Cross-talk between Paracrine-Acting Cytokine and Chemokine Pathways Promotes Malignancy in Benign Human Prostatic Epithelium

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Abstract
The present study explores the mechanisms by which human prostatic carcinoma-associated fibroblasts (CAF) induce tumorigenesis in initiated but nonmalignant human prostatic epithelial cells (BPH-1). CAF express elevated levels of both transforming growth factor-β1 (TGF-β1) and stromal cell-derived factor-1 (SDF-1/CXCL12). TGF-β inhibits the growth of BPH-1 cells in vitro, but was found to be necessary for the tumorigenic response to CAF. This counterintuitive result suggested that the TGF-β signaling system was involved in other processes relating to tumorigenesis. The SDF-1 receptor, CXCR4, is expressed at low levels in benign prostate tissue and in BPH-1 cells in culture. However, CXCR4 levels increase during prostate cancer progression. CXCR4 was found to be induced and localized to the cell membrane in BPH1 cells by CAF-conditioned medium and by CAF cells in tissue recombinants. TGF-β was both necessary and sufficient to allow the detection of membrane-localized CXCR4 in BPH1 cells. Suppression of epithelial cell CXCR4 expression abrogated the tumorigenic response to CAF. SDF-1, secreted by CAF, acts via the TGF-β-regulated CXCR4 to activate Akt in the epithelial cells. This mechanism elicits tumorigenesis and obviates the growth-inhibitory effects of TGF-β. Thus, tumor stroma can contribute to carcinogenesis through synergism between TGF-β, SDF-1, and CXCR4. These experiments suggest mechanisms by which TGF-β can shift its role from an inhibitor to a promoter of proliferation during tumor progression. Both the TGF-β and SDF-1 pathways are targets of drug discovery efforts; these data suggest potential benefits in the cotargeting of these pathways. [Cancer Res 2007;67(9):4244–53]

Introduction
Because the bulk of human tumors are carcinomas, much attention has been paid to the epithelial cell type. However, inflammation and interactions with the surrounding stromal microenvironment are critical for cancer initiation and progression (1–3). Stromal alterations during tumorigenesis have been shown to be involved in tumors, including breast, colon, lung, and prostate (4). Compared with normal stroma, the tumor microenvironment often exhibits an increased number of fibroblasts, enhanced capillary density, and deposition of type I collagen and fibrin.

The fibroblasts within the tumor stroma commonly have a myofibroblastic phenotype similar to the granulation tissue seen in wound healing (5, 6) and have been designated as activated or carcinoma-associated fibroblasts (CAF; refs. 7, 8). The presence of a reactive stroma is most clearly evident, and generally best described, in tumors such as breast cancer, where myofibroblastic cells are not commonly seen in the normal stroma. Under these circumstances, the initiation of smooth muscle actin expression is obvious. In comparison, in an organ such as the prostate, the normal stroma is fibromuscular, and therefore, comparable changes are inherently less apparent. Despite these limitations, human prostatic stroma has been shown to be activated in response to cancer progression, resulting in the formation of a reactive stroma the presence and nature of which has a prognostic value (9–11).

Fibroblasts are recognized as prominent modifiers of cancer progression (12, 13). Using tissue recombination and renal capsule xenografting, Cunha’s group showed that human prostatic CAF induced tumor formation from initiated but nontumorigenic human prostatic epithelial cells, whereas normal prostatic fibroblasts (NPF) did not, although the mechanisms involved were unclear (7, 14).

Transforming growth factor-β (TGF-β) has been suggested to play an important role in the generation of the reactive stroma (9, 10). In prostatic CAF, TGF-β secretion is elevated compared with NPF (15). In an orthotopic xenograft model to reconstruct human mammary gland, Kupferwasser et al. (16) found that overexpressing TGF-β in mouse fibroblasts could induce the initiation of breast cancer from the normal human epithelium. A conditional knock-out mouse, in which fibroblasts from the same carcinoma in the gastrointestinal tract (17). Additionally, in a mammary tissue recombination model, fibroblasts from the same TGF-β knock-out mice expressed elevated stromal hepatocyte growth factor (HGF), macrophage-stimulating protein (MSP), and TGF-α resulted in the activation of MET, RON, ERBB1, and ERBB2 in the adjacent epithelium (18).

