Transforming Growth Factor-β Signaling in Prostate Stromal Cells Supports Prostate Carcinoma Growth by Up-regulating Stromal Genes Related to Tissue Remodeling

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Abstract

Increasing evidence points to an active stromal involvement in cancer initiation and progression. Cytokines derived from tumor cells are believed to modulate stromal cells to produce growth and angiogenic factors, which in turn provide the tumor with the necessary microenvironment for expansion and invasion. Transforming growth factor β (TGFβ) has been implicated as a candidate cytokine to mediate this communication. However, how its signaling in stromal cells regulates tumorigenesis and tumor progression remains unresolved. We show that normal, presenescent fibroblasts or prostate stromal cells cotransplanted with prostate carcinoma cells s.c. into nude mice reduced tumor latency and accelerated tumor growth. When their TGFβ signaling was blocked, the fibroblasts and stromal cells still stimulated tumor initiation but no longer supported tumor growth as control cells did. The loss of the tumor growth–promoting activity of the stromal cells with attenuated TGFβ signaling was not associated with altered cellular senescence or tumor angiogenicity. TGFβ and the medium conditioned by the prostate carcinoma cells stimulated myofibroblast differentiation of the intact stromal cells, but not the stromal cells with attenuated TGFβ signaling. Gene microarray and quantitative reverse transcription-PCR analyses showed that TGFβ up-regulated a host of genes in stromal cells that are involved in tissue remodeling and wound healing. Thus, our study provides evidence for TGFβ as a supporting agent in tumor progression through the induction of a perpetual wound healing process in the tumor microenvironment. [Cancer Res 2007;67(12):5737–46]

Introduction

Prostate cancer is a serious health concern for men in the United States. Whereas various risk factors for prostate cancer, such as age and family history, have been identified for sometime, the underlying cellular and molecular mechanisms that contribute to the progression from normal prostate tissue to prostate intraepithelial neoplasia and finally metastasis to distant organs are less clear. Historically, tumor cells have been extensively studied as the sole determinants supporting cancer growth and metastasis. Recent studies have shown that factors in the tumor microenvironment play key roles in prostate carcinogenesis (1). The stroma, which was believed to play only a structural role, has revealed itself as an active participant in tumor growth and progression. The highest risk factor associated with prostate cancer is age, and there is evidence to suggest that aging may lead to changes in the stroma, including up-regulation of secreted factors, which ultimately create a microenvironment favorable for tumor growth. Whereas cellular senescence may be tumor suppressive early in life (2), it may contribute to tumorigenesis later on (3). In prostate cancer, a reactive stroma has been described to support the formation of prostate intraepithelial neoplasia and eventually invasive prostate carcinoma. Consistent with the observation that tumors are like “wounds that do not heal” (4), the reactive stroma is similar to a wound-healing stroma. The reactive stroma is characterized by an increased presence of myofibroblasts with concurrent up-regulation of cytokines, growth factors, and extracellular matrix components. Tumor-stromal cell interactions are being increasingly studied and it is believed that soluble factors secreted by carcinoma cells may act in a paracrine fashion on the neighboring stromal cells to stimulate the production of growth, angiogenic, and extracellular matrix remodeling factors (5). A candidate cytokine suspected of playing a major role in this communication is transforming growth factor β (TGFβ), which has been shown to directly mediate the differentiation of fibroblasts to myofibroblasts (6) and is a central factor in the wound healing process (7, 8).

TGFβ is a multifunctional cytokine mediating many cellular processes (9, 10). TGFβ regulates cellular proliferation and differentiation, as well as the processes for embryonic development, cell adhesion, wound healing, and angiogenesis, in a tissue-specific manner. In the canonical TGFβ signaling pathway, TGFβ binds to the TGFβ type II receptor (RII), which then heterodimerizes with the type I receptor (RI), causing its activation through transphosphorylation. RI then initiates intracellular signaling by phosphorylating receptor-Smad proteins, Smad2 and Smad3, which complex with Smad4 and ultimately translocate to the nucleus where they act as transcription factors for target genes. Perturbed TGFβ signaling leads to many different disease states in humans such as atherosclerosis, fibrosis, and cancer (11). Mutations in TGFβ receptors have been described, particularly in association with the development of cancer (12). A hallmark of prostate cancer progression is the loss of TGFβ growth inhibition in the epithelium due, in part, to the down-regulation of TGFβ receptors (13). Furthermore, loss of TGFβ signaling in epithelial cells has been shown to promote carcinoma formation (14), whereas restoring TGFβ signaling to carcinoma cells with down-regulated or mutated TGFβ RII suppressed tumorigenicity (15, 16).
Thus, TGFβ functions as a strong tumor suppressor during early carcinogenesis.

