Altered TGF-β Signaling in a Subpopulation of Human Stromal Cells Promotes Prostatic Carcinogenesis

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Abstract

Carcinoma-associated fibroblasts (CAFs) play a critical role in malignant progression. Loss of TGF-β receptor II (TGF-βR2) in the prostate stroma is correlated with prostatic tumorigenesis. To determine the mechanisms by which stromal heterogeneity because of loss of TGF-βR2 might contribute to cancer progression, we attenuated transforming growth factor beta (TGF-β) signaling in a subpopulation of immortalized human prostate fibroblasts in a model of tumor progression. In a tissue recombination model, loss of TGF-βR2 function in 50% of the stromal cell population resulted in malignant transformation of the nontumorigenic human prostate epithelial cell line BPH1. Mixing fibroblasts expressing the empty vector and dominant negative TGF-βR2 increased the expression of markers of myofibroblast differentiation [coexpression of vimentin and alpha smooth muscle actin (αSMA)] through elevation of TGF-β1 and activation of the Akt pathway. In combination, these two populations of stromal cells recapitulated the tumor inductive activity of CAFs. TGF-βR2 activity in mixed stromal cell populations cultured in vitro caused secretion of factors that are known to promote tumor progression, including TGF-β1, SDF1/CXCL12, and members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families. In vivo, tissue recombination of fibroblasts overexpressing TGF-β1 and SDF1/CXCL12 not only induced transformation of BPH1 cells, but also promoted a robust growth of highly invasive cells, similar to effects produced by CAFs. While the precise nature and/or origin of the particular stromal cell populations in vivo remain unknown, these findings strongly link heterogeneity in TGF-β signaling to tumor promotion by tumor stromal cells. Cancer Res; 71(4); 1272-81. ©2011 AACR.

Introduction

The tumor microenvironment includes fibroblasts, smooth muscle, nerves, blood vessels, and components of the inflammatory/immune system. While smooth muscle is the predominant stromal cell type associated with normal prostatic acini, fibroblasts and myofibroblasts are associated with cancer cells and are prominent modifiers of cancer progression (1–3). Cancer-associated fibroblasts (CAFs), isolated from within the tumors of cancer patients, are important elements in tumor growth and progression (4). Studies suggest that these fibroblasts are recruited or activated following injury and that this reactive stroma provides the oncogenic signals that promote tumorigenesis (5). The role of CAF in carcinogenesis has been examined by studies in which human prostatic CAF promoted carcinogenesis in the immortalized but nontumorigenic BPH1 prostatic epithelial cell line (6–8).

Molecular markers of fibroblast subpopulations are poorly defined. Generally, fibroblasts are identified by their spindle-shaped morphology and the overlapping expression of various indicators of their fibroblastic phenotype, such as vimentin and fibroblast specific protein 1 (FSP1). Other markers, which are not expressed in all fibroblasts have been identified (9, 10). This suggests multiple populations of fibroblasts whose specific phenotype may be conditioned by factors, such as developmental stage and status (rest versus activation, as in a wound healing or cancer). Such cells may reflect different lineages or may be the result of site-specific differentiation (3).

Stroma can elicit instructive, permissive, or inductive (reactive) effects on the parenchymal epithelium (11). For example, embryonic mesenchymal cells can instruct epithelial cells to form functional, differentiated glands (12). In contrast, a permissive stroma supports a previously induced epithelial phenotype. Tumor stroma can be considered inductive or reactive because it secretes factors that can stimulate tumor progression in suitably initiated epithelial cells (13).

Transforming growth factor beta (TGF-β) can both induce and suppress differentiation and tumorigenesis in a dose-
context-dependent manner (14). Loss of the TGF-β type II receptor (TGFβR2) has been observed in the stroma of more than 60% of human prostate cancer patients (15). Ablation of TGFβR2 from approximately 40% of the stromal cells in a mouse resulted in cancer of the forestomach and premalignant prostatic lesions (16). This demonstrated that changes in TGF-β receptor activity in fibroblastic subpopulations can have profound effects on adjacent epithelial morphology.

The present study was designed to test whether stromal heterogeneity has direct consequences on tumor progression in a human prostatic tissue recombination model. Here, we report that heterogeneous expression of TGFβR2 in prostate stromal subpopulations elicits changes consistent with the malignant phenotype induced by CAF.