In normal adult tissues, TGF-β is generally seen as a tumor suppressor, inhibiting cell growth and maintaining differentiation. However, as cancer progresses, TGF-β takes on a tumorigenic role, inducing angiogenesis, suppressing apoptotic activity, or enhancing cell motility (19–22). Target cells respond to TGF-β through a number of specific downstream signaling pathways. The overall nature of the response is determined by cellular integration incorporating data from different pathways, the cell context, and
also the adjacent microenvironment (23). Our previous results showed that TGF-β may stimulate invasion via the induction of epithelial to mesenchymal transformation (EMT) in tumorigenic prostatic epithelial cells via phosphoinositide-3-kinase/Akt signaling (22). Collectively, these reports suggest that TGF-β plays an important bidirectional role in the communication between the epithelia and the stroma.

The present study aimed to investigate the mechanisms by which CAF induce tumorgenesis. To approach this question, we studied stromal-epithelial interactions in vivo and in vitro using a model of prostate cancer progression. The data show that TGF-β produced by CAF can induce tumorgenesis by modifying the response of adjacent epithelial cells to other aspects of the tumor microenvironment, specifically to elevated levels of stromal cell–derived factor-1 (SDF-1). This work provides a model for prostate cancer progression that fits well with previous observations of clinical samples and gives a mechanistic explanation for the switch from growth-inhibitory to growth-promoting effects of TGF-β, which are commonly associated with carcinogenesis.

Materials and Methods

CAF and NPF cell generation. Human prostatic CAF and NPF were prepared as previously described (7). For this study, CAFs and NPFs from three separate patients were used to ensure consistency. Following preparation, cells were qualified using a tissue recombination bioassay (7) to confirm that the CAF cells elicit tumorgenesis, whereas the NPF cells do not. Aliquots of CAF and NPF were frozen in liquid nitrogen. Cells were used at low passage (less than 10).

Cell proliferation assay in cell culture. A total of 3,000 cells per well were seeded in 24-well plates in serum-free RPMI 1640 (Life Technologies). After attachment and growth overnight, the culture medium was replaced, and the cells were treated with reagents diluted in conditioned medium (CM) collected from CAF or NPF cells or in serum-free RPMI. About 10 μg/mL TGF-β (1,2,3) blocking Ab (Clone 2G7), 5 ng/mL TGF-β1 ligand (R&D Systems, Inc.), and 100 ng/mL SDF-1 (Peprotech, Inc.) were added as indicated in the figure legends.

Immunofluorescence staining. Paraffin-embedded tissue samples were cut in 5-μm sections and then placed on glass slides. After deparaffinization with Histoclear (National Diagnostics), the antigen was unmasked by heating samples in unmasking solution (Vector Laboratories, Inc.), and the reaction was quenched using 0.3% hydrogen peroxide and blocked with Clean Vision (ImmuVision Technologies). Samples were then incubated with primary antibodies against human CXCR4 (R&D Systems), SV40 Tag (Santa Cruz Biotechnology), p-Smad2 (Cell Signaling Technology), or Ku70 (Abcam). After washing in PBS, the slides were incubated in FITC-conjugated anti-mouse secondary antibodies (Sigma) or biotinylated anti-rabbit secondary antibody (Dako) for 1 h. After extensive washing, the slides were mounted with Vectashield mounting medium with propidium iodide (Vector) or 4′,6-diamidino-2-phenylindole (DAPI). For phosphorylated Smad2 staining, the slides were incubated in ABC solution (Vector) to amplify the signals before visualizing with 3,5-diaminobenzidine (DAB). Hoechst 33258 staining was done and visualized as previously described.

To visualize CXCR4, cells were cultured serum-free with and without TGF-β1 (5 ng/mL). Cells were fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.2% Triton for 5 min at room temperature, and blocked in 10% normal donkey serum. CXCR4 expression was probed with mouse monoclonal antibody (MAB711, Clone 44708, R&D Systems) and visualized with Cy2 donkey anti-mouse secondary antibody (715-225-150, Jackson Immunoresearch). Confocal images were acquired with an LSM 510 inverted confocal microscope at an optical slice thickness of 0.5 μm.

Conditioned medium. CAF or NPF were seeded with 5% FCS in RPMI 1640 at a density of 500,000 per 75-cm² flask, allowed to grow, and attached overnight. Medium was then replaced with serum-free RPMI. The cells were incubated at 37°C for a further 24 h. The medium was collected, centrifuged, passed through a 0.45-μm filter (Millipore), and stored at −80°C for later use.