Paradoxically, TGFβ signaling has also been shown to promote tumor progression. The expression and production of TGFβ isoforms are invariably up-regulated in carcinomas. This is believed to result in selective growth of TGFβ-resistant tumor cells, TGFβ-induced epithelial-to-mesenchymal transdifferentiation of carcinoma cells, and TGFβ signaling in the stroma leading to a microenvironment conditioned to support tumor growth and progression. Thus, in isogenic models, blockade of TGFβ signaling stimulated tumorigenicity of less aggressive mammary tumor cells but inhibited the metastatic potential of more aggressive cells (17).

The tumor-promoting activity of TGFβ is further shown through the successful use of TGFβ inhibitors to halt tumor progression in various model systems (18). However, it remains unresolved whether the blockade of TGFβ signaling in tumor stromal cells contributed to the tumor inhibitory activity of TGFβ antagonists. In fact, conditional knockout of TGFβ RI in fibroblasts has provided evidence to support a tumor-suppressive role for TGFβ in tumor stroma (19, 20). Selective deletion of TGFβ signaling in fibroblasts was shown to cause carcinogenesis in various organs. A caveat to this approach is the ubiquitous role of TGFβ throughout development. Disrupting TGFβ signaling in the knockout animals throughout development may contribute to a predisposition for tumor formation. The fact that knocking out TGFβ signaling in fibroblasts promoted the formation of carcinomas, but not fibrosarcomas, underscores the complexity of tumor-stroma communication and the importance of temporal and spatial activity of TGFβ signaling during development.

To directly determine how stromal cells may modulate tumor progression when they are exposed to excessive stimulation by TGFβ from carcinoma cells, we studied the effect of blockade of TGFβ signaling in stromal cells on tumorigenicity and tumor growth when they are co-transplanted with carcinoma cells. Cotransplantation of the stromal cells s.c. with prostate carcinoma cells dramatically reduced tumor latency independent of TGFβ signaling in the stromal cells. However, the attenuation of TGFβ signaling greatly reduced the ability of the stromal cells to promote the growth of the transplanted tumor cells. This is associated with diminished stimulation of a myofibroblast differentiation marker by TGFβ or the medium conditioned by the carcinoma cells in the stromal cells with attenuated TGFβ signaling. Gene expression profiles and quantitative reverse transcription-PCR (RT-PCR) analysis revealed that TGFβ modulated the expression of a host of genes in the stromal cells whose products are involved in tissue remodeling and wound healing. Thus, TGFβ produced by carcinoma cells seems to stimulate stromal myofibroblast differentiation, resulting in a significant alteration of the extracellular milieu in favor of tumor expansion. Our interpretation of how TGFβ-modulated gene expression in the stromal cells may lead to a perpetual wound without healing is discussed.

Materials and Methods

Cell culture. BJ fibroblasts were derived from human newborn foreskin and were maintained in MEM (Life Technologies, Inc.) with 1% nonessential amino acids (Mediatech) and 10% fetal bovine serum (FBS; Mediatech). Human prostate stromal cells (PrSc) were purchased from Cambrex and the human prostate carcinoma cell line DU145 was originally purchased from the American Type Culture Collection. These cells were maintained in McCoy’s medium supplemented with L-serine, L-asparagine, L-glutamine, sodium pyruvate, MEM nonessential amino acids, MEM amino acids without t-glutamine, MEM vitamins, penicillin, streptomycin, gentamicin, sodium bicarbonate, and 10% FBS, as previously described (21). Cells were maintained at 37°C in a 5% CO2 humidified incubator.

Constructs for stable transfection. DU145 cells were stably transfected with the enhanced green fluorescent protein expression vector pEGFP-N1 (Clontech Laboratories, Inc.) to aid in the identification of these cells in animal tissue. Stable BJ and PrSc cell lines were generated to express the puromycin resistance vector pLPCX (Clontech Laboratories) or the same vector modified to express a TGFβ dominant negative type II receptor (DNRII) which lacks the kinase domain (14).

