivation of the *Fgfr1^{fllox}* mice by embryo transfer and initiation of *Fgfr1^{fllox}* mice (ICR background) in TMF pathogen free facility.
- >8 generations of crossing *Fgfr1^{fllox}* mice into FVB background. Now 0.39% ICR background. Ready for crossing Task 2 and 3.
- >8 generations of crossing *Fgfr1^{fllox}* mice into C57BL/6 background. Now 0.39% ICR background. Ready for crossing in Task 2 and 3.

Task 3:

- Acquisition of homozygous TRAMP mice and initiation of colony in TMF facility. Ready for crossing in Task 3.
- 6 generations of crossing *Fgfr1^{fllox}* mice with C57BL/6 (4 generations) and then crossing with TRAMP (2 generations). Now at less than 2% ICR background.

Reportable Outcomes:

During this project we have published several manuscripts and one chapter that focuses on the role of TGF- β , FGF-2 and CTGF in prostate cancer reactive stroma as discussed below.

- Most recently, we published a manuscript that shows that TGF- β receptor II in stromal cell required for the tumor-promoting activity of TGF- β in xenograft mouse models of prostate cancer (3). In addition, this manuscript showed that much of the TGF- β effects were attributed to the induction of FGF-2 in the responding reactive stroma. This study showed that TGF- β upregulated FGF-2 message and, importantly, TGF- β also induced FGF-2 protein release in a Smad3 mediated manner in prostate stromal cells.
- We have had an invited Chapter entitled "Reactive Stroma and Evolution of Tumors: Integration of Transforming Growth Factor- β , Connective Tissue Growth Factor, and Fibroblast Growth Factor-2 Activities" recently published that discusses the role of FGF-2 signaling in cancer associated reactive stroma (8). This chapter is in the Textbook

entitled: "Transforming Growth Factor-beta in Cancer Therapy" edited by Sonia B. Jakowlew at the NIH. This is both relevant to and supported by this project as this chapter discusses use of the FAP gene for targeting and the targeting of the FGF-2 signaling axis as a putative therapeutic.

- We published a manuscript that addresses the role of CTGF in prostate reactive stroma biology (4). Publication of this data is relevant to this project as we show that TGF- β stimulates expression of both FGF-2 and CTGF in reactive stroma. This paper represents the CTGF arm of this regulation and the present project represents the FGF-2 signaling arm. The reprint is attached. Title: "Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis". The reprint is attached. Finally, we showed that engineered re-expression of FGF-2 in stromal cells that were null for TGF- β receptor resulted in a restored angiogenesis and tumor growth. These data fully support the present project since it was our original hypothesis that FGF-2 signaling in cancer associated stroma promotes tumor growth. Generation of the engineered mouse lines from this study will allow us to probe specific mechanisms with novel models.

Conclusions:

The purpose of this study was to address the role of FGF receptor 1 signaling in carcinoma-associated stroma by generating and using a novel mouse model. A central goal here is the targeted gene expression specifically to sites of reactive stroma. The use of the FAP promoter is a novel concept and generation of the FAP(Cre) mouse will be a resource for all investigators who study reactive stroma tumor microenvironment, since FAP gene expression is observed in reactive stroma of all the major adenocarcinomas.

Although we have had major delays to unanticipated issues with our primary approach, we have been able to focus the project using alternative approach techniques that have worked much better. These approaches were disclosed as an alternative plan in previous Progress Reports. We anticipate no further time delays and we will complete the study as proposed even though formal funding has ended.

Completion of this work and reporting of results will be the first time a transgene will be expressed specifically in the reactive stroma compartment of a tumor mouse model and represents the first time a gene will be conditionally knocked out in tumor associated stroma. This will make it possible to study the biology of engineered gene expression in the tumor microenvironment. The concept that a cancer might be targeted therapeutically via targeting the cancer cell niche in reactive stroma is an important concept to translate into the clinics. Our laboratory is committed to seeing this become reality. Funding of this project by the DOD has made this possible. These studies permit the evaluation of a microenvironment that was previously not possible. As an additional benefit, the FAP-Cre mouse will be valuable to other investigators using mouse models of cancer as the inducible Cre can be used to manipulate gene expression via either overexpression or attenuation of expression) gene expression. We are fully committed to completion of this project as we feel this will represent a major advancement.

Bibliography of Publications Supported by the Project:

1. Yang, F., Strand, D. W., and Rowley, D. R. Fibroblast growth factor-2 mediates transforming growth factor-beta action in prostate cancer reactive stroma. *Oncogene*, 27: 450-459, 2008. (Attached in Appendix)
2. Yang, F., Tuxhorn, J. A., Ressler, S. J., McAlhany, S. J., Dang, T. D., and Rowley, D. R. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res*, 65: 8887-8895, 2005. (Attached in Appendix)
3. Rowley, D. R. Reactive stroma and evolution of tumors: Integration of transforming growth factor-beta, connective tissue growth factor, and fibroblast growth factor-2 activities. *In*: S. Jakowlew (ed.), *Transforming Growth Factor-beta in Cancer Therapy*, Vol. (In Press). Towta, NJ: Humana Press, 2008.

Personnel Supported by the Project:

1. David R. Rowley, Ph.D.
Professor
Molecular and Cellular Biology
2. Feng Yang, Ph.D.
Instructor
Molecular and Cellular Biology
3. Steven J. Ressler, Ph.D.
Postdoctoral Associate
Molecular and Cellular Biology
4. David A. Barron, B.S.
Graduate Student
Molecular and Cellular Biology

References:

1. Tuxhorn, J. A., Ayala, G. E., Smith, M. J., Smith, V. C., Dang, T. D., and Rowley, D. R. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res*, 8: 2912-2923., 2002.
2. Tuxhorn, J. A., McAlhany, S. J., Yang, F., Dang, T. D., and Rowley, D. R. Inhibition of TGF- β activity decreases angiogenesis in a human prostate cancer reactive stroma xenograft model. *Cancer Res*, 62: 6021-6025, 2002.
3. Yang, F., Strand, D. W., and Rowley, D. R. Fibroblast growth factor-2 mediates transforming growth factor-beta action in prostate cancer reactive stroma. *Oncogene*, 27: 450-459, 2008.
4. Yang, F., Tuxhorn, J. A., Ressler, S. J., McAlhany, S. J., Dang, T. D., and Rowley, D. R. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res*, 65: 8887-8895, 2005.
5. Mathew, S., Scanlan, M. J., Mohan Raj, B. K., Murty, V. V., Garin-Chesa, P., Old, L. J., Rettig, W. J., and Chaganti, R. S. The gene for fibroblast activation protein alpha (FAP),

- a putative cell surface-bound serine protease expressed in cancer stroma and wound healing, maps to chromosome band 2q23. *Genomics*, 25: 335-337., 1995.
6. Park, J. E., Lenter, M. C., Zimmermann, R. N., Garin-Chesa, P., Old, L. J., and Rettig, W. J. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem*, 274: 36505-36512., 1999.
 7. Trokovic, R., Trokovic, N., Hernesniemi, S., Pirvola, U., Vogt Weisenhorn, D. M., Rossant, J., McMahon, A. P., Wurst, W., and Partanen, J. FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmus signals. *Embo J*, 22: 1811-1823, 2003.
 8. Rowley, D. R. Reactive stroma and evolution of tumors: Integration of transforming growth factor-beta, connective tissue growth factor, and fibroblast growth factor-2 activities. *In*: S. Jakowlew (ed.), *Transforming Growth Factor-beta in Cancer Therapy*, Vol. (In Press). Towata, NJ: Humana Press, 2007.

ORIGINAL ARTICLE

Fibroblast growth factor-2 mediates transforming growth factor- β action in prostate cancer reactive stromaF Yang¹, DW Strand¹ and DR Rowley*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA*

Transforming growth factor- β (TGF- β) is overexpressed at sites of wound repair and in most adenocarcinomas including prostate cancer. In stromal tissues, TGF- β regulates cell proliferation, phenotype and matrix synthesis. To address mechanisms of TGF- β action in cancer-associated reactive stroma, we developed prostate stromal cells null for TGF- β receptor II (T β RII) or engineered to express a dominant-negative Smad3 to attenuate TGF- β signaling. The differential reactive stroma (DRS) xenograft model was used to evaluate altered stromal TGF- β signaling on LNCaP tumor progression. LNCaP xenograft tumors constructed with T β RII null or dominant-negative Smad3 stromal cells exhibited a significant reduction in mass and microvessel density relative to controls. Additionally, decreased cellular fibroblast growth factor-2 (FGF-2) immunostaining was associated with attenuated TGF- β signaling in stroma. *In vitro*, TGF- β stimulated stromal FGF-2 expression and release. However, stromal cells with attenuated TGF- β signaling were refractory to TGF- β -stimulated FGF-2 expression and release. Re-expression of FGF-2 in these stromal cells in DRS xenografts resulted in restored tumor mass and microvessel density. In summary, these data show that TGF- β signaling in reactive stroma is angiogenic and tumor promoting and that this effect is mediated in part through a T β RII/Smad3-dependent upregulation of FGF-2 expression and release.

Oncogene (2008) 27, 450–459; doi:10.1038/sj.onc.1210663; published online 16 July 2007

Keywords: transforming growth factor- β ; fibroblast growth factor; prostate cancer; stroma; angiogenesis

Introduction

Several studies suggest that a reactive stroma microenvironment affects rate of carcinoma progression, although key factors and signaling mechanisms are poorly understood. Transforming growth factor- β

(TGF- β) is overexpressed in most carcinomas and regulates diverse functions of stromal cells through both Smad-dependent and -independent signaling pathways (Coffey *et al.*, 1986; Roberts *et al.*, 1986; Roberts and Sporn, 1996; Derynck and Zhang, 2003). TGF- β modulates stromal cell phenotype, promotes matrix remodeling, promotes angiogenesis and affects immune responses at sites of tumor formation (Rowley, 2007). Most of these actions are considered tumor promoting. TGF- β has also been considered a tumor suppressor, as it inhibits proliferation of normal epithelial cells; however, many carcinoma cells become refractory to the growth inhibitory activity of TGF- β due to loss or mutation of various TGF- β signaling pathway components in these cells during tumorigenesis (Akhurst and Derynck, 2001). Therefore, understanding the net effects of TGF- β in carcinoma progression is complicated. Net response to TGF- β is likely to be dynamic during evolution of a tumor and will reflect the combined effects of this factor on both carcinoma cells and on all components of the reactive stroma microenvironment.

We have previously shown that a reactive stroma co-evolves early in human prostate cancer during the formation of pre-malignant prostatic intraepithelial neoplasia (PIN) (Tuxhorn *et al.*, 2002a). This reactive stroma was typified by carcinoma-associated fibroblasts, myofibroblasts and matrix remodelling. Elevated TGF- β expression was observed in PIN epithelia and prostate cancer cells. We also showed that TGF- β could induce human prostate fibroblasts to a myofibroblast phenotype *in vitro* with elevated expression of tenascin, a marker of reactive stroma (Tuxhorn *et al.*, 2002a). Our previous studies have developed a xenograft model (differential reactive stroma, DRS) that recombines LNCaP prostate carcinoma cells with engineered prostate stromal cells (Tuxhorn *et al.*, 2002b,c; Yang *et al.*, 2005). In these studies we have shown that reactive stroma promotes experimental prostate cancer progression and much of this was due to stromal regulation of angiogenesis (Tuxhorn *et al.*, 2002b). Using this same experimental xenograft model, we have also reported that both TGF- β and connective tissue growth factor (CTGF), a downstream mediator of TGF- β action, stimulate angiogenesis and promote tumorigenesis (Tuxhorn *et al.*, 2002c; Yang *et al.*, 2005). In contrast, under different conditions with different models and carcinoma/stromal cell lines, other studies showed that

Correspondence: Dr DR Rowley, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, 325D, Houston, TX 77030, USA.

E-mail: drowley@bcm.tmc.edu

¹These authors have contributed equally to this work.

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loss of TGF- β signaling in stroma resulted in elevated expression of tumor-promoting factors (TGF- α and HGF) and a net tumor progression (Bhowmick *et al.*, 2004; Cheng *et al.*, 2005). Together, these studies support the concept that the actions of TGF- β and specific signaling pathways, including downstream mediators expressed in reactive stroma, is an important area for further study. In addition, although the role of Smad3-mediated TGF- β signaling in tissue fibrosis or wounding has been studied (Roberts *et al.*, 2003; Lakos *et al.*, 2004), it is not known whether Smad3 mediates TGF- β induction of angiogenesis and reactive stroma during carcinoma progression. Accordingly, in order to more clearly define the complicated regulation of carcinogenesis by TGF- β action in stroma, the purposes of this study were to determine whether Smad3-mediated TGF- β signaling is a key pathway in cancer-associated stroma and to subsequently assess candidate downstream mediators of TGF- β /Smad3 biological action.

In this report, we use the LNCaP carcinoma/prostate stromal cell recombined DRS xenograft model to show that loss of TGF- β signaling in prostate stromal cells through either a targeted knockout of TGF- β receptor II (T β RII) or by expression of a dominant-negative Smad3 results in an inhibition of the angiogenesis- and tumor-promoting function of reactive stroma. In addition, we show that the angiogenic and tumorigenic function of stromal TGF- β signaling is mediated, in part, by induced expression and release of FGF-2 from prostate stromal cells in a T β RII/Smad3-dependent manner.

Results

Attenuated TGF- β response in prostate stromal cells

The T β RII^{lox/lox}H, T β RII KO, T β RII CT, Smad3 Ctrl and Smad3 DN prostate stromal cell lines were generated as described in Materials and methods. Table 1 summarizes the designated name for each prostate stromal cell line and how each cell line was derived and/or engineered. In T β RII^{lox/lox}H and T β RII CT control cells, TGF- β 1 (50 pM) stimulated a seven- to eight-fold expression of p800Luc (PAI-1 promoter), a three-fold expression of α -SMAp-luc (smooth muscle α -actin promoter) and a three- to four-fold expression of pVim-luc (vimentin promoter) (Figure 1a). In contrast, there was no significant TGF- β 1-induced promoter activity with any of these constructs in the T β RII KO cells. TGF- β 1 also induced a 250- to 270-fold expression of (CAGA)₁₂MLP (Smad binding sequence) in control cell lines, whereas this induction was restricted to 2.6-fold in T β RII KO cells (Figure 1a). Our previous study showed that TGF- β 1 induces a myofibroblast/smooth muscle phenotype in prostate stromal cells *in vitro* (Tuxhorn *et al.*, 2002a). Concordantly, TGF- β 1 induced smooth muscle α -actin filament formation in T β RII CT control cells; however, T β RII KO cells were refractory to TGF- β -induced filament formation (Figure 1b).

Table 1 Derivation and engineering of prostate stromal cell lines

Prostate stromal cell line	Method of cell line generation
T β RII ^{lox/lox} H	Parent cell line derived from Tgfb ^{lox/lox} E2 ^{lox/lox} mouse
T β RII KO ^a	T β RII ^{lox/lox} H cells infected with Cre recombinase
T β RII CT ^a	T β RII ^{lox/lox} H cells infected with mutant Cre recombinase
C57B	Parent cell line derived from C57BL/6 mouse
Smad3 DN ^b	C57B cells infected with pLPCX-Smad3 Δ SSVS
Smad3 Ctrl ^b	C57B cells infected with empty pLPCX vector
T β RII	T β RII KO cells infected with pBMN-FGF-2-GFP
KO+FGF-2 ^c	T β RII KO cells infected with empty pBMN-I-eGFP vector
T β RII	T β RII KO cells infected with empty pBMN-I-eGFP vector
KO+Ctrl ^c	Smad3 DN cells infected with pBMN-FGF-2-GFP
Smad3	Smad3 DN cells infected with empty pBMN-I-eGFP
DN+FGF-2 ^d	Smad3 DN cells infected with empty pBMN-I-eGFP
Smad3	Smad3 DN cells infected with empty pBMN-I-eGFP
DN+Ctrl ^d	Smad3 DN cells infected with empty pBMN-I-eGFP

^aTumor data compared in Figure 3a. ^bTumor data compared in Figure 3b. ^cTumor data compared in Figure 6c. ^dTumor data compared in Figure 6d.