ELISA for TGF-β1 and SDF-1. About 800,000 CAF or NPF were plated in 75-cm² flasks, and after overnight attachment and growth, the cells were washed thrice with PBS and fed with serum-free RPMI 1640. CM was collected after 48 h, centrifuged at 13,000 rpm for 15 min to pellet debris, and stored at −80°C. TGF-β1 and SDF-1 were assessed according to the manufacturer’s protocol (human TGF-β1/SDF-1 Quantikine [R&D Systems, Inc.]), and each experiment was done in triplicate.

Wound-healing assay. Cells were seeded in six-well plates (Falcon) in RPMI 1640 with 5% FCS. After overnight attachment and growth, cells were starved in serum-free RPMI medium for 24 h before incubation with CM from CAF or NPF and, as appropriate, with or without 5 ng/mL TGF-β1, 1.5 μg/mL TGF-β–neutralizing antibody 2G7 or 20 μg/mL CXCR4 neutralizing antibody (clone 12G5, R&D Systems). When they attained a confluent monolayer, the cell sheets were wounded with a plastic pipette tip. This generated a gap in the monolayer, and the ability of cells to migrate into the cleared section was observed and recorded photographically at different times at fixed points in the wound (24). All the experiments were done in triplicate.

Western blotting. Cells were detached by trypsinization after washing with cold PBS, lysed with TNT buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% NP40, (pH, 7.4)) containing proteinase inhibitor (Roche) and phosphatase inhibitor I and II (Sigma). The whole cell lysates were clarified at 13,000 rpm for 20 min at 4°C, the total protein levels were quantified, and lysates were stored for use. About 20 μg proteins per well were loaded and electrophoresed through 10% NuPAGE Bis-Tris gel (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1 h in PBS-T (PBS, 0.1% Tween 20) containing 5% nonfat dry milk and incubated with primary antibody in PBS-T containing 4% nonfat dry milk overnight at 4°C. Washed 30 min in PBS-T, followed by the horseradish peroxidase-conjugated secondary antibody (1:1,000; Amersham-GE Healthcare) diluted in PBS-T containing 2.5% nonfat dry milk for 1 h at room temperature. Amersham ECL plus detection reagent (GE Healthcare) was used to visualize protein bands. Antibodies against CXCR4 (1 ng/mL; R&D Systems, clone 47088), P-Akt (1:500; Cell Signaling) were used. β-actin (1:5000; Sigma) was used for loading control.

Reverse transcription-PCR. RNA was isolated using the RNeasy kit (Qiagen) and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). Signal was amplified to detect CXCR4 using primer sets and conditions as previously described (25).

Flow cytometry for CXCR4. Cells were harvested and resuspended in L-15 medium (Sigma) at a concentration of 2 × 10⁴/mL, and a 50-μL cell suspension was incubated with 50 μL monoclonal mouse anti-human CXCR4 (R&D Systems, Inc.; 10 μg/mL dilution in L-15) for 1 h on ice. After washing thrice, the cells were incubated with allophycocyanin (APC)-labeled anti-mouse secondary antibody (BD PharMingen) for 30 min on ice. Washed thrice further, and analyzed using flow cytometry at the Vanderbilt-Ingram Cancer Center Flow Cytometry and Cell Sorting Core.

Retroviral and lentiviral infections. The BPH-1-DN cells were generated essentially as described in ref. (22) using the dominant negative TβRII (26, 27). Viral supernatant was generated, centrifuged at 3,000 rpm for 5 min, and passed through a 0.45-μm filter. Polybrene (Sigma) was added to the viral suspension at 5 μg/mL and used to infect target cells. Successive rounds of infection over 5 days were employed. The infected cells were selected by fluorescence-activated cell sorting (FACS). Following selection, the activity of the dominant negative receptor construct was confirmed by using a 3TP-luciferase reporter (28). The empty vector construct was used to generate a negative control.

