Materials and Methods

Plasmid Construction and Lentiviral Infection. A dominant negative TGFβ receptor II retroviral vector DNTßRII-LZRS-EGFP (15) and the empty vector construct were used to establish the stable cell lines BHPrS1$^{-DNTßRII}$ and BHPrS1^{-EV}, respectively (1). Viral supernatant was generated, centrifuged at 3000 rpm for 5 min and passed through a 0.45 μm filter before frozen at -80°C until used. Polybrene (Sigma-Aldrich, St Louis, MO, USA, H9268) was added to the viral suspension at 5μg/mL to increase the efficiency of the transduction. GFP-expressing cells were selected by fluorescence-activated cell sorting (FACS). Following selection, the activity of the dominant negative receptor construct was confirmed by luciferase assay using a 3TP-luc reporter. The active form of TGF-ß1 cloned into a pMSCVpuro-IRES-EGFP vector (a generous gift from Dr Anna Spagnoli, Vanderbilt University) was expressed in BHPrS1 cells to generate the BHPrS1$^{-TGFß1}$ cells (2). The pBABE-puro-SDF1α construct was purchased from the non-profit plasmid repository Addgene (Plasmid #12270 http://www.addgene.org) (3). Expression of the TGF-ß1 and SDF1α transgenes by fibroblasts in the culture media were assessed by ELISA. BHPrS1$^{-SDF1α}$ cells were selected with puromycin (Invitrogen) and BHPrS$^{-TGFß1}$ cells expressing GFP were isolated by FACS. The pBABE-hygro-TERT retroviral construct was obtained from Robert Weingberg.

Co-Culture and Conditioned Media experiments. Briefly, cells were seeded at a density of 750,000 cells in a 10 cm dish (200,000 BPH1 cells + 500,000 fibroblasts, or 500,000 fibroblasts alone), and allowed to grow and attach overnight. Cells were washed twice with PBS to remove excess serum, and medium was replaced with serum-free RPMI-1640. Cells were incubated for an additional 48 hrs before the conditioned medium (CM) was collected, centrifuged and passed through a 0.45μm filter (Millipore SLHVM33RS, Billerica, MA), and stored at -80°C for later use.

Cell proliferation assay. BPH1 cells were seeded at 3,000 per well in 96-well plates and cultured in RPMI-1640 with 5% CCS until attached. Medium was changed to serum-free RPMI-1640 for overnight incubation. CM from different co-culture conditions was added to the cells in triplicate and serum-free RPMI-1640 was added to the control wells. Cell growth was analyzed

at 0, 1, 3 and 5 days with CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) reagent (Promega G5421 Madison, Wisconsin) added 2 hr before taking the spectrophotometric reading according to the manufacturer's instructions.

Reverse Transcription (RT)-PCR and PCR Array. RNA was isolated using the RNeasy kit (Qiagen 74104) and reversed transcribed using Superscript-III Reverse Transcriptase (Invitrogen 18080-051). Primer sets and conditions are provided in Supplemental Table 1. PCR analysis was performed using the RT^2 profiler PCR Array (human growth factor PAHS-041, SA Biosciences Corp.) following manufacturer's instructions. RNA from each sample was applied to each PCR template, and the threshold cycle number for each of the 84 genes was normalized to four built-in housekeeping gene controls using the RT^2 Profiler PCR Array Data Analysis web-based software (http://www.sabiosciences.com/pcr/arrayanalysis.php, SA Biosciences Corp.). The normalized and averaged values were reported as fold changes over the control $BHPrS1^{-EV}$.

Western Blotting Antibodies against phospho-Smad2 (Ser465/467), Smad2, phospho-Smad3, phospho-Akt, pan-Akt and phospho-p44/p42 MAPK Erk1/2 were from Cell signaling, vimentin, αSMA and against ß-actin from Sigma (Sigma-Aldrich, USA), and CD90 from eBioscience.
Briefly, after the media was aspirated and washed with cold PBS, cells were lysed by scraping in cold RIPA buffer [50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate and 0.1% SDS] containing complete Mini Protease inhibitor cocktail tablet (Roche 04693124001) and Phosphatase Inhibitor Cocktail I and II (Sigma-Aldrich, P2850 and P5726). The whole cell lysates were sonicated on ice and clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Total protein concentration was quantitated by ECL, and lysates were stored at -80°C for later use. About 30 µg proteins per well were loaded and electrophoresed through 10% NuPAGE Bis-Tris gel (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. After blocking for 1 hr in PBS-T (PBS, 0.1% Tween 20) containing 5% non-fat dry milk at room temperature, membranes were incubated with primary antibody in PBS-T 5% milk overnight at 4°C. After 3 x 5 min washes, membranes were incubated with the horseradish peroxidase (HRP) conjugated secondary antibody (1:1000); Amersham-GE Healthcare) diluted in PBS-T containing 5% milk for 1 hr at room temperature. Amersham ECL plus detection reagent (Amersham GE Healthcare) was used to visualize protein bands. Densitometric

quantitation of western blot films was accomplished by scanning the original films and converting the tiff files to grayscale images. Images were inverted using Photoshop, and mean band intensities were measured using ImageJ (National Institutes of Health).