Materials and Methods

Cells

BPH1 (a nontumorigenic human prostate epithelial cell) was from our stocks (17). De-identified human prostatic tissue samples were obtained from the Vanderbilt Tissue Acquisition Core via the Department of Pathology consistent with Vanderbilt IRB protocols. Benign human prostate stromal cells (BHPrS1) were isolated from a benign prostate surgical sample, also used to establish the BHPrE1 cell line (18). BHPrS1 cells were immortalized using human telomerase reverse transcriptase. Carcinoma-associated fibroblasts (CAF) were isolated and bioassayed as previously described (6). Cells were maintained in RPMI-1640 (Gibco) with 1% antibiotic/antimycotic (Life Technologies) and 5% Cosmic CAF Serum (CCS).

ELISA for TGF-β1 and SDF1α

Around 800,000 cells were plated in 10 cm dishes, and after overnight attachment and growth, cells were washed twice with PBS and fed with serum-free RPMI-1640. Conditioned medium (CM) was collected after 48 hours, centrifuged at 13,000 rpm for 15 minutes to pellet debris, and stored at −80°C for later use. Quantification of TGF-β1 and SDF1α in the CM were assessed by ELISA according to the manufacturer’s protocol [human TGF-β1 (DB100B) and human SDF1α (DS400) Quantikine from R&D Systems, Inc, Minneapolis, MN]. Each experiment was carried out in triplicate.

Human prostate tissue array

Human prostate tissue samples from 90 patients with diagnosed prostate carcinoma representing different Gleason scores were used to generate a tissue microarray. The microarray contained 0.6 mm core samples, two from the peripheral tumor and one from the peripheral zone (PZ) away from the cancer-involved area. The original diagnostic H&E stained slide as well as the Gleason score were available in all cases. Immunohistochemically stained microarray slides were reviewed by a pathologist (MPR) blinded to the Gleason score, and results were expressed in a semiquantitative manner. Percentage staining was scored on a scale of 0–4, where 0 = no staining, 1 = less than 25% of cells staining, 2 = 25% to 50% cells staining, 3 = 50% to 75% cells staining, 4 = more than 75% cells staining. Epithelial and stromal cells were scored separately.

Tissue recombinants and subrenal capsule xenografts

Rat urogenital sinus mesenchyme (rUGM) was prepared from 18-day embryonic Sprague-Dawley rats fetuses (Harlan) as previously described (19). To prepare tissue recombinants, rUGM and CAF cells were mixed in five different ratios (100% rUGM, 75% rUGM: 25% CAF, 50% rUGM: 50% CAF, 25% rUGM: 75% CAF, and 100% CAF) for a total of 250,000 cells and recombined with 100,000 BPH1 cells. After overnight incubation at 37°C, tissue recombinants were grafted under the renal capsule of intact male CB17/crl-Scid-severe combined immune deficient (SCID) mice (Harlan) supplemented with 5 mg testosterone pellets placed in the subcutaneous compartment. For the experiments involving engineered cells, 100,000 BPH cells were recombined with 250,000 stromal cells for BHPrS1−/−/BHPrS1−/−, or BHPrS1−/−. The BHPrS1−/−/BHPrS1−/−, BHPrS1−/− mixtures were composed of 125,000 BPHPrS1−/−/BHPrS1−/− + 125,000 BPHPrS1−/− cells to obtain a 50/50 ratio. Both BHPrS1−/−/− and BHPrS1−/−/− cells were mixed at a density of 250,000 cells with BPH1 cells. Host mice were sacrificed after 8 weeks. The kidneys were removed, grafts cut into halves, and imaged before processing for histology. Graft dimensions were measured and the resultant tumor volume was calculated using the formula: volume = length x width x depth x π/6. This formula underestimates the volume of invasive tumors, and thus tends to underestimate the invasive characteristics of large invasive tumors.

Results

Cancer-associated fibroblasts counteract the organizational effect of embryonic mesenchymal cells on prostatic remodeling

To date, the study of stromal-epithelial interactions in vivo has assumed each tissue to have a broadly homogeneous nature. A study of stromal heterogeneity requires the presence of at least two different cell types in the stromal compartment. We examined whether rUGM could suppress the epithelial malignant phenotype resulting from exposure to CAF. To test this, we recombined CAF and rUGM cells in five different ratios with BPH1 epithelial cells.