The shRNA-CXCR4 or shRNA-Auxin/like proteinaceous fluorescent protein (GFP) control plasmids (kindly provided by Dr. Robert Weinberg, Massachusetts Institute of Technology, Cambridge, MA) were engineered into BPH-1 cells by lentivirus infection as described in ref. (29). Activity of the construct was confirmed using Western blot analysis, which consistently showed a 73% to 76% reduction in CXCR4 expression compared with the levels seen in shGFP control cells.
Tissue recombinants and kidney capsule xenograft. All work involving animals was done under protocols reviewed and approved by the Vanderbilt Institutional Animal Care and Use Committee. Rat urogenital sinus mesenchyme (rUGM) was prepared from 18-day embryonic fetuses of pregnant Sprague-Dawley rats (Harlan) as previously described (30). UGM was then additionally reduced to single cells by a 90-min digestion at 37°C with 187 units/mL collagenase (Life Technologies). After digestion, the cells were washed extensively with RPMI 1640. Viable cells were then counted using a hemacytometer.

To prepare tissue recombinants, rUGM or CAF cells were mixed with BPH-1 cells at a ratio of 250,000 stromal cells to 100,000 BPH-1 cells in a sterile microcentrifuge tube in RPMI 1640. The cell mixture was pelleted and resuspended in 50 μL of rat-tail collagen (pretitrated to pH, 7.4). After polymerization, the collagen was overlaid with growth medium. After incubation at 37°C overnight, the tissue recombinant was grafted under the renal capsule of intact male CB17cr/Hsd-severe combined immune deficient (SCID) mice (Harlan). Hosts were sacrificed either 6 weeks (2G7 blocking antibody studies) or 8 weeks (all other studies) postgrafting. The kidneys were removed and imaged before processing for histology. The graft dimensions were measured, and tumor volume was calculated by the ellipsoidal formula: volume = width^2 x length x 0.52. Note that this formula underestimates the volume of invasive tumors, and thus, we tend to underestimate the significance of size differences between small noninvasive and larger invasive tumors.

Treatment of the host mice with TGF-β neutralizing Antibody (2G7). Host SCID mice were i.p. injected with mouse monoclonal 2G7 (300 μg per mouse) in a 0.2-ml volume via a 26-gauge needle (31), starting 1 day before xenografting surgery and subsequently injected twice weekly at the same dose until sacrifice.

Results

TGF-β signaling is a critical component in the induction of a tumorigenic response to carcinoma-associated fibroblasts. Previous studies have shown that human prostatic CAF can elicit malignant changes in initiated but nontumorigenic human prostatic epithelium (7, 14). To investigate the mechanism underlying this process, we compared the effects of CAF and NPF populations on target epithelia in vitro. Initial studies (data not shown) showed that cells grown in NPF CM proliferated faster than cells grown in serum-free medium, and that proliferation was further increased in CAF CM.

In wound-healing assays, BPH-1 cells were grown in CM from either NPF or CAF until reaching confluence, the monolayers were then wounded with a pipette tip, and the rate of wound closure was recorded. In this case, wound closure was significantly faster in CAF CM than in NPF CM (Fig. 1A).

We examined the effects of abrogation of TGF-β signaling in these epithelial cells on wound healing. Cells expressing a dominant negative TGF-β (DNTβRII) ligand blocking antibody (2G7). In both cases, the overall tumor

To determine whether the observed effects of TGF-β on proliferation and motility in vitro could contribute to tumorigenesis in vivo, we used tissue recombination approaches. Consistent with our experience over many years, the rate for all grafts was extremely high in both test and control groups [overall 97.5% (78/80) in this study].

First, as shown in Fig. 1A, BPH-1 cells were infected using retroviruses containing either DNTβRII or control empty vector (EV). Following selection of cells expressing stably integrated constructs, tissue recombinants were generated with CAF cells. In a complementary experiment, shown in Fig. 2B, BPH-1 + CAF recombinants were grafted into mice preloaded with a TGF-β ligand blocking antibody (2G7). In both cases, the overall tumor

