Identification of SFRP1 as a Candidate Mediator of Stromal-to-Epithelial Signaling in Prostate Cancer

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Abstract

Genetic changes in epithelial cells initiate the development of prostatic adenocarcinomas. As nascent tumors grow and undergo progression, epithelial tumor cells are intimately associated with stromal cells. Stromal cells within the tumor microenvironment acquire new properties, including the capacity to promote phenotypic and genetic progression in adjacent epithelial cells. Affymetrix microarrays were used to identify 119 genes differentially expressed between normal-derived and carcinoma-derived prostatic stromal cells. These included 31 genes encoding extracellular proteins that may act as stromal-to-epithelial paracrine signals. Further investigation of one of these genes, secreted frizzled related protein 1 (SFRP1), revealed that its expression parallels prostatic growth with high expression during prostatic development, low expression in the adult prostate, and elevated expression in prostatic tumor stroma. In addition, as prostatic epithelial cells progressed to a tumorigenic state under the influence of tumor stroma, SFRP1 became overexpressed in the progressed epithelial cells. To further understand the roles of SFRP1 in the prostate, we tested the effects of increased SFRP1 levels on prostatic tissues and cells. Treatment of developing prostates with SFRP1 in culture led to increased organ growth. Treatment of a human prostatic epithelial cell line with SFRP1 led to increased proliferation, decreased apoptosis, and decreased signaling through the Wnt/β-catenin pathway in vitro and increased proliferation in vivo. These data suggest that overexpression of SFRP1 by prostatic tumor stroma may account for the previously reported capacity of prostatic tumor stroma to provide a pro-proliferative paracrine signal to adjacent epithelial cells. (Cancer Res 2005; 65(22): 10423-30)

Introduction

Prostate cancer is the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer death in men (1). Considerable progress has been made toward understanding the genetic changes within prostatic epithelial cells that occur during cancer progression (2). However, the molecular basis for abnormal cell-cell communication between the tumor epithelium and the tumor stroma remains poorly understood. Although prostatic adenocarcinomas are epithelial in origin, stromal cells adjacent to prostatic adenocarcinomas are phenotypically different from stromal cells in disease-free areas (3–5), raising the possibility that abnormal cell-cell communication between the tumor stroma and the tumor epithelium is an important feature of cancer progression. Experiments that manipulated the stromal microenvironment in prostate tumor models support this hypothesis. When normal prostatic mesenchyme/stroma was combined with prostatic Dunning tumor cells, normal stroma promoted differentiation and suppressed invasive growth of the Dunning tumor (6, 7). In contrast, other stromal cell types, including prostatic tumor stroma, stimulated prostate cancer progression in adjacent epithelial cells (8–13).

In one study, primary stromal cells were isolated from human prostate tumors [carcinoma-associated fibroblasts (CAF)] and from prostates without malignant disease [normal-associated fibroblasts (NAF)]. CAFs and NAFs were grown in vitro and in vivo with either primary normal human prostatic epithelial cells (NPRE) or with the BPH1 prostate epithelial cell line (12). Although the BPH1 cell line was immortalized by stable integration of the SV40 large T-antigen, BPH1 cells are nontumorigenic (14). In grafting experiments, both CAFs and NAFs caused the formation of benign prostatic ducts when grown with NPRE cells. However, CAFs stimulated the formation of invasive tumors by BPH1 cells, whereas NAFs promoted the formation of benign ducts by BPH1 cells. Other changes in epithelial behavior included increased proliferation and decreased apoptosis for BPH1 cells grown with CAFs relative to BPH1 cells grown with NAFs using in vitro cocultures (12). These experiments showed that paracrine signals from prostatic tumor stroma can cause a phenotypic progression from a nontumorigenic to a tumorigenic state in adjacent initiated epithelial cells.