TGFβ sensitivity assay. For the measurement of the transcriptional activity of TGFβ with a TGFβ responsive promoter-luciferase construct, pBS4-Luc, cells were seeded at 2.0 × 104 per well in 12-well plates. When cells were 80% confluent, they were cotransfected with 1.0 μg of pBS4-Luc and 0.5 μg of a β-galactosidase expression plasmid using 4.5 μL of Lipofectamine 2000 (Invitrogen) in serum-free medium following the manufacturer’s protocol. After 4 h, the medium was replaced with the serum-containing medium and TGFβ3 was added at 5.0 ng/mL. The cells were lysed after overnight incubation in buffer (100 mmol/L K2HPO4, 1 mmol/L DTT, and 1% Triton X-100) and the luciferase activity in the cell lysate was measured as previously described (22). Luciferase activity was normalized for transfection efficiency by β-galactosidase activity.

Preparation of cell extracts and Western blot analysis. Cells were grown to confluence in 60-mm tissue culture dishes and then treated in the presence or absence of 5.0 ng/mL TGFβ3 for 1 h. Cells were then lysed in buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, and a protease inhibitor cocktail] and total protein concentration was equalized using the BCA protein assay (Pierce). Equal amounts of protein were separated on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes. The blotted membranes were incubated with rabbit anti-phosphorylated Smad2 (1:1,000; Cell Signaling), mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Ambion, Inc.) as a loading control, horseradish peroxidase–conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology), and horseradish peroxidase–conjugated goat anti-mouse (1:5,000; Santa Cruz Biotechnology). To detect α-smooth muscle actin (α-SMA), PrSc were grown to confluence in the full medium and then grown in a serum-free medium for 24 h. The cells were treated with 5.0 ng/mL TGFβ3, conditioned medium from DU145 cells, or untreated for a total of 72 h. Cell extracts were prepared as described above and the blotted membrane was incubated with mouse anti-α-SMA (1:200; Sigma-Aldrich) and mouse anti-GAPDH as a loading control. Conditioned medium from DU145 cells was obtained by growing cells in 100-mm dishes to confluence in the complete medium. The cells were grown in serum-free medium for 24 h, the medium was then changed and the cells were grown in serum-free medium for another 48 h. The conditioned medium was filtered through a 0.45-μm syringe filter, mixed 1:1 with a fresh serum-free medium, and applied to the cells for treatment.

Senescence-associated β-galactosidase assay. The assay was essentially done as described (23). Cells at the indicated passages were seeded at 2.0 × 104 per well in a four-well tissue culture plate. After 24 h, the cells were washed twice with PBS and fixed with 3% formaldehyde in PBS for 5 min. The cells were washed twice with PBS and exposed to a solution containing 1.0 mg/mL X-gal, 40 mmol/L citric acid/Na2HPO4 (pH 6.0), 5 mmol/L potassium ferrocyanide, 150 mmol/L NaCl, and 2 mmol/L MgCl2. The cells were incubated at 37°C for 2 h and then viewed for positive β-galactosidase staining at ×200 magnification by light microscopy.

Animal study. Four- to five-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc.) were used for this study. Animals were maintained under the care and supervision of the Laboratory Animal Research facility at the University of Texas Health Science Center, San Antonio, Texas. The animal protocol was approved and monitored by the Institutional Animal Care and Use Committee. Animals were randomly sorted into cages forming three groups. Tumor cells were inoculated s.c., with or without stromal cells, in the rear flank in a total volume of 100 μL of medium. Animals were monitored weekly for tumor incidence indicated by a palpable mass at the site of injection. Tumor growth was monitored weekly by external caliper measurement and tumor volume was determined in cubic millimeters using...
the formula $V = \frac{(L \times W^2)}{2}$, where $L$ is the length and $W$ is the width of a tumor. At the end of each experiment, animals were euthanized by carbon dioxide inhalation. Tumors were excised and individually weighed. Tumor tissue was frozen in liquid nitrogen for future studies or fixed in 10% buffered formalin for paraffin embedding and sectioning. Tissues from the local lymph nodes, liver, lung, bone, and brain were analyzed for metastases under fluorescence microscopy by searching for the presence of green fluorescent protein—expressing DU145 cells.

**Tumor analysis.** Formalin-fixed tumor samples were embedded in paraffin, sectioned, and stained with H&E and immunostained for the vascular endothelial cell marker CD31. Frozen tumor samples were weighed and pulverized in liquid nitrogen. Protein was then extracted in TEKCl (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0, and 80 mmol/L KCl) and hemoglobin content was measured as previously described (24). Total protein in the extract was determined using the BCA protein assay (Pierce) and hemoglobin content was normalized to total protein.