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**Figure 1.** TGF-β enhances wound healing by BPH-1 cells in vitro. A, BPH-1 cells grown in CAF CM for 3 d show enhanced migratory activity in a wound-healing assay as compared with cells grown in NPF CM (P < 0.01). Expression of a dominant negative TGF-β receptor in the epithelial cells (BPH1-DN) significantly reduces migration in CAF CM (P < 0.01), suggesting that TGF-β plays a role in this process. Columns, mean of all experiments done in triplicate, with significance determined using Student’s t test; bars, SD. B, to further examine the effects of enhancing or blocking TGF-β signaling on the ability of BPH-1 cells to migrate in a wound-healing assay, the wound-healing closure rates were measured in the presence or absence of either the TGF-β blocking antibody 2G7 or an excess of TGF-β ligand. Consistent with the data shown in (A), the blocking or enhancing TGF-β did not affect wound closure in NPF CM. However, in CAF CM, whereas additional TGF-β did not enhance the wound closure rate, blocking its action reduced the rate significantly to levels similar to those seen in NPF. Columns, mean of all experiments done in triplicate, with significance determined using Student’s t test; bars, SD.
volume was significantly reduced, and invasive malignant histology ablated in situations where TGF-β signaling was suppressed (DN and 2G7). This contrasted sharply to EV and untreated controls, where (consistent with previous studies) rapidly growing tumors were produced. H&E staining showed that the control tumors were composed of poorly differentiated, irregular epithelia. In some areas, epithelium formed small glandular nests, whereas in other areas, epithelium appeared as single cells that were intermingled within fibrous stroma. Ku70 (which, in our hands, is human specific) and Hoechst33258 staining confirmed that the majority of the stromal cells in these tumors were of human origin, although occasional host-derived stromal cells could also be seen (data not shown). Most epithelia contained large, pleomorphic nuclei with large nucleoli. These data were consistent with the in vitro observations and showed that TGF-β signaling is necessary for CAF-induced tumorigenesis in this model.

We have previously shown that TGF-β acts upon BPH-1 cells to inhibit proliferation, but does not otherwise alter the motility or invasive potential of these cells (22, 32); however, the data presented here suggest a role for TGF-β signaling in tumorigenesis. This is consistent with previous observations of the role of this pleiotropic growth factor, which is known to play both tumor-suppressive and tumor-promoting roles at different disease stages (33). Our data clearly suggest that additional TGF-β-regulated pathways play a role in this model of tumorigenesis.

Both CAF CM and TGF-β can affect epithelial CXCR4. A preliminary analysis of pathways potentially regulated by TGF-β involving paracrine-acting factors thought to be up-regulated in CAF versus normal stroma suggested SDF-1 as a candidate for investigation. SDF-1 signals through the G-protein coupled receptor CXCR4. Induction of CXCR4 in prostatic epithelial cells has been correlated with malignant progression in the prostate, with little or
no expression in benign epithelium and elevated levels seen in cancer and particularly in bony metastases (34–36). TGF-β has been shown to play a role in the regulation of CXCR4 in macrophages and T cells (37, 38). SDF-1 is elevated in human breast cancer stroma where signaling through this pathway plays a role in tumor growth (8).

To determine whether a soluble factor could be involved in inducing expression or changing localization of CXCR4, we grew BPH-1 cells in CAF CM for 72 h. FACS analysis (which detects protein at the cell surface) showed an approximately 5-fold increase in CXCR4 under these conditions (Fig. 3A).

Given our suspicion that TGF-β might be sufficient to regulate CXCR4 action, we examined the expression of CXCR4 on BPH-1 cells grown in serum-free medium containing 5 ng/ml TGF-β for 0, 24, 48, and 72 h. This resulted in a time-dependent elevation in CXCR4 at the cell surface, as detected by FACS analysis, which was similar to that seen in CAF CM (data not shown). Following 72 h of TGF-β treatment, the percentage of cells expressing an empirically determined high level of CXCR4 on their surface membrane (as determined by FACS analysis) increased from <0.5% to nearly 14% (Fig. 3B). However, further analysis determined that CXCR4 mRNA

Figure 3. Microenvironmental signals can alter CXCR4 expression. A, CAF CM elicits up-regulation of CXCR4 detection in BPH-1 cells in vitro. FACS analysis shows increased detection in CXCR4 at the surface of BPH-1 cells following 72 h of exposure to CM from three different CAF populations. B, quantitation of the increase in CXCR4 detection following 72 h of TGF-β treatment showed that the (empirically determined) population of high CXCR4-expressing cells increased approximately 27-fold over controls during treatment. C, immunofluorescent staining of CXCR4 expression in BPH-1 cells cultured either in serum-free medium with or without 5 ng/mL TGF-β1. In the untreated cells, there is a diffuse low level of CXCR4 detectable throughout the cytoplasm. In the presence of TGF-β1, expression becomes more intense and localizes to the cell membrane. D, Western blot analysis of CXCR4 expression in BPH-1 cells cultured in either CAF CM or NPF CM in the presence or absence of the TGF-β1-blocking antibody 2G7. Left, raw data; right, quantitation. Overall levels of the protein were found to be highest in the CAF CM and were reduced by approximately one-third by 2G7 to levels similar to those seen in NPF CM. CXCR4 levels in cells grown in NPF CM were also reduced further by 2G7.
was detectable by reverse transcription-PCR analysis (data not shown), and protein was seen to be present by Western blot analysis.