Subsequent studies have examined the changes that occur in BPH1 cells that have been grown with CAFs and have formed a tumor in vivo (15, 16). A series of BPH1 derivative cell lines, designated BPH1CAF lines, have been isolated from BPH1 + CAF tumors. A comparison of the BPH1 cell line with the BPH1CAF cell lines showed that the BPH1CAF cell lines had undergone a permanent progression and acquired the ability to form tumors when grafted into nude mice independent of their stromal microenvironment (15). In addition, the genomes of BPH1CAF cells had been extensively rearranged relative to the parental BPH1 cell line (16). These experiments showed that paracrine signals from CAFs promoted genomic instability and genetic progression within adjacent epithelial cells.

We used Affymetrix microarrays to identify 119 genes differentially expressed between CAFs and NAFs. In subsequent
experiments, we determined that one of these genes, SFRP1, is both overexpressed by CAFs relative to NAFs and overexpressed by BPH1CAFTD cell lines relative to the parental BPH1 cell line. SFRP1 is one of five genes encoding secreted proteins that have an NH2-terminal domain homologous to the cysteine-rich domain of frizzled proteins (17). Frizzled proteins are multiaxial transmembrane proteins that serve as coreceptors for Wnt ligands (18). SFRP1 has been shown to bind directly to Wnt1 and to the Drosophila Wnt homologue Wingless. Upon binding, SFRP1 modulates the ability of these ligands to activate signal transduction cascades (19, 20). In addition to elevated expression in CAFs and BPH1CAFTD cell lines, we show that SFRP1 is highly expressed during prostatic development, expressed at low levels in the normal adult prostate, and expressed in human prostatic adenocarcinomas. In addition, SFRP1 stimulated the growth of developing prostatic organ rudiments, increased the proliferation rate of BPH1 cells, and decreased the apoptosis rate of BPH1 cells in vitro. These changes were associated with decreased signaling through the Wnt/β-catenin pathway. Furthermore, BPH1 cells expressing SFRP1 exhibit increased proliferation in vitro. Together, these data suggest that SFRP1 is a growth-promoting gene during prostatic development that is reactivated during the progression of prostate cancer.

Materials and Methods

Cell and organ culture. Cells were grown in a 5% CO2, 37°C incubator. Cell culture medium was RPMI 1640 containing 25 mmol/L HEPES, 1-glutamine, 5% charcoal-stripped fetal bovine serum, 10 units penicillin, 10 μg/mL streptomycin, 25 μg/mL amphotericin, and 1 × 10−6 mol/L testosterone. For organ cultures, ventral prostates were dissected from neonatal Sprague-Dawley rats into basal culture medium at 4°C. Organs were cultured on Millicell-CM membranes (Millipore, Bedford, MA) floating on the surface of culture medium in a 5% CO2, 37°C incubator. Culture medium was a 1:1 mixture of DMEM H-16/DMEM F-12 medium containing NEAA supplements, 1.4 g/L glucose, 0.37 g/L 1-glutamine, 42 μg/mL linoleic acid, 1.2 g/L NaHCO3, 10 μg/mL insulin, 1 μg/mL transferrin, and 1 × 10−6 mol/L testosterone. Culture medium was changed every other day during the culture period.

GeneChip analysis. RNA was prepared from three NAF and four CAF cell isolates. For each cell population, two probes were made from independent RNA preparations. Each probe was hybridized to Affymetrix Hgu95a DNA microarrays. Data were analyzed by ANOVA to identify expression differences that were statistically significant (P < 0.05).

Reverse transcription and real-time PCR. RNA was isolated from cell lines and tissues using the Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. Reverse transcription reactions were conducted on 1 μg of DNase-treated total RNA using 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1 mmol/L each deoxy- nucleotide triphosphate, 150 ng/μL random hexamer primers, 100 units MuLV reverse transcriptase (Invitrogen), and 20 units RNasin (Promega, Madison, WI). Real-time PCR reactions (Figs. 1 and 2) were run using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using the SYBR green reagent system (Applied Biosystems) according to the manufacturer’s instructions. Additional real-time PCR reactions (experiments in Fig. 3) were run using a Roche Light Cycler (Indianapolis, IN). An initial denaturation step of 95°C for 2 minutes was followed by 45 cycles of denaturation at 94°C for 5 seconds, annealing at 58°C for 10 seconds, and extension at 72°C for 10 seconds. Reactions contained 1× Platinum SYBR Green qPCR Supermix UDG (Invitrogen), 3 mmol/L MgCl2, 500 mmol/L F gene-specific forward primer, and 500 mmol/L gene-specific reverse primer. Gene-specific primer sequences are listed in Supplementary Table 2. Relative expression values were calculated as 2ΔCt, where ΔCt = experimental gene Ct value – control gene Ct value.