Expression of dominant-negative Smad3 in prostate stromal cells also attenuated TGF- β signaling. Expression of the Flag-Smad3 Δ SSVS construct was verified by anti-Flag immunofluorescence (Figure 2a). Attenuation of Smad3 signaling was verified by a significant reduction of TGF- β 1-induced expression of the Smad3-responsive (CAGA)₁₂MLP reporter in the Smad3 DN cells compared to control (Figure 2b).

Attenuated stromal TGF- β signaling in LNCaP + stroma xenografts

Construction of LNCaP/prostate stromal cell xenografts and evaluation time points followed protocols identical to what we have published previously (Tuxhorn *et al.*, 2002b,c; Yang *et al.*, 2005). Xenografts that were constructed with LNCaP plus T β RII KO stromal cells resulted in a 44.1% decrease in microvessel density ($P < 0.0001$, $n = 72$ fields from 12 xenografts in each group) and a 49.8% decrease in tumor mass compared to LNCaP plus T β RII CT control stromal cells (Figure 3a) ($P = 0.0167$, $n = 18$) at the 4-week end point. Histopathology of xenografts in all cases revealed clusters of LNCaP cells adjacent to reactive stroma and vessels similar to what we have reported previously for this model. No obvious histological differences were observed in experimental compared to control xenografts. To compare the morphology of current experiments (Figures 7a and b) with subsequent experiments, Figure 7 shows histology for all xenografts in this study. To confirm the histological observations, carcinoma cell to stromal cell ratios were determined in xenografts as described in Materials and methods. No statistically significant differences were observed in carcinoma cell to stromal cell ratios between experimental and control xenografts within each data set (data not shown).

Similar decreases in mass and microvessel density were observed in LNCaP plus Smad3 DN xenografts

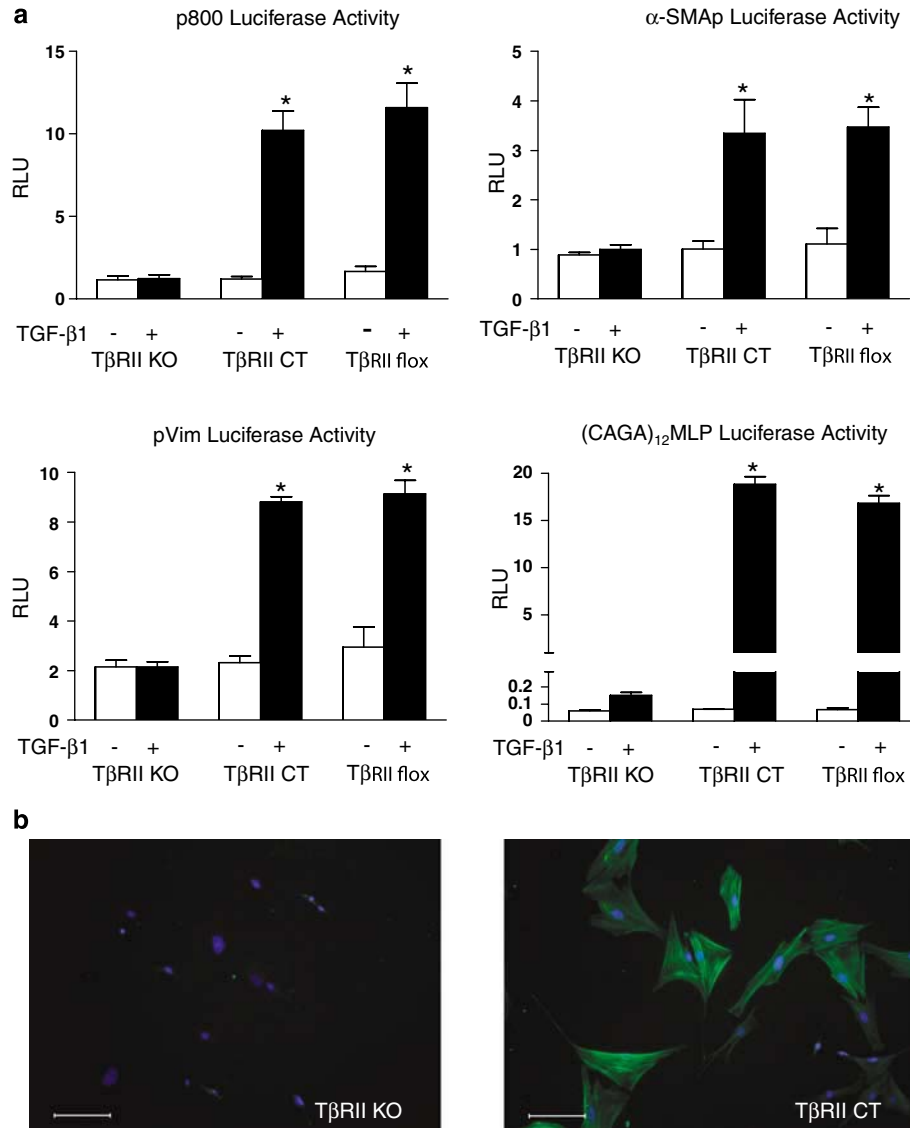


Figure 1 Attenuation of TGF- β signaling in T β RII KO prostate stromal cells. (a) T β RII KO, T β RII CT and the parent T β RII^{flox/flox}H prostate stromal cells (T β RII flox) were transfected with the TGF- β -responsive luciferase reporters, p800Luc, α -SMAp-luc, pVim-luc or (CAGA)₁₂MLP. Cultures were then exposed to either vehicle control (–) or TGF- β 1 (50 pM) (+) for 24 h and assayed for luciferase activity, which was normalized to the co-transfected pRL-null-expressed Renilla luciferase activity. Data shown are mean $\pm$ s.e.m. derived from replicate-independent experiments. *Significant difference ($P < 0.05$). (b) In addition, smooth muscle α -actin filament formation was stimulated by TGF- β in T β RII CT, but not T β RII KO cells. TGF- β , transforming growth factor- β ; T β RII, TGF- β receptor II.

evaluated at both 2-week (day 14) and 4-week (day 28) end points. LNCaP/Smad3 DN xenografts exhibited a 24.5% decrease in mean mass at 2 weeks compared to control LNCaP/Smad3 Ctrl xenografts ($P = 0.0014$, $n = 18$) (data not shown). At the 4-week end point, a 31.4% decrease in microvessel density ($P = 0.0089$, $n = 72$ fields/12 xenografts) and a 40.6% decrease in mean wet weight ($P = 0.0216$, $n = 18$) was observed in LNCaP/Smad3 DN xenografts relative to LNCaP/Smad3 Ctrl xenografts (Figure 3b). As discussed above, no differences in histology or carcinoma cell to stromal cell ratios were observed (Figures 7d and e).

TGF- β 1 induces FGF-2 mRNA and protein expression in stromal cells

Stromal cells were examined by quantitative real-time PCR (qPCR) to assess whether TGF- β 1 stimulated FGF-2 mRNA expression. TGF- β 1 stimulated expression of FGF-2 mRNA by 2- to 7-fold in both the T β RII CT ($P = 0.0332$) and Smad3 Ctrl control ($P = 0.0133$) prostate stromal cells (Figure 4a). In contrast, FGF-2 mRNA remained at basal levels and was refractory to TGF- β 1 stimulation in both the T β RII KO and Smad3 DN prostate stromal cells. Consistent with this observation, stromal FGF-2 immunoreactivity was greatly decreased in LNCaP/T β RII KO xenografts

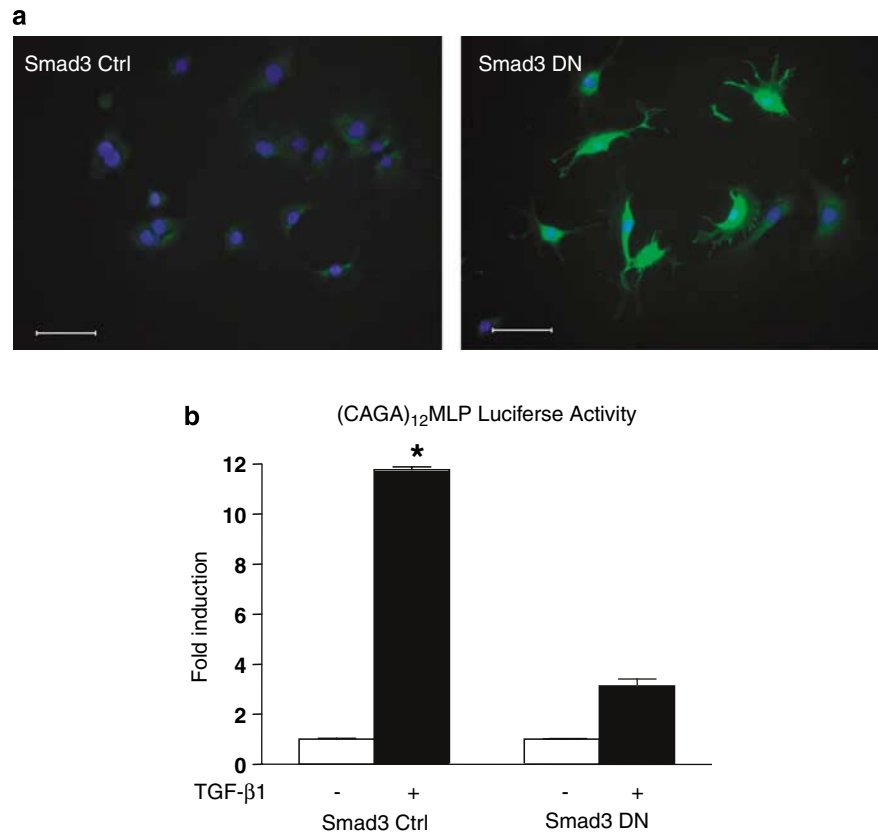


Figure 2 Attenuation of TGF- β signaling in C57B-Smad3 DN prostate stromal cells. **(a)** Flag-Smad3 Δ SSVS expression was verified by immunofluorescence using an anti-Flag antibody. **(b)** Smad3 Ctrl and Smad3 DN prostate stromal cell lines were subsequently transfected with the Smad3-responsive luciferase reporter, (CAGA)₁₂MLP, and treated with TGF- β . Smad3 DN expression significantly attenuated (CAGA)₁₂MLP responsiveness to TGF- β stimulation. TGF- β , transforming growth factor- β .

compared to control LNCaP/T β RII CT xenografts (Figure 4b).

TGF- β 1 induces FGF-2 release in stromal cells

Extracts from stromal cells stimulated with TGF- β 1 showed that total cellular FGF-2 protein was significantly elevated in both T β RII CT and Smad3 Ctrl cells as determined by enzyme-linked immunosorbent assay (ELISA) ($P=0.0166$ and $P<0.0001$, respectively; Figure 5a). This effect was abrogated in T β RII KO and Smad3 DN cells. Detection of FGF-2 released into conditioned media by control cells was below the ELISA detection threshold. Hence, to determine whether TGF- β 1 also induces FGF-2 protein secretion/release, T β RII CT, T β RII KO, Smad3 Ctrl and Smad3 DN prostate stromal cells were each engineered to overexpress an 18 kDa FGF-2-GFP (green fluorescent protein) fusion protein. Under these conditions, TGF- β 1 induced a significant, dose-dependent secretion/release of FGF-2 protein into the conditioned media in both the control T β RII CT+FGF-2 ($P=0.0472$) and the Smad3 Ctrl+FGF-2 cells ($P=0.0085$) (Figure 5b). Conversely, TGF- β 1 had no effect on FGF-2 release from the T β RII KO+FGF-2 or Smad3 DN+FGF-2 cells. As a control, western blot results show that ectopic FGF-2

was expressed at equivalent levels in control cells and cells with attenuated TGF- β signaling (Figure 5b, inset).

T β RII/Smad3-mediated FGF-2 expression in prostate stromal cells promotes angiogenesis and xenograft tumor growth

To determine whether the tumor-inhibiting effects of attenuated TGF- β signaling in stromal cells could be attributed to decreased FGF-2 expression, xenografts were constructed with LNCaP cells plus either T β RII KO or Smad3 DN cells engineered to overexpress the 18 kDa FGF-2-GFP fusion protein (T β RII KO+FGF-2 and Smad3 DN+FGF-2, respectively) or the empty vector control (T β RII KO+Ctrl or Smad3 DN+Ctrl) as described above. LNCaP/T β RII KO+FGF2 xenografts exhibited a recovery of stromal FGF-2 immunostaining (Figure 6a) and this was due to the expression of the FGF-2-GFP fusion protein as these cells were also positive for GFP (Figure 6b). Overexpression of FGF-2 under these conditions produced a 40.0% increase in xenograft mass in LNCaP/T β RII KO+FGF2 xenografts compared to LNCaP/T β RII KO+Ctrl xenografts at the 2-week end point ($P=0.0127$, $n=18$) (data not shown). This expanded to a 141.1% increase in xenograft mass in LNCaP/T β RII KO+

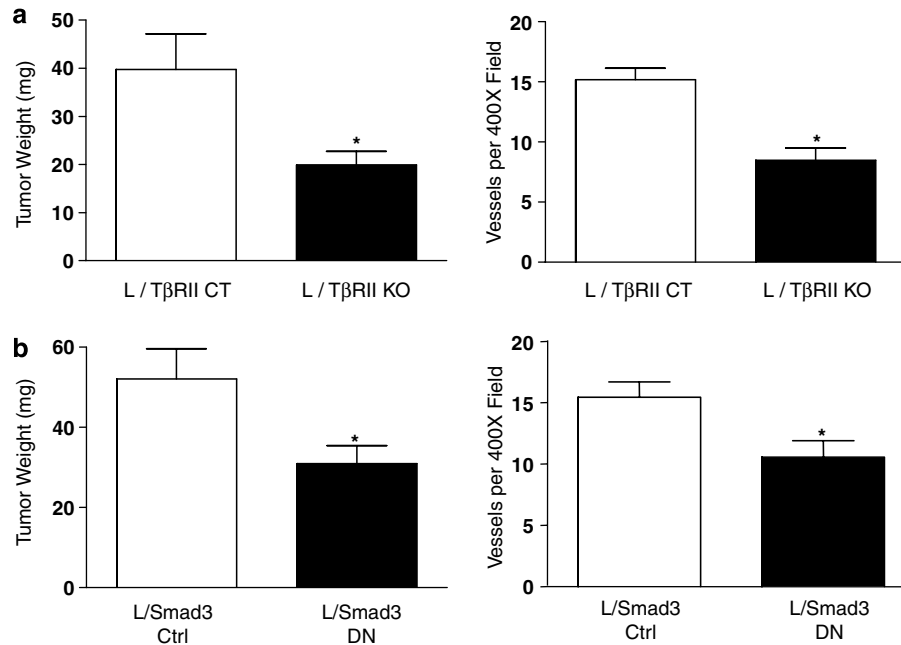


Figure 3 Attenuation of TGF- β signaling in stromal cells inhibits angiogenesis and tumor growth in LNCaP xenografts. (a) A significant decrease in xenograft mass was observed at day 28 in LNCaP/T β RII KO xenografts relative to control LNCaP/T β RII CT xenografts. LNCaP/T β RII KO xenografts also exhibited a significant decrease in the mean microvessel density compared to control LNCaP/T β RII CT xenografts. (b) Similarly, a significant decrease in xenograft weight was observed at day 28 in LNCaP/Smad3 DN xenografts relative to control LNCaP/Smad3 Ctrl xenografts. Concordantly, LNCaP/Smad3 DN xenografts exhibited a significant decrease in mean microvessel density compared to control LNCaP/Smad3 Ctrl xenografts. *Significant difference ($P < 0.05$). TGF- β , transforming growth factor- β ; T β RII, TGF- β receptor II.