Immunofluorescence analysis of BPH-1 cells in serum-free culture revealed a low-level diffuse expression of CXCR4 (Fig. 3C). This was consistent with our flow cytometry observations (which measure only receptor on the cell surface; Fig. 3B, control). Cells in serum-free culture in the presence of TGF-β had a slightly smaller area than untreated cells and showed concentration of stain along the cell membrane (Fig. 3C). This result was in agreement with the FACS analysis, which showed increased levels of the protein detected at the cell surface.

To examine whether TGF-β alters overall expression of CXCR4 mRNA and protein, we used RT-PCR and Western blot analysis. These techniques showed that CXCR4 mRNA (data not shown) and protein could be detected when BPH-1 cells were grown in the either CAF or NPF CM (Fig. 3D). Culture of the epithelial cells in CAF CM resulted in elevations of the total protein levels as compared with culture in NPF CM, and further culture for 72 h in the presence of the TGF-β blocking antibody 2G7 decreased the detectible amount of CXCR4 protein in either CM (Fig. 3D). Taken together, these data suggest that whereas TGF-β can exert some short-term effects on the quantity of CXCR4, it can also play a role in regulating the localization of the protein and, thus, its biological availability.

To examine whether CAF induce CXCR4 in vivo, we examined tissue recombinants prepared using BPH-1 cells recombined with CAF (malignant development) compared with either rUGM or NPF, which induce benign prostatic cords reminiscent of developing human prostate (7, 39). Immunofluorescence staining revealed minimal expression of CXCR4 in epithelial cells of the benign recombinants (Fig. 4A), in marked contrast to the widespread expression seen in the malignant recombinants involving CAF (Fig. 4B). These observations are consistent with published data relating to human clinical samples (36).

**TGF-β is necessary for continued epithelial CXCR4 expression.** The *in vitro* experiments described above show that whereas CXCR4 levels are decreased in the short-term absence of TGF-β, the protein does not disappear. To examine whether the elevated CXCR4 levels seen in recombinants with CAF is related to TGF-β
consistent with the epithelial cells was suppressed, although CXCR4 expression was presence of 2G7, the immunodetection of CXCR4 in the human circumstances, TGF-β antagonists from the 2G7-treated hosts described above. Under these action, we did immunofluorescence staining in tissue recombinants from different patients. This is consistent with previous studies in clinical samples showing that CXCR4 protein expression is significantly elevated in localized and metastatic prostate cancers, breast cancer, and thyroid cancer (36, 40, 41).

Phosphorylation of Akt is a downstream consequence of the activation of a number of chemokine and cytokine receptors, including TGF-β receptors and CXCR4. Uncontrolled activation of this pathway is linked to malignant changes. Specifically, in this model, as well as in others, elevated P-Akt has been linked to EMT and increased cellular invasion and to suppression of nuclear localization of Smad3 and p21, potentially allowing cells to acquire an ability to avoid the growth-inhibitory effects of TGF-β (22). CAF express a number of growth factors that can activate Akt. To determine whether SDF-1 signaling through CXCR4 contributes to Akt activation, we used an antibody (12G5) to the external domain of CXCR4 to block SDF-1 signaling. Following exposure to CAF CM, there was a steady increase over a 72-h period in the level of Akt caused by the CM. Blocking of CXCR4 activity showed a reduction in Akt activation (Fig. 5B). This is consistent with both immediate effects resulting from the activation of receptors by ligands present in the CM and by the activation of receptors, which are expressed and activated as a result of the CM. Blocking of CXCR4 activity showed that by 72 h, this receptor contributed ~48% of the total activation of Akt caused by the CM. Wound-healing assays showed that for cells grown in CAF CM, there was a significant reduction in wound closure rate in the presence of the 12G5 blocking antibody, supporting the idea that signaling through CXCR4 plays a role in...
this process. This supports the contention that TGF-β in CM can contribute to the activation of protumorigenic pathways (specifically by allowing CXCR4 activity), which can then contribute to suppression of the growth-inhibitory activities of TGF-β itself.