In situ hybridization. A segment from the mouse Sfrp1 mRNA (bases 889-1106 of Genbank BC094662) was used to synthesize digoxigenin-labeled sense and antisense RNA probes with a digoxigenin RNA Labeling Kit (Roche) according to the manufacturer’s instructions. Fresh tissues were dissected in PBS at 4°C and immediately frozen in optimum cryamab流星 transferase freezing compound (Tissue-Tek). Tissue sections 10 μm thick were cut at −20°C using a cryostat and mounted on Superfrost-plus microscope slides (Fisher, Pittsburgh, PA). RNA probes were then hybridized to the sections and visualized using a previously described protocol (21).

Human tissue collection. Both fresh and formalin-fixed, paraffin-embedded human prostate tumors were obtained from the University of Minnesota Cancer Center’s Tissue Procurement Facility.

Immunostaining. Paraffin-embedded tissues were cut into 6-μm sections using a microtome, dewaxed with CitriSolv, and rehydrated through a series of graded ethanol solutions. Cell lines were cultured in four-chamber slides (BD Biosciences, San Jose, CA) and were fixed in cold 100% ethanol, air-dried, and rehydrated in PBS. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide and rinsed with PBS. Samples were incubated in 2.5% sheep serum in PBS to bind nonspecific sites. Sections were then incubated with primary antibodies overnight at 4°C. Rabbit polyclonal anti-active caspase-3 antibody was used at a 1:250 dilution (Promega), rabbit polyclonal anti-phospho-histone-H3 antibody used at a 1:100 dilution (Upstate Cell Signaling Solutions, Lake Placid, NY), rabbit polyclonal anti-cytokeratin antibody used at a 1:100 dilution (DakoCytomation, Carpinteria, CA), and mouse monoclonal anti-Ki67 antibody was used at a 1:100 dilution (DakoCytomation, Carpinteria, CA). After washing in PBS containing 1% Tween 20 (PBT), sections were incubated in biotinylated species-specific anti-IgG antibody (Vector Laboratories, Burlingame, CA) at a 1:500 dilution. Slides were washed in PBT and...
SFRP1 expression in the rodent prostate. Real-time RT-PCR analysis of SFRP1 mRNA expression relative to cytokeratin 18. A, SFRP1 expression was detected in urogenital sinus of both male and female embryos at similar levels (left). SFRP1 expression was high in mouse ventral prostate at postnatal day 7 (p7) and low in the adult ventral prostate; P < 0.0001 (right). B, in situ hybridization using SFRP1 antisense RNA probes detected expression at postnatal day 1 (p1) in all lobes of the rate prostate (data not shown). Organ cultures of postnatal day 1 rat ventral prostates were grown a basal medium (n = 8) and the presence of BSA (n = 8) or recombinant SFRP1 [0.1 μg/mL (n = 8), 1 μg/mL (n = 8), and 10 μg/mL (n = 4)]. C, examples of an organ at the culture start and of an organ after 5 days of treatment with a control protein or with 10 μg/mL recombinant SFRP1. D, organs treated with recombinant SFRP1 showed a significant increase in growth (the increase in size for each organ was normalized to its own initial size) relative to control BSA-treated organs. P < 0.001 for all pairwise comparisons between SFRP1-treated and control organs by ANOVA test with least significant difference post hoc analysis.

developed using the avidin-biotin complex (ABC) method and 3,3'-diaminobenzidine substrate kits (Vector Laboratories) according to manufacturer's instructions. Slides were rinsed in water, counterstained with hematoxylin, and dehydrated in graded alcohol solutions. Control sections containing no primary antibody were processed in parallel.