**Microarray.** Oligo microarray chips were generated from 48,958-mer probe set obtained from the Illumina HEEBO (Human Exonic Evidence Based Oligonucleotide). The set covers 26,121 unique RefSeq genes, 24,048 unique Ensembl genes, and 25,416 unique Unigene genes. The set contains a total of 4,650 controls (housekeeping genes; positive controls, negative controls; empty wells and random sequences). The details of the whole protocol were previously described (25). Briefly, total RNA was extracted from near confluent PrSC with and without 24-h treatment with 5.0 ng/mL TGFβ/3. The RNA was purified using a RiboPure Kit from Ambion following the manufacturer’s suggested protocol. Ten micrograms of each total RNA sample were reverse transcribed with random hexamers in the presence of an aminoallyl dUTP. After purification, the cDNAs were coupled with either Cy3 or Cy5 for 1 h and then purified using the PCR cleaning kit from Qiagen. The labeled cDNAs were then hybridized to the slides in a 1× final In Situ Hybridization buffer from Agilent Technologies. The hybridization was done overnight at 60°C in a rotisserie oven (Agilent Technologies). Triplicate sample hybridizations were done for each comparison. The slides were then washed in a series of SSC/SDS buffers and dried by centrifugation as previously described (25). The raw data were Lowess normalized to obtain fold changes of mRNA expression and are accessible at National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE7389).6

**Quantitative real-time PCR.** Cells were grown to near confluence in 100-mm tissue culture dishes and then treated with 5.0 ng/mL TGFβ/3, medium conditioned by DU145 cells, or untreated for a total of 48 h. Cells were lysed using Trizol reagent and total RNA was extracted with phenol/chloroform and precipitated in ethanol. Equal amounts of RNA were reverse transcribed using random primers and the resulting cDNA was used to quantify gene expression. Primers specific for the indicated genes were used to amplify targets using the Brilliant SYBR Green QPCR system from Agilent Technologies. The primers used were MMP1 forward 5′-CTCTGCTGGGAGCAAAAAC-3′ and MMP1 reverse 5′-CTTGGCACAATCTGGCGTGA-3′; MMP3 forward 5′-ACAAGAGATACAACAGGGACCAA-3′ and MMP3 reverse 5′-TAGAGTGGTAGATCATACAAAGCTCATG-3′; PDGFC forward 5′-CAAGGACAGAACGAGTACAAGA-3′ and PDGFC reverse 5′-CCATACACATTTCTC-TACTGCTCATAACTCT-3′; TNC forward 5′-CTCTGACATCATACTGAAACATAC-3′ and TNC reverse 5′-ATGCCGCTGAATCCTCAATG-3′; and β-actin forward 5′-CCGGCAGCTACATGATTGAT-3′ and β-actin reverse 5′-TGACCCTGCCCACCCATAC-3′.

**Results**

TGFβ signaling is effectively knocked down in stromal cells by the expression of DNRII. In our first experiment, we used primary BJ foreskin fibroblasts as a representative stromal-derived cell model. Subsequently, we used prostate-derived primary stromal cells (PrSC) as a more appropriated model for human prostate stroma. The two stromal cells were stably transfected with a puromycin resistance control vector or a DNRII expression vector. Western blot analysis showed that the DNRII-transfected cells expressed the truncated RI as shown in Fig. 1A. The DNRII-expressing cells were shown to have a reduced TGFβ responsiveness as indicated by the attenuation of TGFβ/3-induced phosphorylation of Smad2 (Fig. 1B) and the reduced transcriptional activity of a TGFβ/3-responsive promoter containing Smad-binding elements (Fig. 1C). Therefore, we concluded that the expression of the DNRII was effective in blocking TGFβ signaling in both stromal cells.

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6 http://www.invitrogen.com/content.cfm?pageid=11001

Stromal cell–promoted tumor growth is dependent on intact TGFβ signaling. For our first animal study, we xenografted three groups of nude mice on the right and left rear flanks with 2.0 × 10⁶ DU145 prostate carcinoma cells (five animals per group). The two test groups were cotransplanted with an additional 1.0 × 10⁶ BJ cells containing either the control vector or the DNRII construct. Tumor incidence was determined on a weekly basis by the appearance of a palpable mass at the injection site (Fig. 2A). Tumor burden was measured weekly with external caliper measurement (Fig. 2B) and the tumor volume was calculated using the formula \( V = \frac{L \times W^2}{2} \). The results of this experiment showed that cotransplanting BJ cells with DU145 cells dramatically reduced tumor latency (100% at 4 weeks) compared with animals receiving DU145 cells alone (70% at 8 weeks). The third group which received BJ cells with attenuated TGFβ signaling exhibited a similar reduction in tumor latency (70% at 4 weeks), but suppressed their growth-promoting effect on the DU145 tumors. Our data suggest that TGFβ signaling in the stromal cells does not play an early role in tumor initiation but functions later to promote tumor progression. To elucidate how stromal cells contributed to tumor progression, we examined several possibilities.