Cellular and Tissue Immunofluorescence. Cells were plated on sialized glass (Superfrost) slides and allowed to attach and grow overnight. After serum starvation for 24 hours, cells were treated with 5 ng/ml TGF-ß1 (R&D) for 24 hours. After fixation in methanol for 5 minutes at -20°C, samples were washed twice in PBS, blocked for 30 minutes with 5% goat serum (Vector Laboratories, Burlingame, CA), and incubated for 24 hour at 4°C with primary antibodies followed by washing for 30 minutes in PBS. Staining was visualized using AlexaFluor fluorescence-conjugated secondary antibodies (Invitrogen). Slides were visualized and imaged using a Zeiss upright microscope with attached Axiocam camera and proprietary software. Positive cells were quantitated by image analysis using NIH ImageJ software (http://rsb.info.nih.gov/ij/). For histologic analysis, 5 μm tissue sections were dewaxed, and the antigen was unmasked by heating samples in unmasking solution (Vector Laboratories). Slides were blocked in 5% goat serum in PBS for 30 minutes at room temperature before incubating with primary antibodies. After 1 hour washing in PBS buffer, slides were incubated with secondary antibodies (1:200; AlexaFlour 488 anti-mouse IgG1 and AlexaFlour 546 anti-mouse IgG2a) for 30 minutes at room temperature. Tissue sections were washed for 30 minutes in PBS, mounted, and visualized.

Immunohistochemistry. Primary antibody against phospho-Smad2 (1:50, Ser465/467), SV40 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), Ki67 (1:200, Dako) and SDF1α (1:200, Santa Cruz Biotechnology) diluted in 1% BSA-PBS were added to the slides and incubated overnight at 4°C. To identify collagen deposition, we stained the tissues using the Masson's Trichrome Stain Kit (DAKO). Five μm sections from the microarray and from formalin-fixed, paraffin embedded tissue blocks were obtained and mounted on positively charged slides. Tissue sections were de-paraffinized with xylene and rehydrated with serial passage through graded alcohol solutions. After antigen retrieval in citrate buffer, endogenous peroxidase was quenched in 3% hydrogen peroxide/PBS for 20 min. After washing with PBS, slides were blocked in 5%

BSA-PBS for 1 hr at room temperature. After incubation with primary antibodies, slides were washed with PBS and incubated with a biotinylated streptavidin-HRP secondary antibody (DAKO) for 60 min followed by a 1 hr washing step. Then slides were incubated in ABC-HRP complex (Vector Laboratories) for 1 hr and washed again in PBS for another hour. Bound antibodies were then visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAKO). Slides were rinsed extensively in tap water, counterstained with hematoxylin, and mounted.

Statstical Analysis. Data was analyzed by one-way ANOVA. Data are presented as the mean± standard deviation (SD). Differences were considered statistically significant when $P<0.05$.

1. Ao M, Franco OE, Park D, Raman D, Williams K, Hayward SW. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. Cancer Res. 2007;67:4244-53.

2. Brunner AM, Marquardt H, Malacko AR, Lioubin MN, Purchio AF. Site-directed mutagenesis of cysteine residues in the pro region of the transforming growth factor beta 1 precursor. Expression and characterization of mutant proteins. J Biol Chem. 1989;264:13660-4.

3. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335-48.

Legends for Supplementary Figures and Tables

Supplementary Figure 1. Characterization of Benign Human Prostate Stromal Cell line BHPrS1.

A. Parental BHPrs0 and h-Tert immortalized BHPrS1 cells were grown in vitro in RPMI 5%FBS and the expression of stromal and epithelial cell markers were assessed. Morphologicallly BHPrS0 (P0) and BHPrS1 (P6) cells adopted the typical stromal fusiform phenotype which was maintained at later passage **(a-c)**. **B.** BHPrS0 cells expressed stromal cell markers vimentin, alpha smooth muscle actin αSMA) and the progenitor cell marker nestin **(a-c)**. Immortalized BHPrS1 cells retained vimentin expression, but gradually lost αSMA and nestin after passage 6 **(d-f)**. **C.** Nuclear expression of Ku70 (a) confirmed the human origin and lacked of the epithelial (widespectrum cytokeratin: WSC) and neuroendocrine (synaptophysin: SnaP) markers indicated the fibroblastic nature of BHPrS1 cells.

Supplementary Table 1. Increased expression of phosphorylated Smad 2 in the vicinity of prostate tumors. The number of TGFß responsive stromal cells increased with Gleason score.

Supplementary Figure 2. RT-PCR of several candidate mediators primers ran on a 2.5% agarose/ethidium bromide gel. BHPrS1^{-EV}, BHPrS1^{-DN} and BHPrS1^{-EV}/BHPrS1^{-DN} cells showed differential expression of several secreted factors. Non-transcribed total RNA was used as negative controls showed an absence of PCR product (data not shown).

Supplementary Table 2. PCR Array Analysis .

Several genes involved in cell differentiation and development shoed a significant change in BHPrS1^{-DN} and the presence of a heterogeneous stromal population composed of BHPrS1^{-EV}/BHPrS1^{-DN} compared to BHPrS1^{-EV} cells.

Supplementary Table 3. Primers and conditions used for RT-PCR