TFG-dependent transcription. Cells were grown to ~70% confluence in 24-well plates. Experimental wells were cotransfected with reporter plasmids TOPFlash (Upstate Cell Signaling Solutions; 0.2 μg/well) and pRL-CMV (Promega; 0.01 μg/well) using Fugene6 (Roche) according to the manufacturer's instructions. In separate experiments, cells were cotransfected with reporter plasmids TOPFlash (Upstate Cell Signaling Solutions; 0.2 μg/well) and pRL-TK (Promega; 0.01 μg/well). Following transfection, cells were returned to their normal growth medium [in a subset of experiments, the medium was supplemented with recombinant human SFRP1 protein or bovine serum albumin (BSA) control protein] and cultured for an additional 48 hours. Protein was then extracted, and sequential measurements of firefly and Renilla luciferase activity were made using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining. Formalin-fixed, paraffin-embedded tissues were cut into 6-μm sections using a microtome, de waxed in Citrisolv, and rehydrated through a series of graded ethanol washes. Sections were processed using the DeadEnd Colorimetric Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling (TUNEL) System (Promega) according to the manufacturer's instructions. Slides were rinsed in water, counterstained with hematoxylin, and dehydrated in graded alcohol solutions. Control sections containing no recombinant terminal deoxynucleotidyl transferase enzyme were processed in parallel.

Plasmid construction, transfection, and generation of SFRP1-expressing lines. A human SFRP1 cDNA (22) was digested with XhoI and XbaI and inserted into the XhoI site of pPRRespur2O (Clontech, Palo Alto, CA). Subconfluent BPH1 cells were transfected in six-well culture plates with the pRESpur2O/SFRP1 plasmid using Fugene6 according to the manufacturer's instructions (Roche). Polyclonal populations of stables were selected for in medium containing 5 μg/mL puromycin. In parallel, BPH1 cells were transfected with the empty pPRRespur2O vector, and control populations of stable transfec tants were established by growth in selective medium.

Western blot analysis. Total protein was extracted from cells using radioimmunoprecipitation assay buffer containing protease inhibitors, separated by SDS-PAGE, and electroblotted to an Immobilon membrane (Millipore). Nonspecific binding sites were blocked with 1% BSA in TBS. Membranes were incubated in primary rabbit polyclonal anti-SFRP1 antibody diluted to 1:500 overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA; H-90). After washing in TBS containing 1% Tween 20 (TBST), membranes were incubated in biotinylated anti–rabbit IgG antibody (Vector Laboratories) at a 1:10,000 dilution, washed in TBST, and incubated in an avidin-horseradish peroxidase complex according to the manufacturer's instructions (ABC kit, Vector Laboratories). Membranes were washed in TBST, rinsed in TBS, and incubated for 5 minutes in an turer's instructions. Slides were rinsed in water, counterstained with hematoxylin, and dehydrated in graded alcohol solutions. Control sections containing no recombinant terminal deoxynucleotidyl transferase enzyme were processed in parallel.

Stromal-to-Epithelial Signaling in Prostate Cancer

Figure 3. A, canonical Wnt/β-catenin signaling in the prostate. Real-time RT-PCR was used to examine WNT1 expression (left) and DKK1 expression (right) in CAF and NAF lines not used in the initial microarray analysis. The trends of increased WNT1 and decreased DKK1 expression observed in the microarray study were also observed by real-time RT-PCR; however, only the expression differences for DKK1 (P = 0.0004) and not WNT1 (P = 0.16) were statistically significant. B, BPH1 cells cocultured on CAFs showed reduced TCF-dependent transcription relative to BPH1 cells coculture on NAFs; P = 0.04 (left). BPH1C(CAT) cell lines all showed reduced TCF-dependent transcription relative to parental BPH1 cells; P < 0.0001 for all pairwise comparisons between BPH1 cells and individual BPH1CAT lines (middle). Treatment of BPH1 cells with recombinant SFRP1 (0.1, 1, or 10 μg/mL) reduced TCF-dependent transcription (right). The differences between control and SFRP1-treated cells were statistically significant for the 1 μg/mL (P = 0.02) and 10 μg/mL (P = 0.01) groups. Ps were calculated using ANOVA with least significant difference post hoc analysis.