These experiments gave broadly predictable results, in that, as the proportion of CAF/rUGM cells increased, the epithelial structures in the recombinants became progressively less organized and the size of the grafts increased. Specifically, the previously described, (20) nonmalignant, well-organized epithelial cords formed under the influence of rUGM became less obvious as the proportion of CAF increased, whereas tumor structures and invasion became more prevalent (Fig. 1A and B). Concurrent with changes in epithelial cell organization, changes were also noted in the stroma. Well-organized smooth muscle surrounding the benign cords became progressively more fibroblastic in the more malignant grafts, with expression of vimentin predominating over markers of prostate stromal differentiation, such as alpha and gamma smooth muscle actin (αSMA and γSMA), desmin, and calponin (Fig. 1A). These results show that the epithelial response is dictated by the overall paracrine signaling environment, and demonstrate that this response can be modified by mixing different populations of stromal cells.
Human prostate cancer stromal cells show heterogeneous TGF-β signaling

Elevated TGF-β expression by CAF are linked to prostatic tumorigenesis (7, 21). Immunostaining for TGFβR2 has been shown to decrease in prostate stroma with increasing cancer grade (15, 22). However, the importance of TGF-β-responsive versus nonresponsive stromal cells has not been addressed properly. We stained for phosphorylated Smad2, as a

Figure 1. Prostate stroma regulates the fate of prostate epithelial cells. Heterotypic tissue recombinants of rUGM and CAF in different ratios with BPH1 cells were subrenal capsule grafted to male hosts mice for 8 weeks before the tissues were harvested. A, i–v, H&E shows differentiation of BPH1 cells to benign cords when combined with 100%UGM. As the ratio of CAF cells increased, malignant transformation of BPH1 cells was observed and the epithelium became progressively less organized and more invasive (ii–v). Immunofluorescence labeling of αSMA/Vimentin, (vi–x), γActin/SV40 (xi–xv), and desmin/cytokeratin (xvi–xx), showed a more muscular stroma surrounding areas with high percentage of rUGM (>75%). Markers of normal stromal differentiation were dramatically decreased when the ratio of CAF/rUGM increased to more than 50%. Hoescht 33258 staining of the nucleus (blue) confirmed the human nature of the stromal cells. B, gross picture of BPH1 cells recombined with rUGM or CAF cells (i and ii) showing epithelial invasion into the kidney parenchyma (*). rUGM induced benign glandular differentiation, which was separated by a thick layer of differentiated γSMA (+) fibroblasts (green) from the underlying kidney. C, Tumor volumes were quantitated, graphical representation of the mean ± SD of the grafts is shown n = 6. The volume of the tumors increased with the percentage of CAF.
surrogate for TGF-β activity, in tissue recombinants composed of BPH1 with: rUGM, BHPrS1, and CAF. BHPrS1 cells were established from a benign human prostate surgical sample and characterized to express both vimentin (fibroblast) and α-SM-actin (smooth muscle) proteins without epithelial and neuroendocrine markers (Supplementary Fig. 1). Recombinants composed of CAF + BPH1 showed the highest percentage of phospho-Smad2 expressing stromal cells (52%) compared with either BPH1 + BHPrS1 (<10%) or BPH1 + rUGM (<25%).

Normal prostate epithelial cells were surrounded by cells expressing low levels of phospho-Smad2. Stromal cells in the tumors as well as those in normal looking areas in the PZ of cancer patients had a phospho-Smad2 SCORE of more than 2.5 representing more than 50% of positive cells (see text for details). C, addition of TGF-β1 ligand to cultured fibroblasts induced the expression of αSMA and phospho-Smad2 in a subpopulation of CAF, whereas the BHPrS1 showed a more homogeneous pattern of expression. Note morphologic changes to CAF with serum compared with BHPrS. This phenomena may be the result of the ability of CAF to remodel collagen matrices.