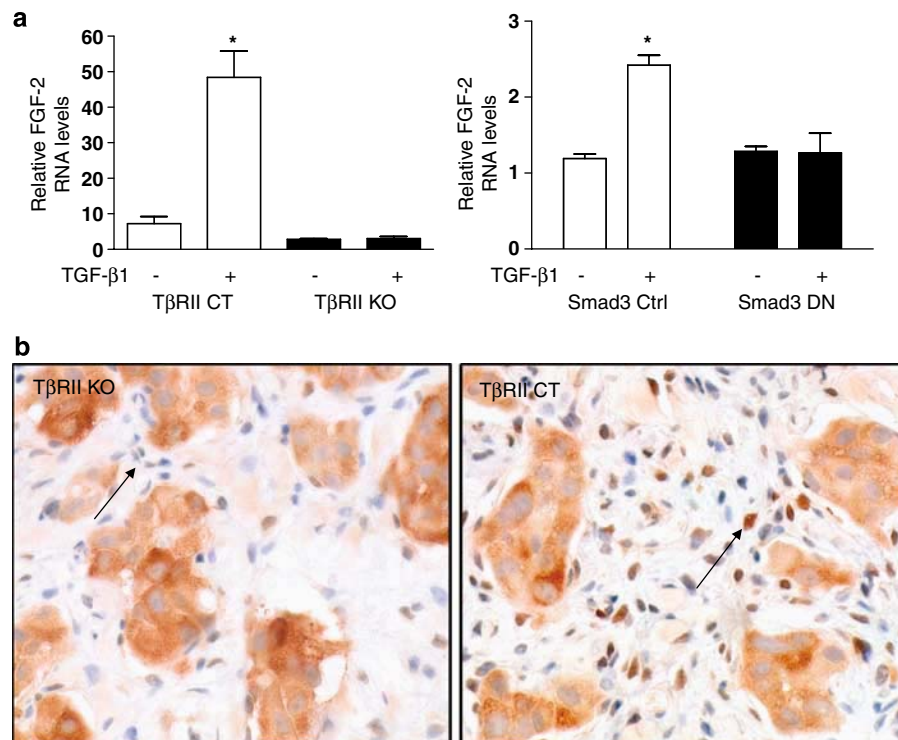


Figure 4 TGF- β 1 induces FGF-2 expression in prostate stroma. (a) qPCR data showed a significant upregulation of FGF-2 mRNA in TGF- β 1-treated T β RII CT or Smad3 Ctrl cells, but not in T β RII KO or Smad3 DN cells. (b) Immunohistochemistry revealed positive FGF-2 staining in T β RII CT cells, but little to no immunoreactivity was observed in T β RII KO stromal cells in LNCaP/Stroma xenografts ($\times 400$). Clusters of immunoreactive cells are LNCaP epithelia and arrows point to stromal cells surrounding epithelia. TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2; qPCR, quantitative PCR; T β RII, TGF- β receptor II.

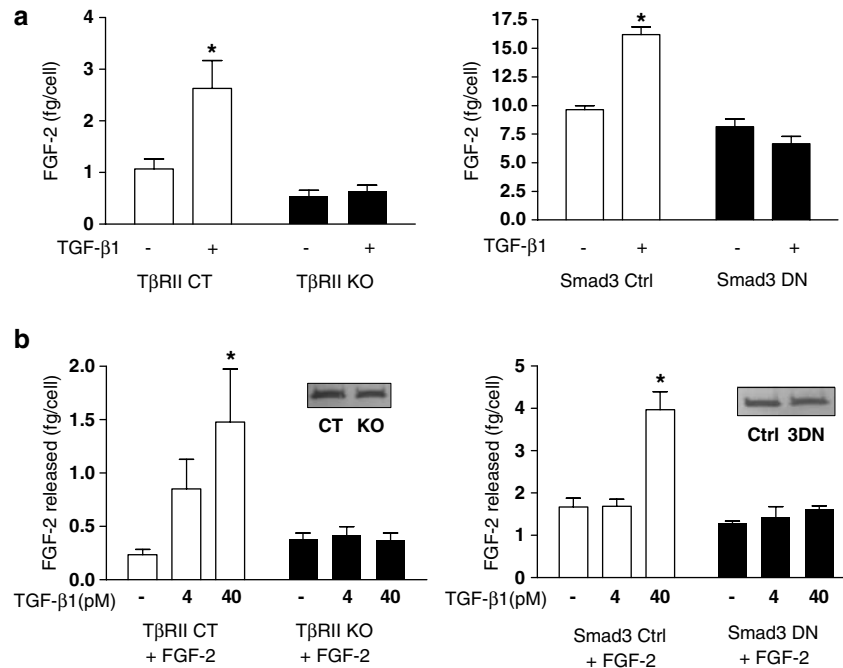


Figure 5 TGF- β 1 induces FGF-2 protein expression and secretion/release in prostate stroma. (a) ELISA results showed a significant increase in FGF-2 protein levels in TGF- β 1-treated T β RII CT and Smad3 Ctrl cells, but not in T β RII KO or Smad3 DN cells. (b) T β RII CT, T β RII KO cells, Smad3 Ctrl and Smad3 DN cells were infected to express equivalent levels of FGF-2-GFP protein, as shown by western blot (anti-GFP, inset). ELISA results revealed a significant increase in FGF-2 protein released from TGF- β 1-treated T β RII CT + FGF-2 and Smad3 Ctrl + FGF-2 cells in a dose-dependent manner, whereas this response was inhibited in either T β RII KO + FGF-2 or Smad3 DN + FGF-2 cells. *Significant difference ($P < 0.05$). TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; T β RII, TGF- β receptor II.

FGF2 xenografts compared to LNCaP/T β RII KO + Ctrl xenografts at the 4-week end point ($P < 0.0001$, $n = 18$). In addition, the average xenograft mass of LNCaP/T β RII KO + FGF-2 xenografts (44.14 ± 5.82 mg) was comparable to the control LNCaP/T β RII CT tumor mass of 39.77 ± 7.36 mg at 4 weeks with native FGF-2 expression with intact TGF- β signaling. Concordantly, LNCaP/T β RII KO + FGF2 xenografts showed a 38.9% increase in microvessel density compared to control LNCaP/T β RII KO + Ctrl xenografts ($P = 0.0307$, $n = 72$ fields/12 xenografts) (Figure 6c). Similarly, overexpression of FGF-2 in Smad3 DN stromal cells produced a 30.4% increase in LNCaP/Smad3 DN + FGF-2 xenograft mass compared to LNCaP/Smad3 DN + Ctrl control xenografts at the 2-week end point ($P = 0.0138$, $n = 18$; Figure 6d). Interestingly, the mean mass in LNCaP/Smad3 DN + FGF-2 xenografts (24.15 ± 1.76 mg) is comparable to the mean mass of 2-week control LNCaP/Smad3 Ctrl xenografts (28.46 ± 1.21 mg) with intact Smad3 signaling. Furthermore, LNCaP/Smad3 DN + FGF-2 xenografts showed a 45.3% increase in microvessel density compared to control LNCaP/Smad3 DN + Ctrl xenografts ($P = 0.0027$, $n = 72$ fields/12 xenografts; Figure 6d). Histopathologic analysis showed that there were no obvious differences in histology between control xenografts and xenografts with attenuated TGF- β signaling or xenografts with attenuated TGF- β signaling and overexpression of FGF-2 (Figure 7). As in previous

experiments, there were no statistically significant differences in carcinoma to stromal cell ratios in these experiments (data not shown).

Discussion

Data presented here represent the first direct experimental evidence that links TGF- β signaling in the stromal compartment with the angiogenic, tumor-promoting effects of reactive stroma in an experimental human prostate cancer model. These data show that tumor-promoting TGF- β signaling in prostate stromal cells is mediated through a Smad3 pathway, although our results do not rule out other TGF- β -activated signaling pathways. In addition, these data show that the biological action of TGF- β signaling in reactive stroma is mediated, in part, through stimulated expression and secretion/release of FGF-2, also in a Smad3-regulated manner. Coordinate TGF- β /FGF-2 signaling in stroma resulted in elevated vessel density and prostate cancer xenograft growth, consistent with pro-reactive stroma and pro-angiogenic activities of both TGF- β 1 (Roberts *et al.*, 1986) and FGF-2 (Dow and deVere White, 2000). These results suggest that TGF- β 1 is a key upstream factor that regulates stromal microenvironment biology during prostate cancer progression and that signaling through Smad3 is a critical pathway. Data

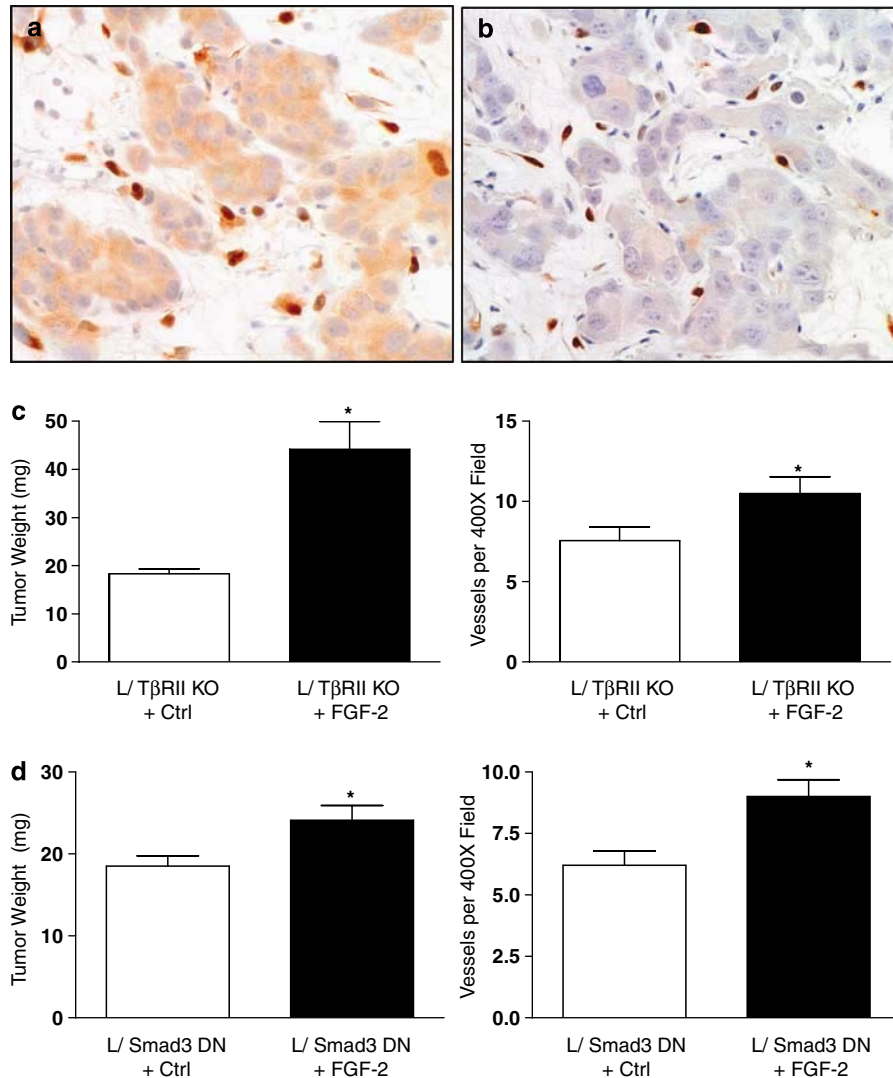


Figure 6 Ectopic expression of FGF-2 in stromal cells with attenuated TGF- β signaling restores LNCaP/Stroma xenograft growth and angiogenesis. Expression of an FGF-2-GFP fusion protein in T β RII KO prostate stromal cells in LNCaP/Stroma xenografts was verified by immunohistochemistry with (a) anti-FGF-2 and (b) anti-GFP antibodies ($\times 400$). LNCaP/Stroma xenografts constructed with T β RII KO (c) or Smad3 DN (d) cells ectopically expressing FGF-2 display a significant increase in tumor mass compared to control xenografts. Concordantly, microvessel density was significantly elevated in T β RII KO + FGF-2 and Smad3 DN + FGF-2 xenografts compared to T β RII KO + Ctrl or Smad3 DN + Ctrl xenografts. *Significant difference ($P < 0.05$). TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2; GFP, green fluorescent protein; T β RII, TGF- β receptor II.

presented here are consistent with the implication of Smad3 in mediating wound healing and fibrosis. As might be predicted, Smad3 null mice were refractory to TGF- β 1-induced pulmonary fibrosis (Bonniaud *et al.*, 2004). However, the function of Smad3 in mediating TGF- β -induced responses in cancer-associated reactive stroma biology is poorly understood. Data here suggest that Smad3-mediated pathways may be common to tissue wound repair, fibrosis and reactive stroma in cancer.