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enhanced chemiluminescence reagent (Bio-Rad, Richmond, CA) before exposing to film.

**Grafting experiments.** Epithelial cells (350,000) of each line were suspended in 50 μL of rat tail collagen gel as previously described (15) and grafted to the kidney capsules of male athymic mouse hosts. After 1 month of growth, the hosts were euthanized, and the grafts were explanted, weighed, fixed in formalin, and embedded in paraffin.

### Results

**Expression profiling of carcinoma associated fibroblasts and normal associated fibroblasts.** To identify genes that were differentially expressed between CAFs and NAFs, RNA was prepared from each and used to probe Affymetrix HgU95A DNA microarrays. Primary cell populations were tested in vivo and in vitro to confirm that they differed in their ability to promote tumor progression by the BPH1 prostatic epithelial cell line as previously reported for CAF and NAF cells (12). Data were analyzed by ANOVA to identify statistically significant (P < 0.05) expression differences. A total of 119 genes were identified with a statistically significant difference in expression between CAF and NAF cells of at least 2-fold (Supplementary Table 1). Among these genes, 31 encode extracellular proteins that may act as stromal-to-epithelial paracrine signals (Table 1).

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**SFRP1** is overexpressed both by carcinoma-associated fibroblasts relative to normal-associated fibroblasts and by BPH1CAPTD cells relative to BPH1 cells. In a previous study, expression profiling of parental BPH1 cells and the derivative BPH1CAPTD-01 cell line identified genes associated with the progression of BPH1 cells from being dependent on CAFs for tumor formation to the acquired ability to form tumors independent of their stromal microenvironment (16). **SFRP1** was overexpressed in CAFs versus NAFs (this study) and overexpressed in BPH1CAPTD-01 cells versus BPH1 cells (16). These observations raised the possibility that elevated **SFRP1** expression may play a central role in the capacity of BPH1 cells to form tumors, acting initially as a paracrine factor from CAFs and later as an autocrine factor in BPH1CAPTD-01 cells. This possibility led us to further investigate **SFRP1**.

We used a real-time reverse transcription-PCR (RT-PCR) assay to confirm the overexpression of **SFRP1** in CAFs relative to NAFs by examining three NAF and eight CAF primary isolates that were not used in the initial microarray study (Fig. 1A). We also examined **SFRP1** expression in primary NPREs, parental BPH1 cells, and seven BPH1CAPTD cell lines by real-time RT-PCR (Fig. 1A). **SFRP1** expression was relatively low in parental BPH1 cells and NPRE cells and overexpressed in seven of seven BPH1CAPTD cell lines. Because these BPH1CAPTD cell lines were derived from four independent

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*Affymetrix probe identifier in the Human Genome U95A Array.
†Positive values indicate increased expression in tumor stroma relative to normal stroma, whereas negative values indicate reduced expression in tumor stroma relative to normal stroma.
²Locus encodes both integral membrane and secreted protein isoforms.
tumors, SFRP1 overexpression seems a common feature of CAF-induced progression in BPH1 cells.

SFRP1 is expressed in primary human prostatic adenocarcinomas. To confirm that SFRP1 expression is also present in human prostate tumors and not merely in cultured prostatic cells, RT-PCR was used to detect SFRP1 transcripts in RNA isolated from prostate tumors (Fig. 1B). Immunohistochemical staining also showed the presence of stromally expressed SFRP1 protein in each of four independent human prostate tumors (Fig. 1C; data not shown).

Expression of SFRP1 parallels growth patterns of the prostate. To address the normal role of SFRP1 in the prostate, we examined SFRP1 expression at developmental and sexually mature stages in mice. Real-time RT-PCR analysis showed relatively high SFRP1 expression in the embryonic day 16.5 urogenital sinus of both male and female mice, indicating that early SFRP1 expression is not androgen dependent (Fig. 2A). High SFRP1 expression was also detected in the developing mouse ventral prostate at postnatal day 7 with low SFRP1 expression levels in the adult ventral prostate (Fig. 2A). In situ hybridization with an SFRP1 antisense probe detected SFRP1 transcripts (dark stain) in all lobes of the developing prostate (Fig. 2B; data not shown). The strongest staining was in a pattern reminiscent of developing epithelial ducts (Fig. 2B, arrow). Staining of adjacent sections with the antisense probe and an antibody against epithelial-specific cytokeratins confirmed that the areas of strong expression were epithelial ducts (data not shown). The less-intense staining observed in the developing prostatic mesenchyme was also above the background seen in negative control sections stained with a sense probe (data not shown), suggesting that SFRP1 is expressed in both the developing prostatic mesenchyme and epithelium.