Figure 2. The prostate tumor stroma shows heterogeneous P-Smad2 expression, a surrogate marker of response to TGF-β. A, Immunohistochemical staining of tissue recombinants for phospho-Smad2. BPH1 + CAF recombinants showed the highest proportion of phospho-Smad2 positive stromal cells (~50%) compared with either BPH1 + BHPrS1 (<10%) or BPH1 + rUGM (<25%). B, phospho-Smad2 expression in human prostate cancer tissue array. Normal prostate epithelial cells were surrounded by cells expressing low levels of phospho-Smad2. Stromal cells in the tumors as well as those in normal looking areas in the PZ of cancer patients had a phospho-Smad2 SCORE of more than 2.5 representing more than 50% of positive cells (see text for details). C, addition of TGF-β1 ligand to cultured fibroblasts induced the expression of αSMA and phospho-Smad2 in a subpopulation of CAF, whereas the BHPrS1 showed a more homogeneous pattern of expression. Note morphologic changes to CAF with serum compared with BHPrS. This phenomena may be the result of the ability of CAF to remodel collagen matrices.
of normal human prostatic fibroblasts affects prostate toward a myofibroblast phenotype, as noted by the increased expression of vimentin and BHPrS1 (7). The combination of BHPrS1 grew faster in the presence of CAF-CM compared with previous observations in a similar system, BPH1 cells and fibroblasts. B, BPH1 þ EV recombinants (vi–ix). The presence of BHPrS1–DN increased the proportion of Ki67 positive cells in the BPH1 þ EV, BHPrS1–DN grafts, no invasion was seen in the other recombinants (v–vii). The adeno-squamous phenotype with invasion into the kidney resembled BPH1–CAEv recombinants. The arrow indicates invasion into the host kidney. Immunofluorescence staining of SV40 confirmed the invasion of BPH1 cells into the kidney parenchyma (7) of BPH1 þ EV/BHPrS1–DN grafts, only act on tumor epithelial cells, but could also exert an effect in a subset of stromal cells that have intact TGF-β signaling.

Loss of TGF-β responsiveness in a subpopulation of prostate stromal cells affects epithelial proliferation and induces tumorigenicity

To test whether changes in TGF-β signaling in a subpopulation of normal human prostatic fibroblasts affects prostate epithelial cells, we fed BPH1 cells with CM from CAF and also from BHPrS1 expressing a dominant negative TGFβR2 (BHPrS1–DN), a control vector (BHPrS1–EV), or a mixture of BHPrS1–DN and BHPrS1–EV in a 50/50 ratio. Consistent with previous observations in a similar system, BPH1 cells grew faster in the presence of CAF-CM compared with BHPrS1-CM (7). The combination of BHPrS1–DN and BHPrS1–EV led to faster proliferation compared with the BHPrS1–EV-CM and similar to the CAF-CM (Fig. 3A). These data suggest that factors secreted by stromal cells heterogeneous for TGF-β responsiveness can affect the proliferation of epithelial cells in a manner similar to CAF.

To further define the role of the heterogeneous stroma in prostate cancer, we tested these cells in vivo. Grossly, BPH1 þ BHPrS1–DN/BN/BHPrS1–EV recombinants formed larger grafts compared with BPH1 þ BHPrS1–DN and BPH1 þ BHPrS1–EV recombinants (Fig. 3B). BPH1 þ BHPrS1–DN/BN/BHPrS1–EV recombinants were 2.5 times larger than BPH1 þ BHPrS1–DN, recombinants composed of BPH1 þ BHPrS1–EV showed minimal growth. The small BPH1 þ BHPrS1–EV grafts were primarily formed of stromal cells and a few epithelial cords without lumen formation [Fig. 3C, i]. BPH1 þ BHPrS1–DN/BN/BHPrS1–EV tissue recombinants had a benign appearance and did not show any sign of invasion into the host kidney (Fig. 3C, iv and v). In contrast, BPH1 þ BHPrS1–DN/BN/BHPrS1–EV grafts (as assessed by H&E staining) resembled a poorly differentiated adenocarcinoma with areas of adenosquamous differentiation. BPH1 cells showed clear invasion into the kidney parenchyma, and there were no clear margins between the kidney and
the grafts as assessed by SV40-T antigen staining (Fig. 3C, i and vi [arrow]). This is similar to previously described patterns in BPH1→CAF recombinants, although the overall tumor size here was smaller (6). The malignant BPH1→BHPs1−DNT/RH/BHPs1−EV recombinants had profound effects on their surrounding stroma. Vimentin and αSMA were elevated compared with BPH1→BHPs1−EV and BPH1→BHPs1−DNT/RH grafts (Fig. 3C, x, xi, and xii). BHPs1−DNT/RH, BHPs1−EV, and BHPs1−DNT/RH/BHPs1−EV mixtures were prepared in order to compare with BPH1 cells. All formed small invasive grafts composed of a thin layer of fibrous connective tissue without any sign of malignant transformation (data not shown) consistent with previous reports (6). These data suggest heterogeneous TGF-β responsiveness the stroma can induce malignant transformation of the initiated prostate epithelial cell line BPH1, in a manner consistent with previous observations of CAF.