Our previous reports have shown that TGF- β is overexpressed in pre-malignant PIN epithelia and that a reactive stroma phenotype co-evolved at sites adjacent to PIN (Tuxhorn *et al.*, 2002a). This initial reactive stroma consisted of carcinoma-associated fibroblasts, myofibroblasts and matrix remodeling, typical of a

wound repair stroma. Matrix remodeling was typified by overexpression of collagen I, tenascin and fibroblast activation protein. TGF- β 1 stimulates synthesis of matrix components by fibroblasts, including collagen type I and promotes angiogenesis in wound repair granulation tissue (Roberts *et al.*, 1986; Roberts and Sporn, 1996). In prostate stromal cells, TGF- β 1 induced synthesis of collagen type I (Fukabori *et al.*, 1997), fibronectin (Butter *et al.*, 2001), tenascin (Tuxhorn *et al.*, 2002c), versican (Sakko *et al.*, 2001) and induced myodifferentiation (Peehl and Sellers, 1998; Tuxhorn *et al.*, 2002a). TGF- β 1 is overexpressed in many carcinomas, including prostate cancer epithelial cells (Eastham *et al.*, 1995), and has been reported as a primary inducer of myofibroblast differentiation in reactive stroma fibroblasts (Desmouliere *et al.*, 1993;

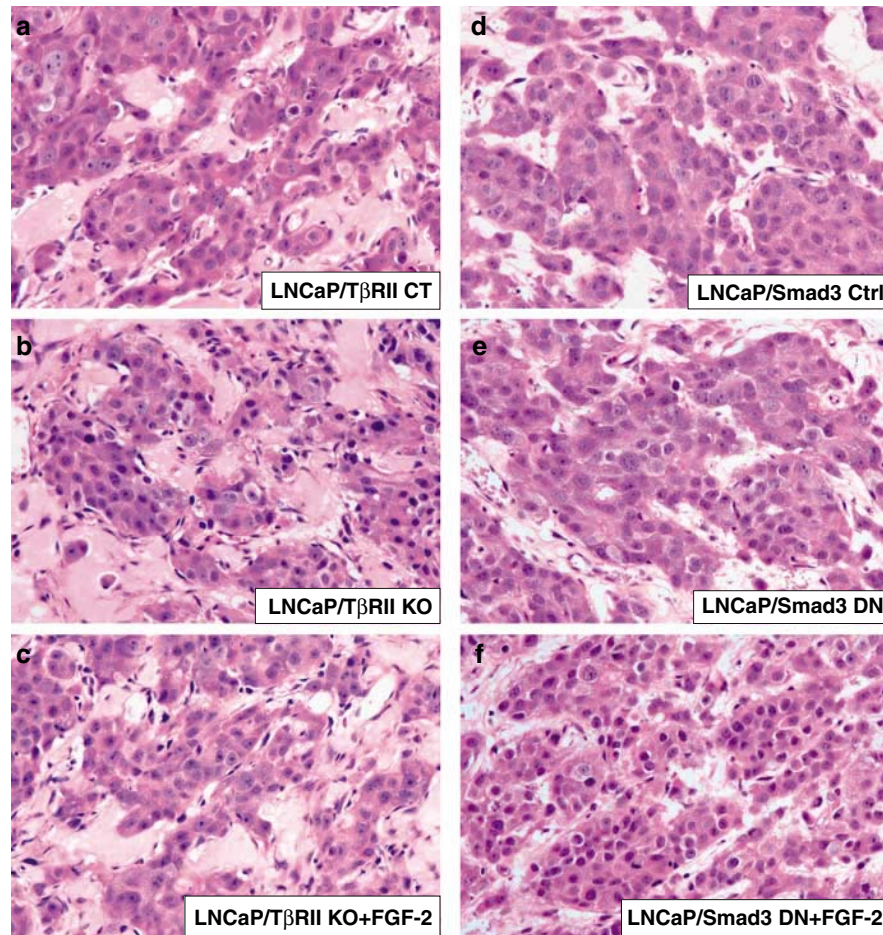


Figure 7 Comparative histology of xenografts. Representative H&E histology ($\times 400$) is shown for each of the following xenografts: (a) LNCaP/T β RII CT, (b) LNCaP/T β RII KO, (c) LNCaP/T β RII KO + FGF-2, (d) LNCaP/Smad3 Ctrl, (e) LNCaP/Smad3 DN and (f) LNCaP/Smad3 DN + FGF-2. H&E, hematoxylin and eosin; T β RII, TGF- β receptor II.

Tuxhorn *et al.*, 2002a). Consistent with this, the myfibroblast is a common stromal cell type in carcinoma-associated stroma in many different epithelial cancers (Ronnov-Jessen *et al.*, 1996; Tuxhorn *et al.*, 2002a).

The carcinoma-associated reactive stroma in human cancer has been characterized as being similar to a wound repair granulation tissue, which commonly has a high myfibroblast population (Dvorak, 1986; Ronnov-Jessen *et al.*, 1996; Rowley, 2007). In granulation tissue, elevated angiogenesis is coordinate with elevated FGF-2 and it is well established that TGF- β promotes granulation tissue formation and angiogenesis (Roberts *et al.*, 1986). FGF-2 is TGF- β -regulated and a mitogen for epithelial and stromal cells while also playing a role in the migration of endothelial cells during blood vessel formation (Dow and deVere White, 2000). Overexpression of FGF-2 in stromal cells is nearly universal in wound repair, fibroses and cancer-associated reactive stroma (Rowley, 2007). In addition to its fibrogenic role during wound repair, a significant upregulation of FGF-2 is seen in a variety of cancers including prostate cancer (Giri *et al.*, 1999; Dow and deVere White, 2000). Concordantly, tumor growth in the TRAMP mouse

prostate cancer model was slowed in an FGF-2 knock-out background (Polnaszek *et al.*, 2003). In addition, FGF receptor 1 (FGFR1), a cognate receptor for FGF-2, is upregulated in epithelia during prostate cancer progression in human cancer (Giri *et al.*, 1999) and in mouse prostate cancer models (Huss *et al.*, 2003). Elevated FGF-2 expression in the stroma might be expected to provide a growth advantage for carcinoma cells with elevated expression of FGFR1. Therefore, it is likely that a TGF- β 1-regulated and Smad3-mediated expression and release of FGF-2 in reactive stroma provides a pro-angiogenic and pro-tumorigenic micro-environment. Accordingly, the TGF- β /FGF-2 signaling axis is likely to be a key regulatory component of carcinoma cell-stromal cell interaction.

Our results do not rule out the involvement of other TGF- β -regulated growth factors. We believe that it is highly unlikely that FGF-2 is the only pathway downstream of TGF- β in stroma promoting LNCaP tumor growth, but that it does partially mediate the angiogenic action of TGF- β . In addition to FGF-2, TGF- β stimulates expression of many other growth factors in stromal cells including vascular endothelial growth factor, heparin-binding-epidermal growth factor, interleukin-6

and CTGF (Igarashi *et al.*, 1993; Pertovaara *et al.*, 1994; Story *et al.*, 1996; Uchiyama-Tanaka *et al.*, 2002; Hayashi *et al.*, 2004; Yang *et al.*, 2005), some of which, similar to FGF2, are Smad3 regulated. Hence, focal overexpression of TGF- β at sites of early cancer might be expected to initiate a local reactive stroma, typified by matrix remodeling, induction of myofibroblasts, collagen deposition and induced angiogenesis, similar to the initiation of these events at sites of wound repair, where platelets release TGF- β . It is also becoming more clear that the net response to elevated TGF- β in cancer is likely to be mediated through a diverse set of downstream factors. Accordingly, the biology induced in the tumor microenvironment may be either tumor-promoting or tumor-inhibiting depending on many local variables. Regardless, co-evolution of a reactive stroma does seem to correlate with tumor progression. Hence, it has been suggested by several that stimulation of a local host reactive microenvironment is a key step during transition of pre-neoplastic foci to overt neoplasia (Ronnov-Jessen *et al.*, 1996; Liotta and Kohn, 2001; Tuxhorn *et al.*, 2001). In this study, we propose that the TGF- β /FGF-2 signaling axis is likely to be one of many coordinate sets of factors that regulate the complex biology of a reactive stroma microenvironment in carcinomas.

Materials and methods

Cell lines

LNCaP and Phoenix E cells were purchased from ATCC (Manassas, VA, USA) and maintained as described previously (Yang *et al.*, 2005). The C57B mouse prostate stromal cell line was generated and cultured as reported previously (Yang *et al.*, 2005). A prostate stromal cell line was initiated from the ventral prostate of an 8-week Tgfr2^{flloxE2/flloxE2} mouse (Bhowmick *et al.*, 2004) carrying loxP sites at introns 1 and 2 of the T β RII gene, following the same procedure as reported previously (Yang *et al.*, 2005) and named the T β RII^{fllox/fllox}H cells. Passages 8–15 were used for all experiments.

Knockout of T β RII alleles in T β RII^{fllox/fllox}H cells

The HR-MMPCreGFP retroviral construct carrying an bioactive Cre-GFP fusion protein with a lox 511 in the 3' LTR U3 region or the control HR-MMPCreGFPY324F carrying a loss-of-function CreY324F-GFP mutant (Silver and Livingston, 2001) were transfected into Phoenix E cells with a Calcium Phosphate Transfection kit (Invitrogen, Carlsbad, CA, USA). Virus was collected, filtered (0.45 μ m) and applied to infect T β RII^{fllox/fllox}H cells as described previously (Yang *et al.*, 2005). Cre-GFP expression in T β RII^{fllox/fllox}H cells excised the floxed T β RII alleles and self-excised the lox 511 flanked Cre-GFP (thus 'Hit and Run'), resulting in T β RII null cells named T β RII KO. CreY324F-GFP did not excise T β RII alleles and the resulting cells were named T β RII CT.

N-Flag-Smad3 Δ SSVS expression in C57B prostate stromal cells

To inhibit Smad3-mediated signaling, either pLPCX-N-Flag-Smad3 Δ SSVS or control pLPCX (Choy *et al.*, 2000) was transfected into Phoenix E cells. Virus was collected, filtered and applied to infect C57B prostate stromal cells. Cells were selected with 2 μ g/ml puromycin. The resulting cell lines were named Smad3 DN and Smad3 Ctrl.

Overexpression of FGF-2

FGF-2-GFP cDNA encoding an 18 kDa isoform FGF-2 fused to GFP protein was subcloned from pRev-TRE2 (Backhaus *et al.*, 2004) into pBMN-LacZ. Functionality of this protein has been described previously (Backhaus *et al.*, 2004). pBMN-FGF-2-GFP or pBMN-I-eGFP control vector was transfected into Phoenix E cells. Virus was collected, filtered and applied to infect T β RII KO or Smad3 DN cells. The resulting cell lines were named T β RII KO + FGF-2, T β RII KO + Ctrl, Smad3 DN + FGF2 and Smad3 DN + Ctrl. Methods for promoter assays, immunocytochemistry, quantitative PCR, ELISA and western blots using cell lines are provided in the Supplementary Information.

Animals and DRS xenografts

Athymic NCr-nu/nu male mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA, USA). All experiments were in compliance with the NIH Guide for the Care and Use of Laboratory Animals according to institutional guidelines of Baylor College of Medicine.

DRS xenografts were generated following procedures published previously (Tuxhorn *et al.*, 2002b,c; Yang *et al.*, 2005). Briefly, 2×10^6 LNCaP cells with 5×10^5 of either T β RII CT, T β RII KO, Smad Ctrl, Smad3 DN, T β RII KO + Ctrl, T β RII KO + FGF2, Smad3 DN + Ctrl or Smad3 DN + FGF2 cells in matrigel were injected subcutaneously in each rear lateral flank of three mice for a total of six tumors per experimental group per injection preparation. A minimum of three independent experiments was performed for each combination tested ($n=18$ tumors). Xenograft tumors were evaluated at either day 14 or 28 after inoculation, as these are optimal intermediate and later stage time points as reported previously (Tuxhorn *et al.*, 2002b,c; Yang *et al.*, 2005). Xenografts were weighed and fixed in 4% paraformaldehyde at 4°C overnight and paraffin embedded. Sections (5 μ m) were mounted onto ProbeOn Plus slides (Fisher, Pittsburgh, PA, USA) and either stained with H&E or processed for immunohistochemistry. Methods for immunohistochemistry, microvessel density counts and determination of carcinoma to stromal cell ratios are included in the Supplementary Information.

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References

- Akhurst RJ, Derynck R. (2001). TGF-beta signaling in cancer—a double-edged sword. *Trends Cell Biol* **11**: S44–S51.
- Backhaus R, Zehe C, Wegehingel S, Kehlenbach A, Schwappach B, Nickel W. (2004). Unconventional protein secretion: membrane translocation of FGF-2 does not require protein unfolding. *J Cell Sci* **117**: 1727–1736.
- Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S *et al.* (2004). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**: 848–851.
- Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M *et al.* (2004). Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. *J Immunol* **173**: 2099–2108.
- Butter S, Laping NJ, Pullen M, Grygielko E, Olson B, Brooks DP. (2001). The role of transforming growth factor-beta and its receptors in human prostate smooth muscle cell fibronectin production. *Eur J Pharmacol* **422**: 47–52.
- Cheng N, Bhowmick NA, Chytil A, Gorska AE, Brown KA, Muraoka R *et al.* (2005). Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* **24**: 5053–5068.
- Choy L, Skillington J, Derynck R. (2000). Roles of autocrine TGF-beta receptor and Smad signaling in adipocyte differentiation. *J Cell Biol* **149**: 667–682.
- Coffey Jr RJ, Shipley GD, Moses HL. (1986). Production of transforming growth factors by human colon cancer lines. *Cancer Res* **46**: 1164–1169.
- Derynck R, Zhang YE. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**: 577–584.
- Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* **122**: 103–111.
- Dow JK, deVere White RW. (2000). Fibroblast growth factor 2: its structure and property, paracrine function, tumor angiogenesis, and prostate-related mitogenic and oncogenic functions. *Urology* **55**: 800–806.
- Dvorak HF. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *New Engl J Med* **315**: 1650–1659.
- Eastham JA, Truong LD, Rogers E, Kattan M, Flanders KC, Scardino PT *et al.* (1995). Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. *Lab Invest* **73**: 628–635.
- Fukabori Y, Nakano K, Ohya A, Yamanaka H. (1997). Stimulative effect of transforming growth factor-beta on collagen synthesis by human prostatic stromal cells *in vitro*. *Int J Urol* **4**: 597–602.
- Giri D, Ropiquet F, Ittmann M. (1999). Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* **5**: 1063–1071.
- Hayashi T, Hideshima T, Nguyen AN, Munoz O, Podar K, Hamasaki M *et al.* (2004). Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* **10**: 7540–7546.
- Huss WJ, Barrios RJ, Foster BA, Greenberg NM. (2003). Differential expression of specific FGF ligand and receptor isoforms during angiogenesis associated with prostate cancer progression. *Prostate* **54**: 8–16.
- Igarashi A, Okochi H, Bradham DM, Grotendorst GR. (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* **4**: 637–645.
- Lakos G, Takagawa S, Chen SJ, Ferreira AM, Han G, Masuda K *et al.* (2004). Targeted disruption of TGF-beta/Smad3 signaling modulates skin fibrosis in a mouse model of scleroderma. *Am J Pathol* **165**: 203–217.
- Liotta LA, Kohn EC. (2001). The microenvironment of the tumour–host interface. *Nature* **411**: 375–379.
- Peehl DM, Sellers RG. (1998). Basic FGF, EGF, and PDGF modify TGFbeta-induction of smooth muscle cell phenotype in human prostatic stromal cells. *Prostate* **35**: 125–134.
- Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O *et al.* (1994). Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* **269**: 6271–6274.
- Polnaszek N, Kwabi-Addo B, Peterson LE, Ozen M, Greenberg NM, Ortega S *et al.* (2003). Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. *Cancer Res* **63**: 5754–5760.
- Roberts AB, Russo A, Felici A, Flanders KC. (2003). Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta. *Ann NY Acad Sci* **995**: 1–10.
- Roberts AB, Sporn MB. (1996). Transforming growth factor- β . In: Clark RAF (ed). *The Molecular and Cellular Biology of Wound Repair*. Plenum Press: New York, NY, pp 275–308.
- Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM *et al.* (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci USA* **83**: 4167–4171.
- Ronnov-Jessen L, Petersen OW, Bissell MJ. (1996). Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev* **76**: 69–125.
- Rowley DR. (2007). Reactive stroma and evolution of tumors: integration of transforming growth factor-beta, connective tissue growth factor, and fibroblast growth factor-2 activities. In: Jakowlew S (ed). *Transforming Growth Factor-Beta in Cancer Therapy*. Humana Press: Towata.
- Sakko AJ, Ricciardelli C, Mayne K, Tilley WD, Lebaron RG, Horsfall DJ. (2001). Versican accumulation in human prostatic fibroblast cultures is enhanced by prostate cancer cell-derived transforming growth factor beta1. *Cancer Res* **61**: 926–930.
- Silver DP, Livingston DM. (2001). Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. *Mol Cell* **8**: 233–243.
- Story MT, Hopp KA, Meier DA. (1996). Regulation of basic fibroblast growth factor expression by transforming growth factor beta in cultured human prostate stromal cells. *Prostate* **28**: 219–226.
- Tuxhorn JA, Ayala GE, Rowley DR. (2001). Reactive stroma in prostate cancer progression. *J Urol* **166**: 2472–2483.
- Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. (2002a). Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* **8**: 2912–2923.
- Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. (2002b). Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. *Cancer Res* **62**: 3298–3307.
- Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. (2002c). Inhibition of TGF- β activity decreases angiogenesis in a human prostate cancer reactive stroma xenograft model. *Cancer Res* **62**: 6021–6025.
- Uchiyama-Tanaka Y, Matsubara H, Mori Y, Kosaki A, Kishimoto N, Amano K *et al.* (2002). Involvement of HB-EGF and EGF receptor transactivation in TGF-beta-mediated fibronectin expression in mesangial cells. *Kidney Int* **62**: 799–808.
- Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. (2005). Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* **65**: 8887–8895.