Introduction of SFRP1 causes increased prostatic growth. As an initial functional test of SFRP1 during prostate development, serum-free organ cultures of developing rat ventral prostates were treated with SFRP1 recombinant protein. SFRP1 substituted for testosterone to stimulate growth of prostatic organ rudiments in a serum-free and hormone-free basal medium (Fig. 2C). These experiments showed that SFRP1 acts as a powerful growth-promoting protein on developing prostatic tissue, more than doubling the growth rate of organ rudiments at the highest concentration tested.

Canonical Wnt/β-catenin signaling is reduced in BPH1 cells cultured with tumorigenic stroma, in tumorigenic BPH1CAPTD cells, and in BPH1 cells treated with SFRP1. Existing data suggest that SFRP1 can either inhibit or enhance signaling via the Wnt/β-catenin pathway dependent on the SFRP1 concentration and cellular context (20). Additional modulators of Wnt signaling, Wnt1 and DKK1, were differentially expressed between normal prostatic stroma and tumor stroma (Table 1). The 2-fold increase of Wnt1 in CAFs relative to NAFs and the 2-fold decrease of DKK1 in CAFs relative to NAFs were confirmed by real-time PCR (Fig. 3A). The changes in Wnt1 and DKK expression suggested an increase in Wnt/β-catenin signaling in response to tumor stroma, whereas the changes in SFRP1 expression could cause a decrease in Wnt/β-catenin signaling in response to tumor stroma. To examine effects of stromal cells and SFRP1 on the Wnt/β-catenin signaling pathway, we used a previously described dual-luciferase reporter system that measures transcription from a promoter under control of the DNA elements (TCF binding sites) that mediate the transcriptional response to activation of the Wnt/β-catenin pathway (23). To determine if stromal cells were capable of altering Wnt/β-catenin signaling in the adjacent epithelial cells, cocultures were done. BPH1 cells were transfected with a reporter plasmid expressing firefly luciferase under control of oligomerized TCF binding sites and a second plasmid expressing Renilla luciferase under control of a constitutive promoter and grown on a confluent layer of either NAFs or CAFs. BPH1 cells cultured with CAFs showed significantly reduced TCF reporter expression relative to BHP1 cells grown with NAFs (Fig. 3B). These data suggested that, despite the increase in Wnt1 and the decrease in DKK1, tumorigenic stroma caused a decrease in Wnt/β-catenin signaling in the adjacent epithelial cells. We also investigated the status of Wnt/β-catenin signaling in parental BPH1 and seven derivative BPH1CAFTD cell lines. Cell lines were cotransfected with the reporter plasmids described above and all seven BPH1CAFTD cell lines had reduced expression from the TCF reporter plasmid relative to the parental BPH1 cell line (Fig. 3B). The ability of exogenous SFRP1 to affect Wnt/β-catenin signaling in BPH1 cells was also examined by treating BPH1 cells with recombinant human SFRP1. Treatment with 0.1, 1, or 10 μg/ml SFRP1 resulted in a reduction in Wnt/β-catenin signaling (Fig. 3B). Interestingly, although the literature suggests that SFRP1 can either enhance or antagonize Wnt/β-catenin signaling dependent on the concentration and cellular context, these experiments suggest that SFRP1 only has antagonist activity in prostatic cells. In addition, no concentration-dependent biphasic response to SFRP1 was observed either in cultured prostatic organ rudiments (Fig. 2D) or in cultured prostatic cells (Fig. 3B) over the range of SFRP1 concentrations where a biphasic response has been observed in other experimental systems.