**SDF-1/CXCR4 signaling is required for CAF-induced tumorigenesis in vivo.** To examine SDF-1/CXCR4 in CAF-induced tumorigenesis, we used retroviral transduction of a shRNA construct (8) to suppress CXCR4 expression in BPH-1 cells. Western blot analysis showed that cells expressing the shRNA construct expressed ~25% of the CXCR4 seen in controls, FACS studies confirmed that these cells did not express detectable levels of membrane-localized CXCR4 following stimulation with either CAFCM or TGF-β (data not shown). The shRNA-expressing BPH-1 cells and control cells expressing a shRNA designed to target GFP were combined with CAFs and grafted to SCID mice. Two months after xenografting, the siCXCR4 BPH-1 cells formed significantly smaller grafts than controls (Fig. 5A). Histologic staining (Fig. 5D) revealed that the control grafts formed the typical adenosquamous tumors previously described. In marked contrast, there was no tumorigenic response in the siCXCR4-expressing cells. These data show that CXCR4 expression is required for CAF-induced tumorigenesis in this model.

**Discussion**

The data presented here provide important insights into the mechanisms by which paracrine signaling can promote tumorigenesis supporting the idea of TGF-β as a master regulator of intercellular communication. Cross-talk between cytokine and chemokine pathways can elicit stromally induced tumorigenic responses in genetically initiated epithelial cells. One important point is that the changes in protein concentrations and localization, which elicit these profound effects, are not massive. The combination of small alterations in a number of related pathways rather than a massive overexpression or loss of a single factor is important. The data also illuminate the change from tumor-suppressive to tumor-promoting actions of TGF-β.

A number of chemokines and cytokines have been proposed to play a role in the microenvironmental promotion of tumorigenesis. Loss of TGF-β receptor function in stromal cells has been shown to elicit malignant changes in some epithelial tissues (17), suggesting that stroma may contribute to, or suppress, tumorigenesis through paracrine interactions. We have shown that human prostatic CAF can induce initiated but nontumorigenic human prostatic epithelial cells to form tumors (7), and that epithelial cells can be permanently transformed by this process (42).

TGF-β has complex and often apparently contradictory effects depending on dose and context. In many normal tissues, TGF-β inhibits proliferation and enhances differentiation (43). In the prostate, TGF-β inhibits the growth of many nontumorigenic prostatic epithelial cell lines and is implicated in the differentiation of prostatic smooth muscle, a key component of prostatic development (22, 44). TGF-β also plays a key role in tumorigenesis where signaling, especially through non-Smad pathways, has been implicated in disease progression (3, 45, 46). We have recently shown that, following transformation caused by exposure to CAF, human prostatic epithelial cells can acquire an ability to ignore the growth-inhibitory effects of TGF-β as a result of elevated activated Akt, which inhibits nuclear translocation of p21 and Smad3 (22). Other published data support the proposal that interactions between Akt and Smad3 vary depending on the relative titers of these proteins. These interactions may be important in avoiding growth inhibition by TGF-β either as a result of changes in Smad3 localization, degradation, or by the inhibition of Smad3 phosphorylation by mTOR-dependent mechanisms (47–49). Given the pleiotropic nature of TGF-β signaling, it is possible, and indeed likely, that the variations noted between these studies reflect the specific details of the various models used.

Our initial findings of elevated expression of TGF-β by CAF were consistent with observations by others working in this field (15) and were of interest because of studies indicating the possible role of TGF-β in enhancing tumor progression (22). The in vivo and in vitro studies described here showed that TGF-β contributes to oncogenic effects on BPH-1 cells. TGF-β was shown to be necessary for CAF-induced tumor formation by BPH-1 cells. However, TGF-β per se is not tumorigenic to these cells. Previous studies showed that TGF-β acts upon BPH-1 cells to inhibit proliferation (22, 32). These observations set up a familiar apparent paradox mirroring that seen in a number of human tumors, where TGF-β is growth inhibitory for normal cells and yet promotes tumor progression (33).