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Stromal Expression of Connective Tissue Growth Factor Promotes Angiogenesis and Prostate Cancer Tumorigenesis

Feng Yang, Jennifer A. Tuxhorn, Steven J. Ressler, Stephanie J. McAlhany, Truong D. Dang, and David R. Rowley

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

Abstract

Our previous studies have defined reactive stroma in human prostate cancer and have developed the differential reactive stroma (DRS) xenograft model to evaluate mechanisms of how reactive stroma promotes carcinoma tumorigenesis. Analysis of several normal human prostate stromal cell lines in the DRS model showed that some rapidly promoted LNCaP prostate carcinoma cell tumorigenesis and others had no effect. These differential effects were due, in part, to elevated angiogenesis and were transforming growth factor (TGF)- β 1 mediated. The present study was conducted to identify and evaluate candidate genes expressed in prostate stromal cells responsible for this differential tumor-promoting activity. Differential cDNA microarray analyses showed that connective tissue growth factor (CTGF) was expressed at low levels in nontumor-promoting prostate stromal cells and was constitutively expressed in tumor-promoting prostate stromal cells. TGF- β 1 stimulated CTGF message expression in nontumor-promoting prostate stromal cells. To evaluate the role of stromal-expressed CTGF in tumor progression, either engineered mouse prostate stromal fibroblasts expressing retroviral-introduced CTGF or 3T3 fibroblasts engineered with mifepristone-regulated CTGF were combined with LNCaP human prostate cancer cells in the DRS xenograft tumor model under different extracellular matrix conditions. Expression of CTGF in tumor-reactive stroma induced significant increases in microvessel density and xenograft tumor growth under several conditions tested. These data suggest that CTGF is a downstream mediator of TGF- β 1 action in cancer-associated reactive stroma and is likely to be one of the key regulators of angiogenesis in the tumor-reactive stromal microenvironment. (Cancer Res 2005; 65(19): 8887-95)

Introduction

Our previous studies have characterized reactive stroma in human prostate cancer progression and have developed the differential reactive stroma (DRS) xenograft model to address the role of reactive stroma in experimental prostate tumorigenesis. These studies have shown that reactive stroma initiates during prostatic intraepithelial neoplasia, exhibits a myofibroblast wound repair stromal phenotype, is tumor promoting, and is mediated, in part, by transforming growth factor (TGF)- β 1 action (1–3). Our

studies have also shown that reactive stroma was essential for inducing early angiogenesis and acted to stimulate both the incidence and rate of LNCaP prostate cancer cell tumorigenesis in DRS model xenografts (2). These studies showed that differential LNCaP tumor progression is based on the type of stroma in the xenograft tumor and the stromal response to TGF- β 1.

Connective tissue growth factor (CTGF) has emerged as a potent mediator of TGF- β 1 action in wound repair stromal responses and in fibrosis disorders (4–6). CTGF is a member of the CCN gene family (for CTGF, Cyr61, and Nov; refs. 7–9). This family includes six structural and functional related proteins: CTGF (10, 11); cysteine-rich 61 (Cyr61; ref. 12); nephroblastoma overexpressed (NovH; ref. 13); and Wnt-1-induced signaling protein (WISP) 1, WISP2, and WISP3 (14). The CCN family members (excluding WISP2) share four conserved structural modules with sequence homologies similar to insulin-like growth factor-binding protein, von Willebrand factor, thrombospondin, and cysteine knot (8). CTGF message is potently stimulated by TGF- β 1 (15–19) and likely mediates TGF- β 1-induced collagen expression in wound repair fibroblasts (20). CTGF is expressed by several stromal cell types, including endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts, and some epithelial cell types in diverse tissues. Consistent with its role in connective tissue biology, CTGF enhances stromal extracellular matrix synthesis (16) and stimulates proliferation, cell adhesion, cell spreading, and chemotaxis of fibroblasts (10, 16, 21). CTGF was also shown to stimulate smooth muscle cell proliferation and migration (22). In addition, CTGF is a potent stimulator of endothelial cell adhesion, proliferation, migration, and angiogenesis *in vivo* (23–25). As might be predicted, CTGF is expressed in the reactive stromal compartment of several epithelial cancers, including mammary carcinoma, pancreatic cancers, and esophageal cancer (26–28). Expression of CTGF is also observed in several stromal cell disorders, including angiofibromas, infantile myofibromatosis, malignant hemangiopericytomas, fibrous histiocytomas, and chondrosarcomas (29, 30). Accordingly, CTGF is considered to be a profibrosis marker (31). Together, these findings suggest that CTGF is a key regulatory factor for stromal tissue biology in wound repair and cancer progression; however, this has not yet been tested *in vivo* using engineered stromal cells.

Expression of TGF- β 1 is elevated in most epithelial carcinoma cells (32) and our previous studies have shown that TGF- β 1 is a critical regulator of carcinoma-associated reactive stroma, angiogenesis, and reactive stroma promotion of tumor progression in LNCaP xenograft tumors (3). Because TGF- β 1 stimulates CTGF expression in stromal cells (15), including human prostate stromal cells (19), CTGF has accordingly emerged as a candidate downstream effector of TGF- β 1 action in reactive stroma.

The DRS model system was specifically developed to evaluate differential gene expression in the reactive stromal compartment

Note: J.A. Tuxhorn is currently in Life Sciences Systems and Services, Wyle Laboratories, Houston, TX 77058.

Requests for reprints: David R. Rowley, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: 713-798-6220; Fax: 713-790-1275; E-mail: drowley@bcm.tmc.edu.

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in xenografts composed of tissue-specific cancer cells and coordinate stromal cells (2, 3). These studies showed that two different human prostate stromal cell lines, HTS-2T and HTS-40C, exhibited differential effects in reactive stroma-induced angiogenesis and tumorigenesis of LNCaP prostate cancer cells (2). The present study was conducted to assess candidate genes responsible for the differential functions. We report here that CTGF was differentially expressed in tumor-promoting prostate stromal cell lines and that CTGF expression is stimulated by TGF- β 1 in prostate stromal cells. In addition, we show that overexpression of CTGF in engineered prostate stromal cells in the DRS LNCaP xenograft model resulted in significantly elevated angiogenesis and LNCaP tumorigenesis *in vivo*.

Materials and Methods

Cell lines. LNCaP human prostate carcinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/mL penicillin, and 100 μ g/mL streptomycin (Sigma, St. Louis, MO). The HTS-2T and HTS-40C normal human prostate stromal cell lines were established in our laboratory (2) and cultured in Bfs medium: DMEM (Invitrogen) supplemented with 5% FBS (Hyclone), 5% Nu serum (BD Biosciences, Bedford, MA), 0.5 μ g/mL testosterone, 5 μ g/mL insulin, 100 units/mL penicillin, and 100 μ g/mL streptomycin (Sigma). The Phoenix E packaging cell line was received from ATCC (by permission from Dr. Gary Nolan, Stanford University, Stanford, CA) and maintained in DMEM with high glucose (Invitrogen) supplemented with 10% heat inactivated FBS (Hyclone), 2 mmol/L glutamine (Invitrogen), and antibiotics as described above.

The mouse prostate stromal cell line, C57B, was derived from an 8-week C57BL/6 male mouse. The ventral prostate was removed, cut into 1 mm³ cubes, and placed in wells of a six-well culture plate in Bfs medium and cultured at 37°C with 5% CO₂. Monolayers of stromal cells extended from the explants and, at confluence, the explants were removed and stromal cells were continued in culture by routine serial passage. C57B cells were positive for androgen receptor, vimentin, and smooth muscle α -actin with low expression of calponin (data not shown), similar to human prostate stromal cell lines we have reported previously (2). C57B cells were used at passages 15 to 25 for all experiments.

The GeneSwitch-3T3 cell line expressing the GeneSwitch regulatory protein from the pSwitch vector was purchased from Invitrogen. GeneSwitch-3T3 cells and derivative engineered cell lines were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 100 units/mL penicillin, 100 μ g/mL streptomycin (Sigma), and Hygromycin B and/or Zeocin (Invitrogen) as described below.

cDNA microarray analysis. HTS-2T and HTS-40C cells were cultured in Bfs medium to 80% confluence. Total RNA was extracted from each cell line with RNA STAT-60 total RNA/mRNA isolation reagent (Tel-test, Inc., Friendswood, TX) following the instructions of the manufacturer. Microarray analysis was done using 30 μ g of total RNA. The cDNA reverse transcription and fluorescent labeling reactions were carried out using Cy3-labeled nucleotides for control (HTS-2T) and Cy5-labeled nucleotides for experimental (HTS-40C) samples as described previously (33). A microarray chip carrying 6,000 human cDNAs obtained from Baylor Microarray Core Facility was used. The hybridized slide was scanned with an Axon 4000A dual-channel scanner (Axon Instruments, Foster City, CA) and the data was analyzed using Gene Pix v. 3.0 software package (Axon). Genes were considered up-regulated if the expression was changed at least 3-fold from the control. Data with low signal intensity, high background, and high variability were eliminated.

Reverse transcription-PCR. Differential expression of CTGF in HTS-2T and HTS-40C cells was assessed by reverse transcription-PCR (RT-PCR) analysis. HTS-2T and HTS-40C cells were cultured in Bfs medium to 80% confluence and total RNA was extracted with the RNeasy Miniprep kit (Qiagen, Inc., Valencia, CA). CTGF amplification with primer 5'-GGTTAC-

CAATGACAACGCCT-3' and primer 5'-TGCTCCTAAAGCCACACCTT-3' were used to monitor CTGF expression, by using the TaqMan one-step RT-PCR kit (Applied Biosystems, Foster City, CA).

To determine the effects of TGF- β 1 on CTGF expression, HTS-2T cells were cultured to 80% confluence, exposed to M₀ serum-free media (MCDB 110 supplemented with insulin, transferrin, and sodium selenite; Sigma Diagnostics) for 24 hours, followed by 100 pmol/L (2.5 ng/mL) porcine TGF- β 1 (R&D Systems, Minneapolis, MN) or vehicle control in M₀ media treatment for an additional 24 hours before total RNA extraction as described above. 18S rRNA amplifications with 18S rRNA primers (provided in the TaqMan one-step RT-PCR kit) were used for total RNA loading control. RT-PCR reactions were carried out in 50 μ L total volume with 80 ng of total RNA and 32 pmol of each primer. First-strand synthesis was done at 48°C for 30 minutes. For CTGF amplification, PCR cycles were run at 95°C for 15 seconds, 60°C for 2 minutes, for a total of 28 cycles. For 18S rRNA amplification, PCR cycles were run at 95°C for 15 seconds, 60°C for 1 minute, for a total of 20 cycles. The PCR products were electrophoresed through a 2% agarose gel, visualized with ethidium bromide, and photographed. A similar RT-PCR procedure was carried out to monitor CTGF expression in HTS-2T and HTS-40C cells, with a total RNA of 200 ng per reaction.

Retroviral infection. The pRc/CMV-CTGF plasmid containing human CTGF cDNA was a kind gift from Dr. Gary Grotendorst (Lovelace Respiratory Research Institute, Albuquerque, NM; ref. 16, 26). For the construction of pBMN-CTGF-I-enhanced green fluorescent protein (EGFP) vector for retroviral delivery of CTGF, the human CTGF cDNA coding sequence was excised with *Eco*RI from pRc/CMV-CTGF vector and ligated into the pBMN-I-EGFP retroviral vector kindly provided by Dr. Gary Nolan with the same restriction site. Clones were sequenced to ensure correct CTGF cDNA orientation and sequence.

The pBMN-CTGF-I-EGFP vector (bicistronic) or pBMN-I-EGFP control vector were transfected into Phoenix E cells with a calcium phosphate transfection kit (Invitrogen) following a modified protocol. In brief, Phoenix cells were seeded at 1.5×10^6 cells in a 6 cm culture plate 24 hours before transfection. For transfection, 10 μ g of DNA and 61 μ L of 2 mol/L CaCl₂ were brought to 0.5 mL with double-distilled water and added dropwise to 0.5 mL of 2 $\times$ HBS, while aerating with a pipette, and followed by 30-minute incubation at room temperature to form fine precipitates. To Phoenix cells in 6 cm plates in 3 mL media, 2 μ L of 50 mmol/L chloroquine stock were added. Five minutes later, DNA/CaHPO₄ precipitates were added dropwise, followed by overnight incubation at 37°C. Medium was replaced 24 hours after transfection and plates were incubated at 32°C. Virus in the supernatant from each retrovirus-producing line was collected 48 hours after transfection and filtered (0.45 μ m). Three milliliters of viral supernatant with additional 5% FBS, 5% Nu serum (BD Biosciences), 0.5 μ g/mL testosterone (Sigma), 5 μ g/mL insulin, and 5 μ g/mL polybrene was applied immediately to C57B prostate stromal cells at 60% to 80% confluence in T25 flask. Infection was carried out at 37°C. Viral supernatant was replaced with fresh Bfs medium 24 hours after infection. Expression of retroviral construct was confirmed by counting the percentage of green fluorescent (GFP positive) C56B cells per $\times 100$ field. Infected cultures with a >90% green fluorescent cells per field were passaged and frozen (−80°C) in 4 $\times 10^6$ cells/vial aliquots for use in DRS xenografts.

3T3 cell GeneSwitch system. The GeneSwitch system (Invitrogen) was used to engineer 3T3 fibroblast cells with mifepristone (RU 486) inducible expression of a V5-His tagged CTGF protein. GeneSwitch-3T3 cells expressing the GeneSwitch regulatory protein from the pSwitch vector were purchased from Invitrogen. For the construction of pGene CTGF-V5-His vector, the human CTGF cDNA was PCR amplified from pRc/CMV-CTGF with primers 5'-CTAGGATCCGCCCGCAGTGCC-3' (*Bam*HI) and primer 5'-TCTCTGGGGCCCTGCCATGTCTCCGTACATCTTC-3' (*Apa*I). PCR cycles were run at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 80 seconds for a total of 20 cycles after first incubation at 95°C for 2 minutes. The PCR reaction was incubated at 72°C for another 10 minutes for final extension. PCR products were purified with QIAquick PCR purification kit (Qiagen). After digestion with *Bam*HI and limited digestion with *Apa*I (to avoid internal *Apa*I site along CTGF cDNA sequence), the 1.1 kb CTGF insert was

gel purified and cloned in frame into the pGene/V5-His A vector (Invitrogen). Fidelity was confirmed by sequence analysis. The pGene CTGF-V5-His vector or pGene/V5-His empty vector control was transfected into GeneSwitch-3T3 cell line (Invitrogen) with FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN), following the protocol of the manufacturer. Stable transfected GeneSwitch-3T3 cells were selected and maintained in media (as described previously) containing 50 µg/mL of Hygromycin B and 200 µg/mL of Zeocin. Mifepristone (100 pmol/L) was used to induce CTGF-V5-His fusion protein expression. Regulated expression was confirmed by Western blot analysis of secreted proteins.