The tumor-promoting effect of stromal cells is not due to senescence. Previous studies have shown that senescent stromal cells were more effective in promoting tumorigenicity than presenescent cells (26, 27). PrSC that were used in our animal study were at passage number 10. Cells that were at passage number 12 displayed a normal morphology and did not express senescence-associated β-galactosidase. In contrast, cells at passage number 22 displayed an abnormal morphology and were positive for senescence-associated β-galactosidase (Fig. 3A). Thus, at the time when the PrSC control and DNRII cells were cotransplanted with DU145 cells into the animals, they were presenescent and approximately at mid-life span, suggesting that the different tumor-promoting activity between the PrSC control and DNRII cells was not associated with a difference in cellular senescence.

TGFβ signaling in the stromal cells did not stimulate angiogenesis. To determine whether the decreased tumor-promoting activity of the PrSC DNRII cells was due to reduced tumor angiogenesis, we compared the hemoglobin levels in the
extracts from tumors formed by DU145 + PrSC control cells and by DU145 + PrSC DNRII cells. As shown in Fig. 3B, the hemoglobin content of tumors from these two groups was not different. Immunohistochemical staining for the endothelial marker CD31 also showed no difference between tumor sections containing DU145 with either PrSC control or DNRII cells (data not shown). Thus, our data suggest that TGFβ signaling in the PrSC did not contribute to angiogenesis.

TGFβ3 and medium conditioned by DU145 cells induced a myofibroblast phenotype in PrSC. Myofibroblasts are characterized by the expression of α-SMA, extracellular matrix components such as collagens and fibronectins, and extracellular degrading enzymes such as MMPs, and are found at sites of tissue remodeling such as areas of wound healing, fibrosis, and tumor-associated stroma (28). Tuxhorn et al. (29) previously showed that myofibroblasts positive for collagen and tenacin production were found to populate the reactive stroma in prostate cancer tissue and were associated with intense TGFβ1 staining. This group further showed that TGFβ1 induced a myofibroblast phenotype in PrSC in vitro and this was blocked by a TGFβ neutralizing antibody. To determine whether TGFβ added exogenously or in the medium conditioned by DU145 cells could induce a myofibroblast phenotype in PrSC, we first determined whether DU145 cells secreted active TGFβ isoforms. Commercial ELISA assays for TGFβ1 and TGFβ2 showed that DU145 cells produced active forms of both isoforms (Fig. 4A). Because PrSC responded equally to all three TGFβ isoforms (Fig. 4B), we determined whether treatment with TGFβ3 or medium conditioned by DU145 cells will stimulate the myofibroblast marker α-SMA. Our results show that TGFβ3 at 5 ng/mL greatly stimulated α-SMA expression in the PrSC control cells and the stimulation was effectively blocked in the PrSC DNRII cells (Fig. 4C). The conditioned medium from DU145 cells contained low concentrations of active TGFβ isoforms (Fig. 4A), which were further diluted by half with a fresh serum-free medium to partially supplement spent nutrients. Therefore, it moderately stimulated α-SMA expression in PrSC control cells but moderately decreased α-SMA expression in PrSC DNRII cells, when compared with the respective control treatment without TGFβ3 and the conditioned medium (Fig. 4C). These results support the notion that TGFβ secreted from DU145 cells can induce PrSC to differentiate to a myofibroblast phenotype, which contributes to the formation of a reactive stroma. To further our knowledge of which genes are up-regulated by TGFβ in PrSC that could contribute to a “reactive” or “wound-healing” phenotype, we did a DNA microarray study to compare the changes in gene expression profile of PrSC after treatment with TGFβ3.

TGFβ3 up-regulated PrSC genes specific to tissue remodeling. Many genes were found to be up-regulated >2-fold in PrSC by the treatment with TGFβ3. We found that several of them could be grouped into three major categories: myofibroblast-related factors, extracellular matrix remodeling factors, and growth factors (Table 1).