Absence of TGF-β signaling in a proportion of stromal fibroblasts induces changes to phenotype and intracellular signaling

We investigated the regulation of TGF-β signaling in human prostate fibroblasts on growth factor and growth factor receptor expression using low cycle number reverse transcriptase PCR (RT-PCR). Of a small initial screen of growth factors and receptors, many transcripts showed consistent patterns in the mixture (always expressed, never expressed, or intermedi- ate between the wild-type and DN components; Supplementary Fig. 2). Some molecules, including SDF1α and TGFBR3, showed patterns in which the overall expression in the BHPs1−DNT/RH/BHPs1−EV mixed population was clearly induced or suppressed compared with the BHPs1−DNT/RH or BHPs1−EV cells alone. Loss of TGF-β signaling in BHPs1−DNT/RH was correlated with increased expression of TGF-β1 (Fig. 4A). We used PCR arrays to examine these cells cultured under normal serum conditions. The results revealed that members of the TGF-β superfamily as well as several fibroblast growth factor (FGF), insulin like growth factor (IGF), and interleukins were highly dysregulated in the BHPs1−DNT/RH/BHPs1−EV mixtures compared with the component strains (Supplementary Table 2). The data also confirmed the RT-PCR observations that TGF-β expression was increased in BHPs1−DNT/RH and BHPs1−DNT/RH/BHPs1−EV cells [Fig. 4B (arrow)]. This again suggests that interactions between stromal cells can modify the overall paracrine signaling environment.

To better understand the molecular mechanisms by which the mixtures of BHPs1−DNT/RH/BHPs1−EV cells can induce a CAF-like phenotype, we focused on several major pathways and as well as markers altered in CAF by Western blot. Cells showed patterns in which the overall expression in the BHPs1−DNT/RH/BHPs1−EV mixtures had an effect on BHPs1−EV with intact TGFBR2. These data can explain the field effect observed in patient samples in which normal prostate fibroblasts respond to the high levels of TGF-β ligand secreted by tumors. Expression of phospho-Akt and p27 were higher in BHPs1−DNT/RH/BHPs1−EV and BHPs1−DNT/RH compared with BHPs1−EV. There were no significant changes in the activation of the MAPK (mitogen activated protein kinase) pathway as assessed by phospho-p44/p42 expression. However, there was increased expression of the tumor stromal markers vimentin and αSMA, as well as the recently described marker of mesenchymal lineage cells, CD90, frequently found in prostate cancer stroma (23). Collectively, these data suggest that changes in TGF-β signaling may regulate the interactions between stromal cells that might help determine not only the CAF-phenotype, but also the overall paracrine signaling environment responsible for the tumor promoting abilities of the cancer stroma.

Overexpression of TGF-β1 and SDF1α in benign human prostate fibroblasts induces in vivo malignant transformation of BPH1 cells