Overexpression of SFRP1 induces increased proliferation and decreased apoptosis in nontumorigenic prostatic epithelial cells in vitro. To further test the potential functional role of SFRP1 in prostatic epithelial cells, we engineered the BPH1 human prostatic epithelial cell line to stably express a human SFRP1 cDNA. Immunoblotting was used to confirm the overexpression of SFRP1 in two separate polyclonal lines (Fig. 4A). Immunocytochemical studies of these two independent polyclonal SFRP1-expressing lines was done with a proliferation-associated antigen phospho-Ser10-histone H3 (24), and the ratio of positive cells to total cells was determined for each cell line. Both SFRP1-overexpressing cell lines had a higher labeling index indicating increased proliferation (Fig. 4B). SFRP1-overexpressing and control cell lines were also examined by immunocytochemistry for the apoptosis-associated antigen active caspase-3 (25). SFRP1 expression resulted in decreased apoptosis relative to the parental cell line and relative to empty vector–transfected control cells (Fig. 4C). BPH1 cells overexpressing SFRP1 were also tested for altered migration/invasiveness using a soft agar colony formation assay and an in vitro wound-healing assay with no differences relative to control cells observed (data not shown).

Overexpression of SFRP1 induces increased BPH1 cell proliferation and apoptosis in vivo. BPH1 cells overexpressing SFRP1 were also analyzed by grafting them under the renal capsules of immunodeficient mice for 1 month. Overexpression of SFRP1 by BPH1 cells was not sufficient to allow BPH1 cells to form large tumors (Fig. 5A). However, immunohistochemical staining for the Ki67 proliferation–associated marker showed that the SFRP1-overexpressing cell lines exhibited increased proliferation relative to controls (Fig. 5B). Unlike their behavior in vitro, TUNEL staining revealed that SFRP1-expressing cell lines grown in vivo also exhibited a marked increase in apoptotic cells relative to the control cells (Fig. 5C and D).
A

\[
\text{SFRP1}
\]

\[
\beta\text{-actin}
\]

B

\[
P_d - \text{gating (FS)}
\]

C

\[
P_c - \text{gating (SS)}
\]

Figure 4. Overexpression of SFRP1 in BPH1 cells leads to increased proliferation and decreased apoptosis in vitro. Two polyclonal SFRP1-overexpressing lines and a polyclonal line with stable integration of the empty vector were generated. A. Increased expression of SFRP1 in the stable transfectants was confirmed by immunoblotting. B, immunohistochemistry for the proliferation-associated antigen phospho-Ser^10-histone-H3 showed that both SFRP1-overexpressing cell lines had an increased proliferation rate relative to control cell lines. P < 0.0001 for all pairwise comparisons between control cells and stable transfectants. SFRP1-overexpressing and control cell lines were also examined by immunohistochemistry for the apoptosis marker active caspase-3. C, both SFRP1 overexpressing cell lines had a lower apoptosis rate than control cells. P < 0.0001 for all pairwise comparisons between control cells and stable transfectants. Ps were calculated using ANOVA with least significant difference post hoc analysis.

Discussion

Previous studies have established that CAFs and NAFs provide different paracrine cues to adjacent prostatic epithelial cells both in vitro and in vivo (12). Epithelial cells grown with CAFs have elevated proliferation and reduced apoptosis relative to epithelial cells grown with NAFs. In addition, CAFs promote the formation of invasive tumors by the otherwise nontumorigenic BPH1 cell line, whereas NAFs do not. We used Affymetrix arrays to identify gene expression differences between CAFs and NAFs (Table 1; Supplementary Table 1). A comparison of the full list of genes differentially expressed between CAFs and NAFs (Table 1; Supplementary Table 1) to data in the literature suggests that many of the observed differences could have functional consequences for tumor growth.