A characteristic of myofibroblasts in tissue remodeling is their contractile property (30), which is induced by TGFβ (31). Several genes involved in cytoskeletal contraction and motility were found to be up-regulated by TGFβ in PrSC. These included actins, KIAA0992 (pallidin), and TPM1 (tropomyosin 1α), as well as MYO10 (myosin X), which is involved in filopodia formation (32). Myofibroblast differentiation is dependent on cell-cell contacts and adhesion (33). We found several relevant up-regulated factors which are involved in wound-healing myofibroblast recruitment and migration including cadherins (34), tenasin (35), and tetraspanins (36).

Myofibroblasts in the reactive stroma are central to extracellular matrix remodeling, which involves both degradation and deposition (29). We found that TGFβ up-regulated the expression of genes involved in extracellular matrix degradation such as ADAMs and MMPs. SH3MD1 (Fish/Tk5) was also up-regulated which has been shown to specifically interact with ADAMs (37) and is required for protease-driven cancer invasion (38). Another protease related to pathologic extracellular matrix degradation is PRSS11/HtrA1 (39). Some protease inhibitors that are associated with invasion and

Figure 3. Cellular senescence and the level of tumor angiogenesis were not altered by the expression of DNRII in PrSC. A, PrSC at passage 12 or passage 22 were seeded at 2 × 10⁴ per well in a four-well tissue culture plate. After 24 h, the cells were washed twice with PBS and then fixed with 3% formaldehyde in PBS. The cells were washed and exposed to a β-galactosidase staining solution. The cells were incubated at 37°C for 2 h and then viewed for positive β-galactosidase staining at ×200 magnification by light microscopy. B, frozen tumor samples were weighed and then pulverized in liquid nitrogen. Protein was extracted in a proportional volume of TEKCI buffer and hemoglobin content was measured relative to a standard curve. Total protein in the extract was determined using the BCA protein assay and hemoglobin content was normalized to total protein. Columns, mean for three representative tumors; bars, SE.
metastasis were also up-regulated including SERPINE1/PAI-1 (40) and SERPINE2/protease nexin I (41). Interestingly, tissue inhibitor of metalloproteinase (TIMP)-3, an inhibitor of MMPs, was up-regulated by 3.6- to 8.8-fold. TIMP3 is believed to be an inhibitor of tumor progression; however, it has also been shown to be a key factor in pathologic fibrosis (42). Several extracellular matrix structural components related to myofibroblast differentiation were also up-regulated by TGF\(\beta\) such as collagens, elastin, fibronectin, and matrix Gla protein (MGP).

Finally, we found that TGF\(\beta\)3 up-regulated several factors in PrSc that are involved in growth regulation including AREG, CTGF, GREB1, INHBA, and PDGFs, as well as a survival factor, CLU. Other growth factors such as epidermal growth factor and insulin-like growth factor (IGF)-I were not up-regulated in PrSc by TGF\(\beta\)3 treatment (data not shown). Interestingly, TGF\(\beta\)3 down-regulated hepatocyte growth factor (HGF) by 3.1- to 6.3-fold in PrSc, which is likely to be significant in the context of TGF\(\beta\)3-stimulated perpetual wound response without healing as discussed below. TGF\(\beta\)3 also up-regulated TGFBR1 by 2.9- to 3.5-fold. In a model of systemic sclerosis, overexpression of TGFBRI in fibroblasts was shown to up-regulate collagen which recapitulates the matrix reaction of the disease state (43). The same group had previously found that a majority of systemic sclerosis fibroblasts have an elevated TGFBR1 to TGFBR2 ratio.

By grouping the TGF\(\beta\) up-regulated genes we found a pattern of gene expression that can promote myofibroblast differentiation, migration, extracellular matrix remodeling and growth. These findings support our hypothesis that TGF\(\beta\) can stimulate PrSc to produce factors that can generate a microenvironment favorable for tumor growth.

**Conditioned medium from DU145 cells induced tissue remodeling factors only in PrSc with intact TGF\(\beta\) signaling.** To confirm the microarray data, we quantified the transcripts of four of the TGF\(\beta\)3-up-regulated genes from our microarray analysis with quantitative real-time RT-PCR. Treatment with TGF\(\beta\)3 up-regulated transcript levels of all four genes tested (Fig. 5). Furthermore, the conditioned medium from DU145 cells also up-regulated the transcripts of these four genes in PrSc with intact TGF\(\beta\) signaling. The stimulation was blocked, however, in the cells that expressed the DNRII. Thus, our findings specifically implicate TGF\(\beta\)3 as the soluble factor in DU145-conditioned medium that stimulates the expression of tissue remodeling factors in PrSc.