We previously reported that blocking either TGF-β or SDF1α in vivo impairs the ability of CAF cells to promote tumorigenici
ty (7). Since these two factors were elevated in BHPs1−DNT/RH/BHPs1−EV, we wanted to know whether the overexpression of TGF-β1 or SDF1α alone in normal prostate fibroblasts could elicit changes that mimic CAF behavior. We therefore generated BHPs1−EV, BHPs1−TGF-β1, and BHPs1−SDF1α cells using a retroviral system. Secretion of constitutively active TGF-β1 and SDF1α by these cells were quantitated by ELISA and compared with BHPs1−DNT/RH and BHPs1−EV grafts in vivo (Fig. 5A, upper). ELISA for TGF-β secretion corroborated RT-PCR observations in which the BHPs1−DNT/RH and BHPs1−DNT/RH/BHPs1−EV cells had higher levels compared with the BHPs1−EV. BHPs1−TGF-β1 showed the highest expression with levels similar to CAF. No significant change in TGF-β1 expression was elicited in BHPs1−EV (Fig. 5A, lower). To further characterize the in vivo effects, BHPs1−EV, BHPs1−TGF-β1 or BHPs1−SDF1α were each recombined with BPH1 cells and grafted under the renal capsule of SCID mice. Both BHPs1−TGF-β1 and BHPs1−SDF1α formed large tumors that were highly invasive into the kidney (Fig. 5B, arrows). Notably, these grafts were smaller than typical for BPH1→CAF (data not shown). BPH1→BHPs1−EV grafts were composed of a large bulk of stromal cells with a few solid epithelial cords (Fig. 5C, i). However, when the stroma was enriched for SDF1α, we observed large areas with adenoma- squamous differentiation pushing into the kidney with minimal invasion (Fig. 5C, ii). BPH1→BHPs1−TGF-β1 developed poorly differentiated adenocarcinomas with a large stromal component, and were highly invasive into the kidney as noted by poor delimitation of the tumor boundaries (Fig. 5C, iii). These tumors were highly proliferative measured by Ki67 staining (Fig. 5C, iv, v, and vi). Masson’s Trichrome staining showed increased collagen deposition in the stroma of BPH1→BHPs1−TGF-β1 compared with BPH1→BHPs1−SDF1α and BPH1→BHPs1−EV grafts, providing corroboration of TGF-β1 secretion (Fig. 5C, vii, viii, and ix). Staining for SDF1α was highest in the stroma of BPH1→BHPs1−SDF1α recombinants followed by BPH1→BHPs1−TGF-β1 with little staining in BPH1→BHPs1−EV grafts (Fig. 5C, x, xi, and xii). Overall our data...
suggest that specific stromally secreted factors resulting from partial loss of TGFβR2 can result in paracrine prostate cancer promotion.

**Discussion**

Malignant tumors are complex caricatures of developing organs in which stromal and epithelial cells are engaged in active communication. The tumor stromal cells, and the extracellular matrix which they deposit, play a key role in restraining or promoting tumorigenesis. The specific nature of the human prostate cancer stromal phenotype has been shown to be an independent clinical prognostic marker (24, 25), underlining the importance of paracrine interactions in human disease. In *vivo* models have been used to demonstrate paracrine mechanisms by which the stromal microenvironment acts to promote tumorigenesis (7, 21, 26). The prostate tumor microenvironment is complex, including cells of many different lineages (27–34). These include, but are not limited to, smooth muscle, various types of fibroblasts, senescent stromal cells, nerves and blood vessels, and a wide variety of immune and inflammatory cell types.

The diversity in the types of cells that compose the tumor stroma makes it difficult to study the contribution of each component. In addition, the lack of stromal cell lines that can retain the tumor-inducing properties shown by CAF cells in *vivo* represents another important problem. In an attempt to demonstrate the impact of a heterogeneous stromal population in prostate cancer progression, we recreated a controlled tumor microenvironment by impairing TGF-β signaling in about 50% of human benign prostate fibroblasts.

Mesenchyme can restrict the growth of some tumorigenic cells. For example, rat urogenital mesenchyme recombined with Dunning Prostate Adenocarcinoma epithelium resulted in the formation of organized glands and loss of tumorigenicity (35). We have also seen that rUGM decreases the invasive properties of the tumorigenic BPH1 *Caldel* cells (unpublished...
with BHPrS1 of the chemokine in grafts composed of SDF1α significantly larger than the controls. Note the invasive characteristic of the tumors (arrows) C. Histological examination revealed malignant transformation of prostate cancer showed around 50% to 75% of phospho-Smad2 staining. Immunohistochemical analysis of human CAF cells, somewhat over 50% of cells showed phospho-Smad2 staining. Changes in epithelial phenotype were accompanied with increased proportions of TGF-β responsive stromal cells, as determined by the surrogate reporter of Smad2 phosphorylation, observed adjacent to areas of malignant transformation. When BPH1 cells were recombined with BHPrS1α-expressing compared with TGF-β1-expressing fibroblasts (x–ix). Intense SDF1α staining corroborated the secretion of the chemokine in grafts composed of SDF1α-expressing compared with TGF-β1-expressing fibroblasts (x–xii).

The presence of 25% CAF cells in the stroma (when mixed with 75% UGM) was sufficient to induce small changes in the overall phenotype of epithelial cells (Fig. 1). These changes were more obvious when half of the population was composed of the tumor promoting CAF stromal cells, suggesting that the restrictive nature of the normal stroma (a feature that prevent tumor progression) is a function of the overall balance of the stromal tissue. The identification of the factor(s) or the type and nature of cells involved in this restriction could be beneficial for targeting the stroma therapeutically.