To render engineered GeneSwitch-3T3 pGene CTGF-V5-His and GeneSwitch-3T3 pGene/V5-His cells less proliferative and less tumorigenic for use in the DRS xenograft model, the cells were irradiated with increasing doses of γ -irradiation. The γ -irradiation dosage of 800 rad was chosen for DRS xenograft tumor experiments because it resulted in viable cells with a low proliferation rate and high expression of mifepristone-inducible CTGF-V5-His protein *in vitro* (Western blot, data not shown).

Western blot analysis. For V5 Western blot, conditioned medium from GeneSwitch-3T3 pGene CTGF-V5-His cells induced with 100 pmol/L mifepristone (or vehicle control) was electrophoresed through a 12% SDS-PAGE gel. Proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and incubated in PBS buffer with 5% nonfat milk at 4°C overnight. Mouse anti-V₅ monoclonal antibody (Invitrogen), diluted at 1:5,000, was used as primary antibody to detect the presence of CTGF-V5-His fusion protein, and incubated for 2 hours at room temperature. Secondary antibody was biotin-conjugated sheep anti-mouse IgG (Sigma), diluted at 1:1,000, and incubated for 1 hour at room temperature. A streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, United Kingdom) diluted at 1:1,000 was incubated for 30 minutes at room temperature. Protein bands were detected by incubation with ECL+ Western blotting detection system (Amersham Biosciences) for 5 minutes at room temperature followed by exposure to Hyperfilm ECL from Amersham Pharmacia Biotech.

For CTGF Western blot, C57B CTGF and control cells were grown in Bfs to 80% confluence, then switched to serum-free M₀ media for 2 days. The media were collected and concentrated 20-fold by Amicon Ultra 4 centrifugation (5000 MWCO; Millipore, Billerica, MA). The concentrated samples were electrophoresed through a 12% SDS-PAGE gel and proteins were transferred onto Immobilon-P (Millipore). The membrane was incubated in PBS buffer with 2.5% normal donkey serum at 4°C overnight. The immunoblot protocol was the same as above, except the primary antibody was goat anti-CTGF antibody L-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted at 1:400, and secondary antibody was biotin-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), diluted at 1:40,000.

Animals and preparation of differential reactive stromal xenografts. Athymic NCr-nu/nu male homozygous nude mice, 6 to 8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All experiments were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and according to the institutional guidelines of Baylor College of Medicine.

DRS xenograft tumors were generated following procedures we have published previously (2, 3, 34). Briefly, frozen aliquots of LNCaP human prostate cancer cells (16×10^6) and the engineered stromal cells—C57B-CTGF (8×10^6 cells), C57B-control (8×10^6 cells), and γ -irradiated GeneSwitch-3T3 pGene CTGF-V5-His cells (4×10^6 cells)—were thawed in a 37°C water bath for 1 to 2 minutes and washed once with 10 mL RPMI supplied with 10% serum (for LNCaP cells) or with 10 mL DMEM supplied with 10% serum (for stromal cells) in 15 mL conical tubes. The cells were pelleted at $1,400 \times g$ for 2 minutes and resuspended in 6 mL RPMI 1640 with 10% FBS. The LNCaP cells were then combined with stromal cells, mixed well, and pelleted again at $1,400 \times g$ for 2 minutes. The supernatant was aspirated to either 300 µL (for Matrigel experiments) or 200 µL [for growth factor-reduced (GFR) matrix mixture experiments] and cells were resuspended in the remaining medium. Cells

were incubated on ice for 1.5 minutes and then combined with either 0.5 mL of Matrigel (Becton Dickinson, Bedford, MA) or 0.6 mL of a GFR matrix mixture composed of a 1:1 ratio of neutralized Vitrogen 100 (99.9% collagen type I; Cohesion, Palo Alto, CA) and GFR Matrigel (Becton Dickinson). In all experiments, the final volume was 800 µL. The cell and matrix mixture was drawn into a 1 mL syringe fitted with a 20-gauge needle. After switching to a 25-gauge needle, 100 µL of the cell-matrix suspension was injected s.c. in each lateral flank of adult NCr-nu/nu male mice.

To induce expression of CTGF-V5-His, mice with DRS xenografts composed of LNCaP cells combined with γ -irradiated GeneSwitch-3T3 pGene CTGF-V5-His cells received mifepristone (Sigma) or vehicle control (sesame seed oil; Sigma) at 0.5 mg/kg administered as 100 µL i.p. injections at the time of tumor injection and repeated every 48 hours until tumors were harvested. This mifepristone dose was based on protocols shown previously to induce consistent gene expression *in vivo* and had no effect on xenograft tumor weight or volume (data not shown). All mifepristone experiments are in accordance with our approved Animal Use Protocols and institutional guidelines of Baylor College of Medicine.

Tumors were collected at different time points between days 10 and 21 postinoculation. For the experimental sets of LNCaP cells combined with C57B-CTGF or C57B-control cells, the tumors were photographed *in situ* for GFP expression to confirm gene expression using a fluorescent dissecting microscope. The tumors were weighed, measured in three dimensions, and fixed in 4% paraformaldehyde (neutral buffered) at 4°C overnight, washed three times in PBS, and processed for paraffin embedding. Tumors were paraffin-embedded and 5 µm sections were cut and mounted onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were either stained with H&E for histologic analysis or processed for immunohistochemistry.

Immunohistochemistry. Primary antibodies were as follows: anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (rat monoclonal MEC13.3; BD PharMingen, San Diego, CA); anti-V5 mouse monoclonal antibody 46-0705 (Invitrogen); rabbit anti-GFP antibody A-11122 (Molecular Probes, Eugene, OR); goat anti-CTGF antibody L-20 (Santa Cruz). Secondary antibodies were as follows: biotin-conjugated goat anti-rat IgG (BD PharMingen) for CD31, biotin-conjugated Universal Secondary (Invitrogen) for V5, biotin-conjugated goat anti-rabbit IgG B8895 (Sigma) for GFP, and biotin-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories). Specificity of each primary antibody has been evaluated previously (refs. 2, 3, 34; and unpublished data).

Immunostaining was done with the MicroProbe Staining System (Fisher Scientific) following our protocol published previously (2, 3, 34). Reagents formulated for use with capillary action systems were purchased from Open Biosystems (Huntsville, AL) and used according to the protocol of the manufacturer. In brief, tissues were deparaffinized using Auto Dewaxer and cleared with Auto Alcohol. Brigati's iodine and Auto Prep were used to improve tissue antigenicity. Antigen retrieval were used in CD31, V5, and CTGF staining. For CD31 staining, tissues were incubated in 0.1% trypsin (Zymed, South San Francisco, CA) for 10 minutes at 37°C; for V5 and CTGF staining, tissues were subjected to high-temperature-steamer treatment in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 minutes. Goat anti-mouse Fab fragment (Jackson ImmunoResearch Laboratories) 1:65 was used for 30 minutes at 37°C for blocking before anti-V5 immunostaining. Sections were then incubated in protein blocker (for V5, CD31, and GFP) or 5% normal donkey serum in universal buffer (for CTGF). Primary antibodies were diluted and used under the following conditions: V5 (1:200), CD31 (1:50), GFP (1:200) in primary antibody diluent, and CTGF (1:100) in 5% normal donkey serum overnight at 4°C. Secondary antibodies were diluted and used under following conditions: biotin-conjugated universal secondary antibody for 4 minutes at 50°C; biotin-conjugated goat anti-rat IgG 1:100; biotin-conjugated goat anti-rabbit IgG 1:500; and biotin-conjugated donkey anti-goat antibody 1:200 for 45 minutes at 37°C. Tissues were treated with Auto Blocker to inhibit endogenous peroxidase activity. For detection, sections were incubated in RTU VectaStain Elite ABC reagent (Vector

Laboratories, Burlingame, CA) and then incubated in stable diaminobenzidine tetrahydrochloride twice for 3 minutes each at 50°C. Tissues were counterstained with Auto Hematoxylin for 30 seconds.

Microvessel density analysis. Analysis was done according to standard procedures we have published previously with DRS tumors (2, 3, 34). Tissue sections were stained for CD31 as described above. Sections were scanned at $\times 100$, and five random areas per tumor section were selected. Vessels in these fields were counted (at $\times 400$) by an observer blinded to experimental conditions. The average vessel count was determined for each specimen.

Statistical analysis. Tumors from each condition were analyzed, and average tumor weight and average microvessel counts were compared with these values from their matching control tumors for statistical relevance using the unpaired *t* test. Statistical analyses used GraphPad Prism for Macintosh version 3.0 (GraphPad Software, San Diego, CA). *P* < 0.05 was considered statistically significant.

Results

Differential expression of connective tissue growth factor in tumor-promoting human prostate stromal cell lines. Our previous studies using the DRS xenograft model showed that several human prostate stromal cell lines differentially promote LNCaP prostate cancer cell tumorigenesis (2). Stromal cell-promoted tumors exhibited a significantly elevated rate of angiogenesis and this was TGF- β 1 regulated (2, 3). Notably, the HTS-40C and the HTS-2T human prostate stromal cell lines exhibited opposing effects. In two-way DRS xenografts constructed of cancer cells and stromal cells in the absence of extracellular matrix (Matrigel), the HTS-40C/LNCaP combinations resulted in a 65% tumor incidence, whereas HTS-2T/LNCaP combinations were nontumorigenic (0% tumor incidence; ref. 2). To address potential mechanisms, gene expression profiles in HTS-40C and HTS-2T stromal cells were compared using cDNA microarray analyses. This analysis showed that 12 previously characterized genes were elevated by 3- to 31-fold in the protumorigenic HTS-40C stromal cell line compared with HTS-2T. These genes are listed in Table 1. Expression of several of these genes is associated with reactive stroma that forms at sites of wound repair, microbial invasion, or carcinoma as we have reported previously (1, 32, 35). Of these, CTGF is a known inducer of angiogenesis (36), is TGF- β 1 regulated in stromal cells (18, 37–39), and has been reported to directly enhance TGF- β 1

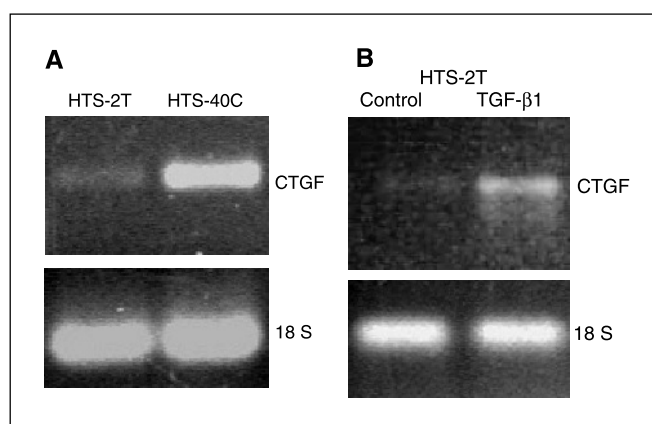


Figure 1. Expression of CTGF message in different prostate stromal cell lines. *A*, RT-PCR-amplified products from HTS-40C cells compared with HTS-2T cells. *B*, HTS-2T cells exposed to TGF- β 1 (100 pmol/L) or vehicle control for 24 hours. In both cases, 18S rRNA amplifications were used as loading control.

receptor-ligand binding (40). Our microarray data suggested that CTGF message was 4.5-fold higher in HTS-40C cells compared with HTS-2T cells. Further analysis confirmed this with RT-PCR and showed that CTGF message expression was severalfold higher in HTS-40C cells relative to HTS-2T cells as shown in Fig. 1*A*.

Although the HTS-2T stromal cell line did not support LNCaP tumorigenesis in matrix-free conditions (two-way tumors), HTS-2T cells did promote LNCaP tumors (incidence, rate of tumorigenesis, and angiogenesis) when combined with Matrigel matrix in three-way DRS xenografts that are constructed with cancer cells, stromal cells, and Matrigel matrix (2, 3). Matrigel matrix is high in TGF- β 1 and we have reported that inhibiting TGF- β 1 activity in Matrigel lowers the rate of tumorigenesis and angiogenesis in three-way DRS tumors (3). Accordingly, we next determined whether TGF- β 1 could induce CTGF expression in human prostate HTS-2T stromal cells. As shown in Fig. 1*B*, HTS-2T cells in control conditions exhibited low expression, whereas HTS-2T cultures exposed to TGF- β 1 (100 pmol/L, 24 hours) exhibited elevated CTGF message expression. This is in agreement with previous reports showing TGF- β 1 regulation of CTGF expression in other stromal cell lines (15, 19).

Table 1. Genes up-regulated in the HTS-40C cells compared with HTS-2T cells

40C/2T	Gene	UniGene no.	Accession no.	Gene description
31.333	<i>PLOD2</i>	Hs.477866	U84573	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
10.800	<i>TRAP1</i>	Hs.30345	U12595	Tumor necrosis factor receptor-associated protein 1
7.666	<i>TP53BP2</i>	Hs.523968	AI123916	Tumor protein p53-binding protein, 2
5.847	<i>ARF3</i>	Hs.119177	M74493	ADP ribosylation factor 3
4.899	<i>CFH</i>	Hs.363396	M12383	Complement protein H
4.637	<i>FMO</i>	Hs.132821*	AL021026	Flavin-containing monooxygenase
4.466	<i>CTGF</i>	Hs.75511	U14750	Connective tissue growth factor
3.841	<i>THBS1</i>	Hs.164226	NM_003246	Thrombospondin 1
3.574	<i>BRAP</i>	Hs.530940	AF035950	BRCA1-associated protein
3.152	<i>ADH1A</i>	Hs.368549	NM_000667	Alcohol dehydrogenase 1A (class I), α polypeptide
3.130	<i>PTSG1</i>	Hs.201978	U63846	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
3.083	<i>PTX3</i>	Hs.546280	M31166	Pentraxin-related gene, rapidly induced by interleukin-1 β

*Retired UniGene number without concise replacement.

Expression of connective tissue growth factor in prostate stromal cells promotes angiogenesis and LNCaP tumorigenesis.

A construct containing the full-length human CTGF cDNA (kindly provided by Dr. Gary Grotendorst) was used to construct a bicistronic retroviral vector (pBMN-CTGF-I-EGFP) containing CTGF followed by an IRES and EGFP for detection of expression. Either vector control (pBMN-I-EGFP) or the CTGF-containing retrovirus preparations were used to infect the mouse prostate

stromal cell line (C57B) and cells were analyzed for fluorescence 48 hours later as described in Materials and Methods. Figure 2A shows infected and EGFP-expressing C57B stromal cells before use in the DRS xenograft. C57B cells routinely exhibited a 90% infectivity rate or higher (data not shown). Western blot analysis showed overexpression of the mature form of CTGF (~38 kDa) in the experimental cell conditioned medium and low endogenous levels in the control infected cultures (Fig. 2B). Shorter fragments were also observed (Fig. 2B, *band A* and *band B*), which have been reported in the conditioned media of CTGF-secreting cells by others (41).