**Discussion**

TGF\(\beta\) has shown opposing roles in carcinogenesis and the mechanism by which it acts as a tumor suppressor or tumor promoter by signaling either in the epithelium and/or stroma remains an active area of research (44). Our study focused on the role of TGF\(\beta\) in tumor-stromal communication. Previous studies, including our own, have shown that administration of various TGF\(\beta\) inhibitors can inhibit tumor growth and metastasis in...
animal models (18, 21, 22). However, whether the blockade of TGFβ signaling in the epithelium or stroma is responsible for the antitumor actions of the inhibitors remains to be clarified. We designed our study to answer this question by blocking TGFβ signaling specifically in the stromal cells. The human prostate carcinoma cell line (DU145) was chosen for our study because it was shown to be weakly tumorigenic in our previous study (21). Thus, we could more easily determine a positive effect of cotransplanted stromal cells on carcinoma cell growth. Our results confirmed that stromal factors do contribute to tumor initiation of transplanted carcinoma cells. More importantly, TGFβ signaling in the stromal cells showed little effect on tumor initiation but was critical for the stromal promotion of carcinoma tumor growth in vivo. Thus, various factors and pathways activated in stromal cells seem to contribute to carcinogenesis at different stages and TGFβ seems to act on stromal cells to promote tumor progression.

Whereas our observation that stromal cells can promote tumorigenicity of transformed epithelial cells is consistent with Table 1. TGFβ-up-regulated PrSC genes involved in tissue remodeling

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Extracellular matrix remodeling factors

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<tr>
<td>F2RL1</td>
<td>coagulation factor II (thrombin) receptor-like 1, PAR-2</td>
<td>2.7–2.9 ± 0.0–0.6</td>
<td>2</td>
</tr>
<tr>
<td>FNI</td>
<td>fibronectin 1</td>
<td>2.9 ± 0.6</td>
<td>1</td>
</tr>
<tr>
<td>MAPK11</td>
<td>mitogen-activated protein kinase 11</td>
<td>2.3 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>MGP</td>
<td>matrix Gla protein</td>
<td>3.3 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>MMP1</td>
<td>matrix metalloproteinase 1 (interstitial collagenase)</td>
<td>5.0 ± 1.6</td>
<td>1</td>
</tr>
<tr>
<td>MMP3</td>
<td>matrix metalloproteinase 3 (stromelysin 1, progelatinase)</td>
<td>3.6 ± 0.2</td>
<td>1</td>
</tr>
<tr>
<td>PRSS11</td>
<td>protease, serine, 11 (IGF binding)</td>
<td>2.4 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>serine (or cysteine) protease inhibitor, PAI-I</td>
<td>4.2 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>SERPINE2</td>
<td>serine (or cysteine) protease inhibitor</td>
<td>4.1 ± 1.6</td>
<td>1</td>
</tr>
<tr>
<td>SH3MD1</td>
<td>SH3 multiple domains 1, Fish/Tk5</td>
<td>2.7 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>TIMP3</td>
<td>tissue inhibitor of metalloproteinase 3</td>
<td>3.6–8.8 ± 0.3–0.4</td>
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</tr>
</tbody>
</table>

Growth factors

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Mean* ± SD</th>
<th>Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG</td>
<td>amphiregulin (schwannoma-derived growth factor)</td>
<td>3.2 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>CLU</td>
<td>clusterin</td>
<td>2.3 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
<td>3.1 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>GREB1</td>
<td>gene regulated by estrogen in breast cancer protein</td>
<td>2.7 ± 0.0</td>
<td>1</td>
</tr>
<tr>
<td>IL-11</td>
<td>interleukin 11</td>
<td>3.4 ± 1.3</td>
<td>1</td>
</tr>
<tr>
<td>INHBA</td>
<td>inhibin, βA (activin A, activin AB α polypeptide)</td>
<td>7.5 ± 1.9</td>
<td>1</td>
</tr>
<tr>
<td>PDGFC</td>
<td>platelet-derived growth factor C</td>
<td>2.2 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>PDGFD</td>
<td>platelet-derived growth factor D</td>
<td>3.4 ± 0.2</td>
<td>1</td>
</tr>
<tr>
<td>TGFBR1</td>
<td>transforming growth factor β receptor 1</td>
<td>2.9–3.5 ± 0.3–0.6</td>
<td>2</td>
</tr>
</tbody>
</table>