Changes in epithelial phenotype were accompanied with increased proportions of TGF-β responsive stromal cells, as determined by the surrogate reporter of Smad2 phosphorylation, observed adjacent to areas of malignant transformation. When BPH1 cells were recombined with CAF cells, somewhat over 50% of cells showed phospho-Smad2 staining. Immunohistochemical analysis of human prostate cancer showed around 50% to 75% of phospho-Smad2 positive stromal cells. We have previously shown that in prostate cancer the level of stromal TGFβ2 decreases with increasing tumor grade (15). This disparity could be because of the fact that Smad2 can serve as a substrate and be phosphorylated not only by TGF-β action, but also by other TGF-β superfamily members including the activin, nodal, BMP, and GDF (36, 37).

Heterogeneity in TGF-β signaling was maintained in isolated CAF cells compared with normal prostate fibroblasts in which almost all cells respond to TGF-β stimulation. More than 90% of the BHPs1 cells demonstrated by αSMA staining in response to TGF-β and co-localization of phospho-Smad2/αSMA in normal fibroblasts was almost universal. In contrast, only half of the CAF cells showed clear TGF-β responses. Similar heterogeneity in αSMA was previously observed in a mouse model of pancreatic and breast cancer (10).

Collectively, these observations demonstrate the heterogeneous nature of TGF-β signaling in the tumor microenvironment. To examine the consequences of a heterogeneous population for tumor promotion, we impaired TGF-β
signaling in 50% of normal prostate stromal cells. This change in the stromal composition resulted in malignant transformation of initiated epithelial cells in a minor but sufficient degree to represent some of the tumor-inductive properties CAF. PCR array was carried out in normal fibroblasts and compared with those lacking TGF-β responsiveness and a mix of the two populations to screen for molecular changes that might contribute to the CAF phenotype. Our results showed significant alterations in the expression of genes associated with the transition of normal fibroblasts to CAF. Several genes involved in development, cell differentiation, and angiogenesis were altered including TGF-β1, which has been shown in several studies to be involved in prostate cancer progression (38). Interestingly, abrogation of TGF-β signaling in fibroblasts resulted in the increase of TGF-β ligand secretion. Thus, a higher BHPrS1^INT/N/RII/BHPrS1^EV ratio caused more TGF-β1 secretion to support the conversion to a myofibroblast phenotype.

Overexpression of the chemokine SDF1α/CXCL12 can support proliferation and invasion of the prostate cancer cell line, PC3 (39). We have previously shown crosstalk between TGF-α and CXCR4, the receptor for SDF1α, which is secreted by CAF in carcinogenesis (7). We found that overexpression of TGF-β1 by normal fibroblasts was sufficient to induce neoplastic changes. Similar observations were described with fibroblasts isolated from reduction mammoplasty (40). Interestingly, SDF1α alone did not induce a robust growth of BPH1 cells, as compared with TGF-β1, but had a dramatic effect on the invasiveness of the cells into the kidney parenchyma. One possible explanation is the absence of complete activation of fibroblasts toward a CAF phenotype, as previously reported (39). It is possible that the high amount of TGF-β produced by these fibroblasts is sufficient to result in the basal alterations in the stroma required for tumor promotion, such us conversion to the myofibroblast phenotype and increased secretion of angiogenic factors (two features commonly found in cancer; ref. 41). Once such a conversion occurs, SDF1α might support progression and invasion at a later stage. These observations indicate that an altered heterogeneous stromal environment can promote human prostate cancer formation by changes in the repertoire of secreted factors.

In this study, we demonstrated that heterogeneity of human prostate stroma may be an important contributor to stromally induced carcinogenesis. Abrogation of TGF-β signaling in a subpopulation of prostate stromal cells is consistent with the previously demonstrated loss of stromal TGFβR2 in prostate cancer (22). While the biological situation is unlikely to be this simple, this model represents a step toward modeling stromal complexity. Further exploration of the intrinsic mechanisms responsible for the conversion to the CAF phenotype, and the epithelial responses to such a phenotype, are necessary for the developing of effective cancer therapies that can target both the epithelial and stromal compartments.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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