To evaluate the effects of CTGF expression from prostate stromal cells in three-way LNCaP tumors in nude mice, we inoculated cell combinations in either complete Matrigel or a modified matrix composed of a 1:1 mix of GFR Matrigel together with neutralized Vitrogen 100 collagen type I (GFR Matrigel/Vitrogen) to reduce bioactive factors in the matrix component. S.c. three-way DRS xenograft tumors were constructed in male nude mice using 2×10^6 LNCaP cells, and 1×10^6 control C57B (EGFP-expressing vector only) or CTGF-expressing C57B prostate stromal cells and the different Matrigel matrix preparations as described in Materials and Methods. Tumors were harvested at day 13 postinoculation because our previous studies have shown that day 10 to day 14 postinoculation is the optimal time frame to assess initial rate of angiogenesis and tumorigenesis (2, 3, 34). It should be noted that control or CTGF-transduced C57B cells inoculated alone or with matrix were nontumorigenic (data not shown) similar to our previous report (2). As shown in Fig. 2C, tumors were fluorescent *in situ* before removal. This confirmed transgene expression and viability of the engineered C57B stromal cells in the tumor xenograft.

Tumors exhibited a typical arrangement of LNCaP carcinoma cell clusters, surrounded by stromal cells, matrix, and vessels as shown in Fig. 3A and B, similar to what we have reported previously (2). There were no particular differences in histology or ratio of carcinoma to stromal cells in experimental tumors compared with control tumors. Prostate stromal cells engineered with the CTGF transgene in tumors were positive for both EGFP (Fig. 3C) and CTGF (Fig. 3D) proteins, and were immediately adjacent to clusters of LNCaP carcinoma cells. Immunostaining for CD31 as an endothelial marker showed an obvious difference in vessels. The density of CD31-positive microvessels in CTGF-expressing xenografts (Fig. 3F) seemed higher compared with control xenografts (Fig. 3E). Microvessel counts confirmed this. In complete Matrigel conditions, LNCaP xenograft tumors constructed with CTGF-expressing prostate stromal cells exhibited a microvessel density of 10.60 ± 1.35 compared with 6.16 ± 1.60 in vector-only control tumors ($n = 25$ fields, five tumors each, mean $\pm$ SE, $P < 0.05$; Fig. 4A). This represented a 72% increase in vessel density in the stromal CTGF-expressing tumors. The increase in vessel density correlated with elevated tumor mass. The mean wet weight of stromal CTGF-expressing LNCaP tumors was 24.42 ± 0.76 mg compared with 18.08 ± 1.54 mg ($n = 5$, mean $\pm$ SE, $P < 0.01$; Fig. 4B) in control tumors, indicating that stromal CTGF expression produced a 35% increase in tumor mass when xenografts are constructed in complete Matrigel conditions.

Significant differences in angiogenesis were even more pronounced in the low growth factor-modified matrix (GFR Matrigel/Vitrogen 100) conditions. CTGF-expressing tumors exhibited an average microvessel density of 10.10 ± 1.73

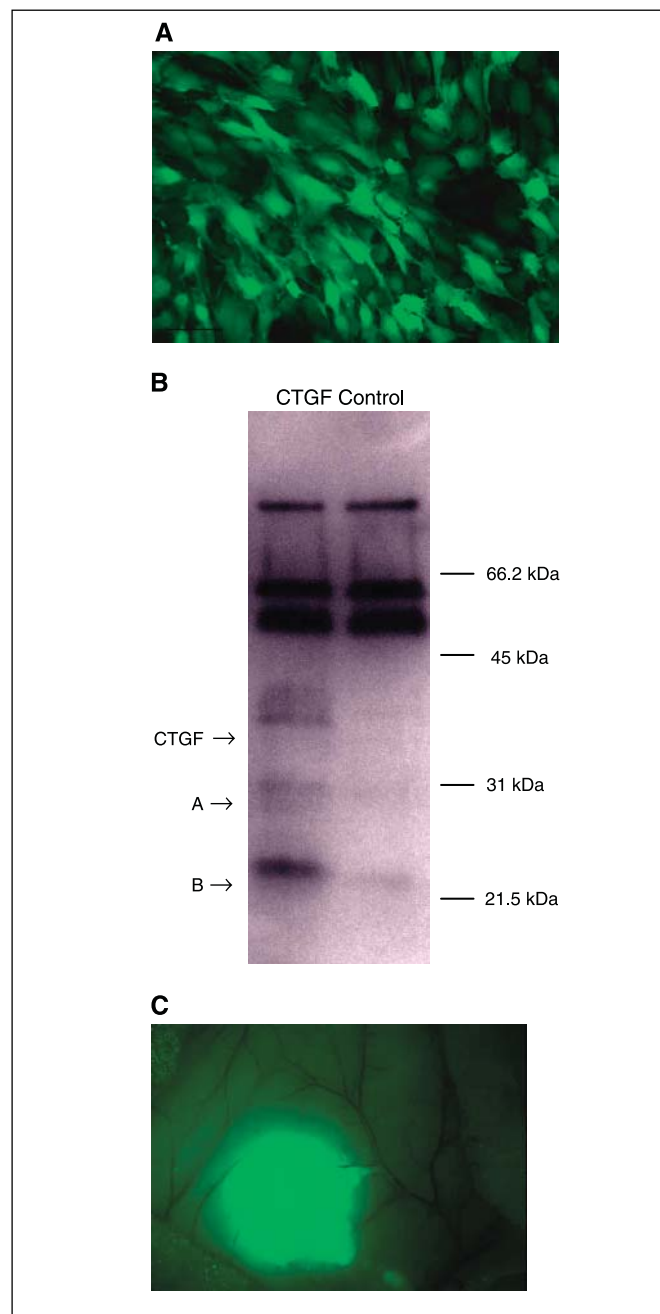


Figure 2. Transgene expression in retroviral-infected C57B prostate stromal cells and DRS tumors. A, GFP fluorescence of retroviral (pBMN-CTGF-I-EGFP) infected C57B cells *in vitro*. Bar, 100 μ m. B, Western blot analysis of CTGF protein in conditioned media of pBMN-CTGF-I-EGFP-infected C57B cells (CTGF) compared with pBMN-I-EGFP control vector infected cells (Control). C, GFP fluorescence of tumors *in situ*. An incision was made in the skin immediately adjacent to the s.c. tumor. The skin flap was turned back and photographed with a fluorescent dissecting microscope.

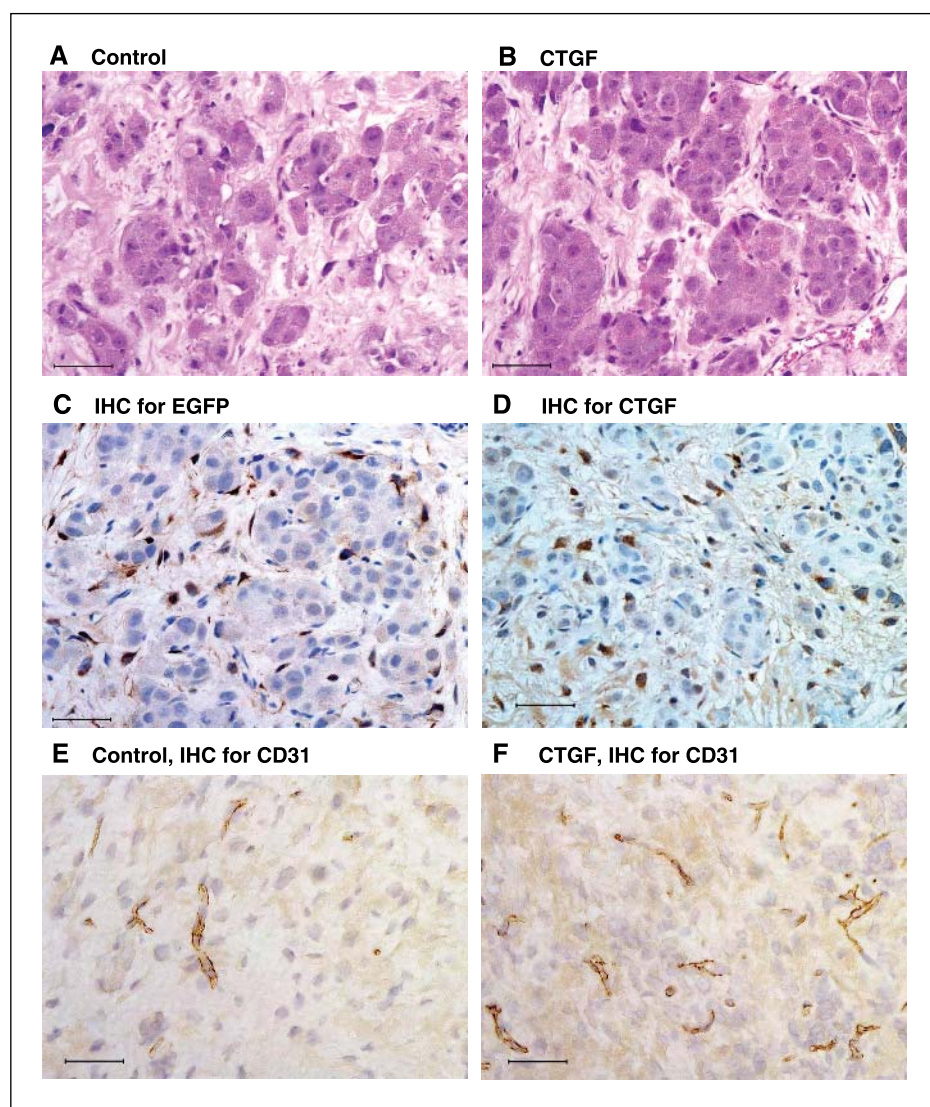


Figure 3. Histologic analysis of three-way LNCaP DRS xenograft tumors constructed with control or CTGF-expressing prostate stromal cells. *A* and *B*, the histology of DRS tumors generated from LNCaP cells combined with control C57B cells (*A*) or CTGF-expressing C57B cells (*B*). *C*, immunohistochemistry of EGFP expression in tumor stromal cells. *D*, immunohistochemistry of CTGF expression in tumor stromal cells. *E*, immunohistochemistry of CD31 expression in vessels from tumors constructed with control stromal cells. *F*, immunohistochemistry of CD31 expression in vessels of tumors constructed with CTGF-expressing stromal cells. Bar, 50 μ m.

compared with 4.70 ± 1.00 in control tumors, representing a 115% increase over control ($n = 30$, from six tumors in each condition, mean $\pm$ SE, $P < 0.01$; Fig. 4C). The stromal CTGF-expressing LNCaP tumors constructed in the GFR-modified matrix showed an average wet weight of 17.58 ± 0.60 mg compared with 12.97 ± 0.71 mg in control tumors ($n = 18$ in the CTGF experimental and $n = 17$ in the control, mean $\pm$ SE, $P < 0.0001$; Fig. 4D), representing a 36% increase in tumor mass.

Regulated expression of CTGF-V5-His in 3T3 fibroblasts promotes LNCaP tumorigenesis. To confirm and extend the findings with retroviral transduced C57B cells, the GeneSwitch System (Invitrogen) was used to engineer 3T3 stromal cell lines with mifepristone-regulated expression of an epitope-tagged CTGF-V5-His (fusion protein). Cultures at 80% to 100% confluence were induced with 100 pmol/L mifepristone for 24 to 48 hours. Western blot analysis for the V5 epitope showed an inducible 41 kDa CTGF-V5-His band in the conditioned media (Fig. 5A). DRS xenograft tumors were generated in nude mice using 2×10^6 LNCaP cells combined with γ -irradiated 5×10^5 GeneSwitch-3T3 pGene CTGF-V5-His cells and complete Matrigel (three-way DRS xenograft conditions). Irradiated engineered 3T3

cells (800 rad) were used because these cells remain viable, exhibit regulated transgene expression, and have a low proliferative rate relative to wild-type NIH 3T3 cells. Mice were given mifepristone or vehicle i.p. every 48 hours as described in Materials and Methods. Our previous studies have shown that this protocol of mifepristone treatment has no ill effect on nude mice and does not affect control tumor biology (2, 34). Resulting tumors were harvested 10 days postinoculation. Immunohistochemistry showed tightly regulated CTGF-V5-His protein expression *in vivo* (Fig. 5B). No expression was noted in tumors derived from vehicle control-treated animals (Fig. 5C). Tumors exhibited a typical carcinoma phenotype similar to the LNCaP/C57B combinations, although the tumors were considerably more heterogeneous with more focal nodules of carcinoma and other areas that seemed to have little carcinoma growth. There was, however, no apparent difference in histopathology noted between vehicle control and mifepristone-treated animals. LNCaP DRS tumors from mifepristone-treated animals exhibited a 25% average increase in wet weight as shown in Fig. 5D. The mean weight of control tumors was 17.91 ± 1.04 mg, whereas tumors from mifepristone-treated animals averaged 22.41 ± 1.76 mg ($P < 0.05$,

$n = 12$ tumors each). The tumors exhibited a very heterogeneous density of microvessels, as might be expected, due to the nodular and heterogeneous histopathology. This was obvious at low-power observation (data not shown). The heterogeneous nature of the vessel density patterns in these tumors was not compatible with the microvessel-counting protocol (see Materials and Methods) as the accuracy of this method is dependent on uniform vessel distribution. Accordingly, no attempt was made to quantitate microvessel density in these tumors as these data would not be accurate.

Discussion

To date, no effective approach exists to manipulate over-expression of a transgene in the stromal compartment in a tissue-specific manner *in situ*. Accordingly, we have used the DRS xenograft tumor approach to test the biological consequences of differential transgene expression in the reactive stroma compartment of an experimental human tumor in a nude mouse host. Our previous studies have shown that use of different human prostate stromal cell lines result in vast differences in LNCaP tumorigenesis *in vivo* (2). Furthermore, we have shown that the endogenous TGF- β 1 activity in complete Matrigel is responsible for this difference in both angiogenesis and tumorigenesis (3). Our current study shows that CTGF may mediate TGF- β 1 actions in the prostate stromal

cells. Expression of a CTGF transgene in the reactive stromal compartment of LNCaP DRS xenograft tumors resulted in enhanced tumorigenesis that was correlated with a more rapid rate of angiogenesis. We conclude from these data that CTGF may be an important regulator of tumor-reactive stroma and angiogenesis.

Our studies and others have suggested that reactive stroma in carcinomas is an important process associated with early events in tumorigenesis, including the formation of a wound repair type of matrix and enhanced angiogenesis (1–3, 32). Reactive stroma is remarkably similar in most carcinomas. Typically, carcinoma-associated reactive stroma is composed of activated fibroblasts and myofibroblasts, characteristic of a wound repair-type stroma (1, 32, 35). A key feature of wound repair stroma is rapid and elevated angiogenesis. In wounding, platelet-released TGF- β 1 and platelet-derived growth factor function to regulate stromal cell phenotype changes and to stimulate stromal cell migration, matrix production, and angiogenesis. TGF- β 1 is overexpressed by cancer epithelial cells in most carcinomas, including prostate cancer (32, 35). Moreover, CTGF is TGF- β 1 regulated in a diverse set of cell types, including human prostate stromal cells as reported here (15–19). In addition, CTGF has been shown to stimulate a wound repair type of stroma in several key studies and has been shown to mediate, in part, TGF- β 1-induced matrix remodeling (20). Hence, it is important to determine whether CTGF mediates a TGF- β 1-stimulated reactive stroma response in cancer and whether this reactive stroma is tumor promoting. Data reported here address this question directly and suggests that TGF- β 1 stimulated CTGF expression in carcinoma-associated reactive stroma, promotes angiogenesis, and results in enhanced tumorigenesis.