*Mean fold increase due to TGFβ treatment from triplicate measurements.
†A range of mean and SD is provided for genes having more than one corresponding probe.
published studies (26, 27), those studies also showed that the tumor-promoting activity was much greater in senescent fibroblasts than in presenescent fibroblasts. The analysis of senescence-associated β-galactosidase activity indicated that both the control and DNRII-expressing PrSC were not senescent at the time of cotransplantation with the carcinoma cells. The morphology of the two cells also seemed to be identical and presenescent before cotransplantation, suggesting that the difference in their tumor-promoting activity was not due to cell senescence. Consistent with this interpretation, Coppe et al. (26) showed the up-regulation of vascular endothelial growth factor (VEGF) in senescent fibroblasts resulting in more numerous and larger blood vessels in the tumors containing senescent fibroblasts than in those containing presenescent fibroblasts. In our study, we did not observe a significant difference in VEGF expression between the control and DNRII PrSC (data not shown). There was no significant difference in tumor hemoglobin levels and the prevalence of CD31 staining between xenografts containing either the control or the DNRII PrSC. Thus, whereas TGFβ has been shown to promote angiogenesis in prostate tumors (21, 45), its signaling in the stromal cells seems to play an insignificant role in tumor angiogenesis.

Although TGFβ signaling in fibroblasts has been shown to play a tumor-suppressive role in the development of epithelial tumors in Tgfb2 knockout mice and fibroblasts (19, 20), how the loss of TGFβ signaling early in the development may alter the phenotype of the fibroblasts and their interaction with epithelial cells remain to be elucidated. It is possible that transgenic animals having TGFβ signaling knocked out in fibroblasts are likely to undergo developmental changes that could lead to a predisposition for cancer formation. Another important difference between our DNRII-expressing fibroblasts and the Tgfb2 knockout fibroblasts is that TGFβ signaling is only partially blocked in the former (see data in Fig. 1B and C) while completely lost in the latter. Thus, whereas significant up-regulation of HGF expression was observed in the Tgfbr2 knockout fibroblasts and believed to contribute to the tumor promotion by the knockout fibroblasts (19, 20), PrSC DNRII cells did not display a different expression level of HGF from PrSC control cells (data not shown), although a 3- to 6-fold down-regulation was observed in the microarray analysis in PrSC control cells when treated with TGFβ. On the other hand, our data strongly support TGFβ signaling in the stroma as tumor promoting. Consistent with what has been reported (31), the treatment with TGFβ or the medium conditioned by the carcinoma cells was shown to stimulate myofibroblast differentiation as indicated by the up-regulation of α-SMA. We also found in our gene microarray analysis that TGFβ can stimulate PrSC to express many genes whose products participate in myofibroblast differentiation and promote reactive stroma formation and carcinoma growth. Thus, although TGFβ inhibits the expression of HGF in stromal fibroblasts, our in vivo data suggest that the growth-promoting factors stimulated by TGFβ more than compensated for the loss of HGF in the model system we used.

Whereas the inhibition of HGF expression by TGFβ may contribute to the tumor-suppressive activity of TGFβ signaling in stromal fibroblasts, it may also cause a tumor to become a wound that does not heal. The wound healing process is characterized by three separate, but overlapping, steps (46). The process begins with hemostasis and inflammation, which is followed by proliferation and then maturation and remodeling. Normally, this process occurs with the chronological appearance and decline of participating factors in the wound, including TGFβ. Myofibroblast differentiation is a critical early step found in normal wound healing but is also found in pathologic fibrosis and cancer-associated stroma and is known to be regulated, at least in part, by TGFβ (30). One could hypothesize a scenario in which TGFβ overexpression in a carcinoma could trigger a wound healing-like reaction in the

Figure 5. TGFβ3 and conditioned medium from DU145 cells up-regulated factors involved in tissue remodeling and reactive stroma formation only in PrSC with intact TGFβ signaling. PrSC control and DNRII cells were grown to confluence in 100-mm tissue culture dishes. The cells were then left untreated (NT) or treated with 5.0 ng/mL TGFβ3 (β3) or medium conditioned by DU145 cells (CM) for a total of 48 h. Cells were lysed using Trizol reagent and total RNA was extracted using phenol/chloroform and precipitated in ethanol. Equal amounts of RNA were reverse transcribed using random primers and the resulting cDNA was used for real-time quantitative PCR. Primers specific for the indicated genes were used to amplify targets using the Brilliant SYBR Green QPCR system from Stratagene. Columns, mean of triplicate measurements; bars, SE.


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References


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