SDF-1 is overexpressed in human breast (8) and prostate CAF (Fig. 5A). SDF-1 can contribute to both tumor growth and angiogenesis (8). We show here that TGF-β or CAF CM in vitro and CAF cells in vivo were able to increase expression and membrane localization of the SDF-1 receptor, CXCR4, by the epithelial cells. TGF-β was shown to be both necessary and sufficient for receptor expression in the nontumorigenic epithelial cells.

Elevation of CXCR4 expression is observed in human prostate cancer progression, with little expression in benign tissue and elevated expression seen in tumors (36). Elevated CXCR4 is also seen in other human tumors and is linked to increased tumor growth and poor prognosis. In this study, we show that SDF-1/CXCR4 signaling is required for CAF-induced tumorigenesis of BPH-1 cells. This work extends previous observations by establishing links between the TGF-β and SDF-1 pathways. The data presented here provide a mechanism by which epithelial cells can use elevated levels of TGF-β found in tumor stromal cells as a triggering mechanism to respond to the effects of elevated stromal levels of SDF-1 found in CAF. As tumorigenesis progresses, tumor epithelial cells tend to express increased levels of TGF-β. Indeed, TGF-β secretion is elevated between 4- and 20-fold in the tumorigenic lines derived from BPH1 as compared with the parental cells. Such tumor cell–derived TGF-β could further contribute, by an autocrine route, to the total exposure of tumor cells from their microenvironment and, thus, enhance tumor promotion. In the model used here, epithelial cell–derived TGF-β would seem to be insufficient to trigger early malignant changes, but could certainly contribute to the overall growth and later progression of tumors.

Although BPH-1 cells show growth inhibition upon TGF-β treatment, the tumorigenic derivative BPH-1CAPTD lines avoid such inhibition via the effects of elevated levels of constitutively active Akt expression, which blocks nuclear translocation of Smad3 and p21 (22). We show here that during malignant transformation, SDF-1 signaling contributes significantly to the elevation of phosphorylated Akt in the target epithelial cells. These observations (summarized in Fig. 6) show a potential feedback loop

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between the TGF-β and SDF-1 pathways. Elevated stromal TGF-β elicits epithelial CXCR4 expression, allowing stromal SDF-1 to activate Akt in the epithelial cells. P-Akt can then contribute to a loss of the growth-inhibitory response to TGF-β. Cells act as signal integrating centers, and this combination of a cytokine and a chemokine acting in concert might be sufficient to push benign but genetically initiated epithelial cells into a cycle of uncontrollable proliferation which would predispose them to irreversible transformation. Such transformed cells would likely express elevated levels of TGF-β, which could further contribute to the malignant process.

In summary, these studies show that relatively small changes in specific molecular signals can alter the manner in which cells see and respond to their microenvironment. In this study, we highlight links between the TGF-β and CXCL12/SDF-1 pathways and show that both of these pathways are linked paracrine arbiters of tumorigenesis in CAF-driven tumorigenesis in vivo. Efforts are currently under way by a number of groups to target both TGF-β and SDF-1 signaling using small molecules, and the data presented here suggest the potential for synergistic links between these approaches.

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3. Bierie B, Moses HL. Tumour microenvironment: integrating centers, and this combination of a cytokine and a chemokine acting in concert might be sufficient to push benign but genetically initiated epithelial cells into a cycle of uncontrollable proliferation which would predispose them to irreversible transformation. Such transformed cells would likely express elevated levels of TGF-β, which could further contribute to the malignant process.

Figure 6. Interactions of TGF-β and SDF-1 pathways in tumorigenesis and tumor progression. This stylized and simplified diagram brings together data from this and other studies and presents a model illustrating how the TGF-β and SDF-1/CXCR4 pathways might interact initially to contribute to tumor formation and to set up conditions that would predispose cells to further malignant progression. In particular, this model suggests a mechanism by which TGF-β could switch from a tumor-suppressive to a tumor-promoting role, via a large increase in Akt activation brought about through TGF-β-induced CXCR4 activation. Activated Akt also inhibits Smad3 activity, allowing the cells to sidestep growth-inhibitory responses to TGF-β while continuing to retain a response to its growth-promoting activity.

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Cross-talk between Paracrine-Acting Cytokine and Chemokine Pathways Promotes Malignancy in Benign Human Prostatic Epithelium

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