It is becoming clearer that the classic regulators of wound repair play an important role in carcinoma-reactive stroma and CTGF biology. For example, both fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor have been reported to stimulate CTGF expression (25, 42). FGF-2 expression is also TGF- β 1 regulated in fibroblasts from the prostate gland and other tissues (43, 44). Hypoxia will induce CTGF expression via a hypoxia-inducible factor-1 α pathway (45). In addition, thrombin and plasma clotting factor VIIa also induce CTGF expression (46). Accordingly, several factors and conditions associated with wound repair are known to affect CTGF expression and many of these factors and conditions are likely to play a role in tumor-associated reactive stroma.

The specific mechanisms of how CTGF or closely related family members directly affect reactive stromal cells in the tumor microenvironment is not fully understood. It is known that both CTGF and Cyr61 promote fibroblast adhesion through integrin α 6 β 1 and that this process requires cell surface heparan sulfate proteoglycans (47). Cyr61 and CTGF also stimulated migration and proliferation of fibroblasts, as well as endothelial cells (24, 48). In addition, CTGF also affects matrix production and remodeling. For example, CTGF was shown to stimulate fibronectin expression via a p42/44 mitogen-activated protein kinase and phosphoinositide 3 kinase/protein kinase B pathway (49). It will be important to dissect key CTGF signaling pathways in reactive stroma associated with tumors. Key components of these mechanisms may be useful as targets of therapeutic approaches directed at the tumor microenvironment.

The DRS model described in this study brings the opportunity to use highly efficient gene delivery and stable gene integration

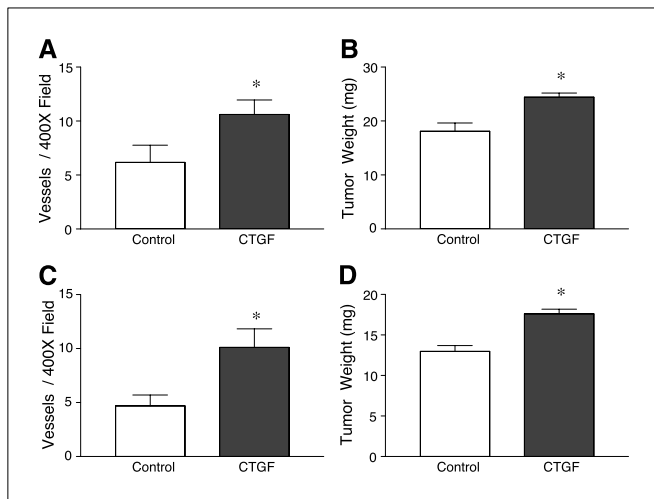
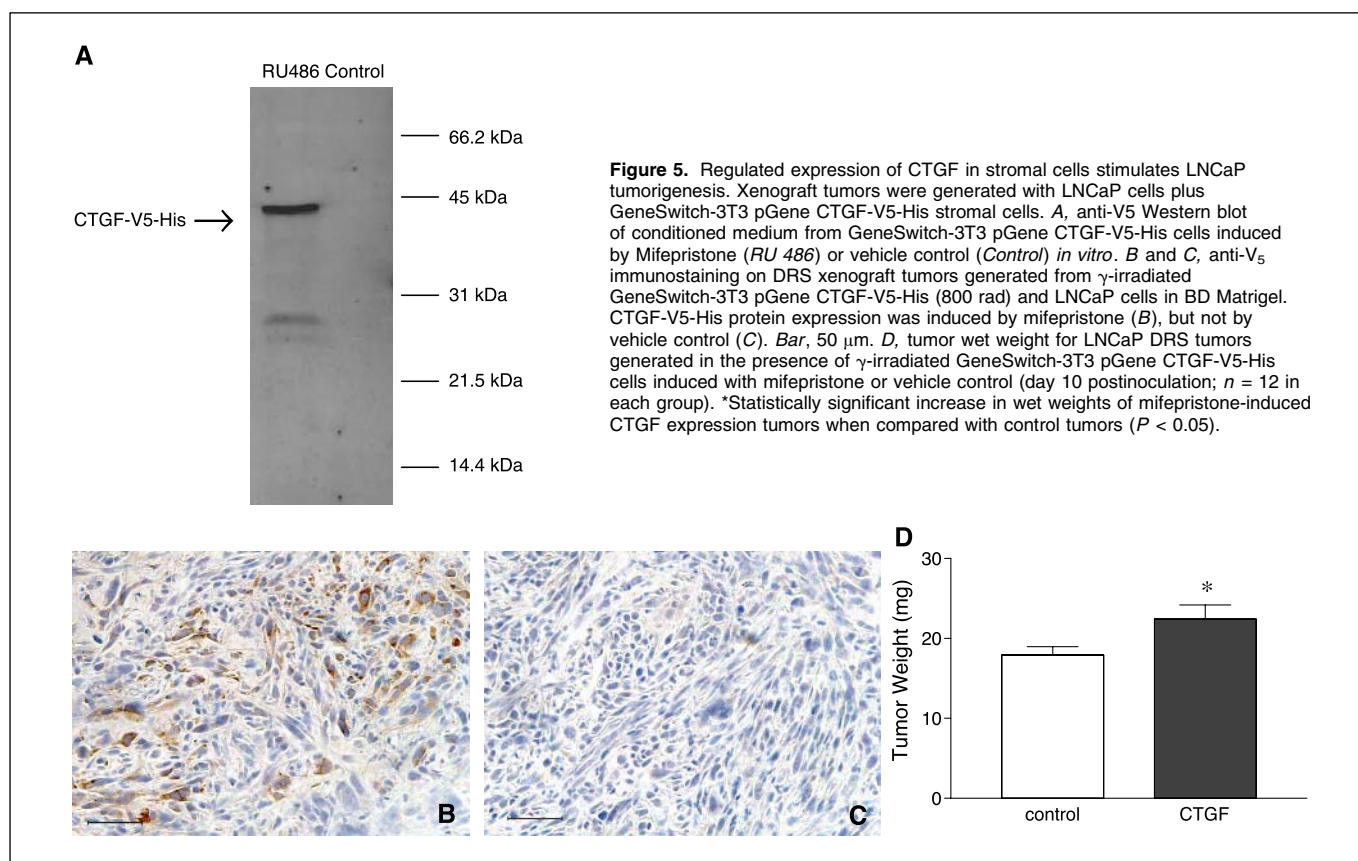


Figure 4. Stromal expression of CTGF stimulates microvessel density and tumor weight in three-way LNCaP DRS tumors constructed in different matrix preparations. A and B, microvessel densities and tumor weights were compared between the LNCaP tumors generated in the presence of C57B prostate stromal cells engineered to express CTGF (CTGF) or vector control stromal cells (Control), in complete BD Matrigel conditions at day 13 postinoculation. A, microvessel density, as assessed by CD31-positive structures, counted by a blinded observer ($n = 25$ fields, five tumors for each group). *Statistically significant increase in tumor microvessel density for DRS tumors generated in the presence of stromal cells expressing CTGF ($P < 0.05$). B, tumor wet weight ($n = 5$). *Statistically significant increase in wet weights of CTGF-expressing tumors when compared with control tumors ($P < 0.01$). C and D, microvessel densities and tumor weight were compared between the LNCaP tumors generated in the presence of C57B cells engineered to express CTGF (CTGF) or vector control C57B stromal cells (Control), in the low growth factor modified matrix (GFR Matrigel/Vitrogen 100) conditions at day 13 postinoculation. C, microvessel density, as assessed by CD31-positive structures, counted by a blinded observer ($n = 30$ fields, six tumors for each group). *Statistically significant increase in tumor microvessel density for DRS tumors generated in the presence of stromal cells expressing CTGF ($P < 0.01$). D, tumor wet weight ($n = 17$ in the control and $n = 18$ in the CTGF experimental). *Statistically significant increase in wet weights of CTGF expression tumors when compared with control tumors ($P < 0.0001$).



of retroviral-infected mouse prostate stromal cell lines to study the roles of epithelial cell-stromal cell interactions in carcinoma tumorigenesis and progression. Accordingly, the DRS model has allowed for the ability to dissect out the roles of individual growth factors in the reactive stroma compartment of a tumor. Data reported here represent the first study to show that expression of CTGF in the tumor microenvironment stromal cells of an experimental epithelial cancer functions to stimulate angiogenesis and tumor growth.

Emerging data supports the concept that the reactive stromal tumor microenvironment functions to affect the rate of tumorigenesis in most epithelial carcinomas studied to date. Accordingly, it is likely that the biological components and specific mechanisms of reactive stroma can be used both as prognostic indicators and as targets of therapeutics. This study

shows that CTGF is a TGF- β 1-regulated and stromal-expressed factor that promotes tumorigenesis and is, therefore, a theoretical target for therapeutics focusing on tumor-associated reactive stroma biology.

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References

1. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 2002;8:2912-23.
2. Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. *Cancer Res* 2002;62:3298-307.
3. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor- β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res* 2002;62:6021-5.
4. Franklin TJ. Therapeutic approaches to organ fibrosis. *Int J Biochem Cell Biol* 1997;29:79-89.
5. Crean JK, Lappin D, Godson C, Brady HR. Connective tissue growth factor: an attractive therapeutic target in fibrotic renal disease. *Expert Opin Ther Targets* 2001;5:519-30.
6. Ihn H. Pathogenesis of fibrosis: role of TGF- β and CTGF. *Curr Opin Rheumatol* 2002;14:681-5.
7. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* 1999;20:189-206.
8. Lau LF, Lam SC. The CCN family of angiogenic regulators: the integrin connection. *Exp Cell Res* 1999;248:44-57.
9. Grotendorst GR, Lau LF, Perbal B. CCN proteins are distinct from and should not be considered members of the insulin-like growth factor-binding protein superfamily. *Endocrinology* 2000;141:2254-6.
10. Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 1991;114:1285-94.
11. Ryseck RP, Macdonald-Bravo H, Mattei MG, Bravo R. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ* 1991;2:225-33.
12. O'Brien TP, Yang GP, Sanders L, Lau LF. Expression of cyr61, a growth factor-inducible immediate-early gene. *Mol Cell Biol* 1990;10:3569-77.
13. Martinier C, Viegas-Pequignot E, Guenard I, et al.

- Physical mapping of human loci homologous to the chicken *nov* proto-oncogene. *Oncogene* 1992;7:2529–34.
14. Pennica D, Swanson TA, Welsh JW, et al. *WTSP* genes are members of the connective tissue growth factor family that are up-regulated in *wnt-1*-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci U S A* 1998;95:14717–22.
 15. Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 1993;4:637–45.
 16. Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 1996; 107:404–11.
 17. Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor β response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ* 1996;7:469–80.
 18. Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF- β in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis. *J Mol Cell Cardiol* 2000;32:1805–19.
 19. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF- β 1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 2005;126:59–69.
 20. Duncan MR, Frazier KS, Abramson S, et al. Connective tissue growth factor mediates transforming growth factor β -induced collagen synthesis: down-regulation by cAMP. *FASEB J* 1999;13:1774–86.
 21. Chen Y, Abraham DJ, Shi-Wen X, Black CM, Lyons KM, Leask A. CCN2 (connective tissue growth factor) promotes fibroblast adhesion to fibronectin. *Mol Biol Cell* 2004;15:5635–46.
 22. Fan WH, Pech M, Karnovsky MJ. Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration *in vitro*. *Eur J Cell Biol* 2000;79:915–23.
 23. Shimo T, Nakanishi T, Nishida T, et al. Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells *in vitro*, and angiogenesis *in vivo*. *J Biochem (Tokyo)* 1999;126:137–45.
 24. Babic AM, Chen CC, Lau LF. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin α v β 3, promotes endothelial cell survival, and induces angiogenesis *in vivo*. *Mol Cell Biol* 1999;19:2958–66.
 25. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 2002;5:153–65.
 26. Frazier KS, Grotendorst GR. Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. *Int J Biochem Cell Biol* 1997;29:153–61.
 27. Wenger C, Ellenrieder V, Alber B, et al. Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene* 1999;18:1073–80.
 28. Koliopanos A, Friess H, di Mola FF, et al. Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World J Surg* 2002; 26:420–7.
 29. Kasaragod AB, Lucia MS, Cabirac G, Grotendorst GR, Stenmark KR. Connective tissue growth factor expression in pediatric myofibroblastic tumors. *Pediatr Dev Pathol* 2001;4:37–45.
 30. Shakunaga T, Ozaki T, Ohara N, et al. Expression of connective tissue growth factor in cartilaginous tumors. *Cancer* 2000;89:1466–73.
 31. Blom IE, Goldschmeding R, Leask A. Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? *Matrix Biol* 2002;21:473–82.
 32. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *J Urol* 2001;166:2472–83.
 33. Polnaszek N, Kwabi-Addo B, Peterson LE, et al. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. *Cancer Res* 2003;63:5754–60.
 34. McAlhany SJ, Ressler SJ, Larsen M, et al. Promotion of angiogenesis by ps20 in the differential reactive stroma prostate cancer xenograft model. *Cancer Res* 2003;63: 5859–65.
 35. Rowley DR. What might a stromal response mean to prostate cancer progression? *Cancer Metastasis Rev* 1998;17:411–9.
 36. Shimo T, Nakanishi T, Nishida T, et al. Involvement of CTGF, a hypertrophic chondrocyte-specific gene product, in tumor angiogenesis. *Oncology* 2001;61:315–22.
 37. Grotendorst GR. Connective tissue growth factor: a mediator of TGF- β action on fibroblasts. *Cytokine Growth Factor Rev* 1997;8:171–9.
 38. Harlow CR, Davidson L, Burns KH, Yan C, Matzuk MM, Hillier SG. FSH and TGF- β superfamily members regulate granulosa cell connective tissue growth factor gene expression *in vitro* and *in vivo*. *Endocrinology* 2002; 143:3316–25.
 39. Kothapalli D, Hayashi N, Grotendorst GR. Inhibition of TGF- β -stimulated CTGF gene expression and anchorage-independent growth by cAMP identifies a CTGF-dependent restriction point in the cell cycle. *FASEB J* 1998;12:1151–61.
 40. Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF- β . *Nat Cell Biol* 2002;4:599–604.
 41. Boes M, Dake BL, Booth BA, et al. Connective tissue growth factor (IGFBP-rP2) expression and regulation in cultured bovine endothelial cells. *Endocrinology* 1999; 140:1575–80.
 42. Suzuma K, Naruse K, Suzuma I, et al. Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. *J Biol Chem* 2000;275:40725–31.
 43. Story MT, Hopp KA, Meier DA, Begun FP, Lawson RK. Influence of transforming growth factor β 1 and other growth factors on basic fibroblast growth factor level and proliferation of cultured human prostate-derived fibroblasts. *Prostate* 1993;22:183–97.
 44. Song QH, Klepeis VE, Nugent MA, Trinkaus-Randall V. TGF- β 1 regulates TGF- β 1 and FGF-2 mRNA expression during fibroblast wound healing. *Mol Pathol* 2002; 55:164–76.
 45. Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of CTGF is directly mediated by Hif-1. *Am J Physiol Renal Physiol* 2004; 287:1223–32.
 46. Pendurthi UR, Allen KE, Ezban M, Rao LV. Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VIIa x tissue factor-induced signal transduction. *J Biol Chem* 2000;275:14632–41.
 47. Chen CC, Chen N, Lau LF. The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J Biol Chem* 2001;276:10443–52.
 48. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci U S A* 1998;95: 6355–60.
 49. Crean JK, Finlay D, Murphy M, et al. The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. *J Biol Chem* 2002;277:44187–94.