Among these genes, we chose to focus on SFRP1 because it is both overexpressed by CAFs relative to NAFs (Table 1; Fig. 1) and overexpressed by BPH1^KAT1^ cells relative to BPH1 cells (Fig. 1; ref. 16). This suggested that SFRP1 might initially act as a stromal-to-epithelial growth promoting paracrine signal from CAFs and subsequently act as an autocrine growth-promoting signal within BPH1 cells. Supporting this possibility, BPH1 cells overexpressing SFRP1 were grafted into immunodeficient mice, both proliferation and apoptosis were higher than in control cells yielding no net increase in overall growth of the grafts (Fig. 5). The different apoptotic responses to SFRP1 in vitro and in vivo likely reflect differences in status of Wnt signaling in tissue culture medium relative to the

\text{in vivo} \text{ graft site. The fact that SFRP1 promoted BPH1 cell proliferation both in vitro and in vivo does support a role for SFRP1 as a pro-proliferative stromal signal during prostate cancer progression. However, for SFRP1 overexpression to contribute to net tumor growth, it likely must cooperate with other changes in the tumor that act to block apoptosis. In BPH1 cells, SFRP1 also acted to limit signaling by the canonical Wnt/\beta\text{-catenin} pathway (Fig. 3). This observation suggests that overexpression of SFRP1 by prostatic tumor stroma may explain some changes in \beta\text{-catenin} activity that have been reported in prostate cancer. Activation of the canonical Wnt/\beta\text{-catenin} pathway is an important mechanism of cancer progression in colon cancer and in many other cancer types (26). Studies in prostate cancer found activating \beta\text{-catenin} mutations in <5% of tumors (27, 28). Subsequent studies that looked for accumulation of nuclear \beta\text{-catenin} in prostate cancer have reported widely varied results. An initial study observed nuclear accumulation of \beta\text{-catenin} in 5 of 24 metastatic prostate cancers examined (29). Another study found that 27 of 48 primary prostatic tumors and 12 of 16 metastatic prostate tumors were positive for cytoplasmic/nuclear \beta\text{-catenin with increasing levels of \beta\text{-catenin associated with progression to metastasis} (30). A third study that examined five normal prostates, 26 benign prostatic hyperplasia cases, 232 localized prostate cancers, and 20 metastatic prostate cancers found that nuclear \beta\text{-catenin was most common in benign prostate hyperplasia, and only 1 of 20 metastatic tumors had nuclear \beta\text{-catenin in >10% of tumor cells} (31). This final study is the only one that correlated nuclear \beta\text{-catenin status with long-term outcome of treatment. They found that lower levels of nuclear \beta\text{-catenin predicted a decreased chance of relapse-free survival. These studies support a role for changes in \beta\text{-catenin signaling in

Figure 5. Overexpression of SFRP1 in BPH1 cells leads to increased proliferation and apoptosis in vitro. A, the wet weights of subcapsular renal grafts of SFRP1-overexpressing lines were not statistically different from control lines after 1 month in vivo. B, immunohistochemistry for Ki67 marker showed increased proliferation in SFRP1-overexpressing cells relative to controls. P = 0.0017 for BPH1 versus SFRP1 line 1; P = 0.0014 for empty vector versus SFRP1 line 1; P = 0.0062 for BPH1 versus SFRP1 line 2; P = 0.0048 for empty vector versus SFRP1 line 2. TUNEL staining revealed a dramatic increase in apoptotic cells (arrowheads in D) for lines overexpressing SFRP1 (D) relative to control cells (C) grown in vivo.
prostate cancer progression. However, they also suggest that the role of β-catenin in prostate cancer is likely to be quite different than its role in colon cancer where activation of β-catenin drives cancer progression in the majority of tumors (26).

A study by Horvath et al. provides a clue as to one possible role for SFRP1 in prostate cancer. In their study, accumulation of nuclear β-catenin was most common in epithelial cells from areas of benign prostatic hyperplasia (BPH). Interestingly, a substantial decrease in nuclear β-catenin was observed in epithelial cells from areas of BPH adjacent to prostate cancers relative to areas of BPH not adjacent to prostate cancers. This suggested that the tumor microenvironment promoted a decrease in nuclear β-catenin.

We observed overexpression of SFRP1 in prostatic tumor stroma (Table 1; Fig. 1), and SFRP1 reduced Wnt/β-catenin signaling in prostatic epithelial cells (Fig. 3). Thus, SFRP1 is a likely candidate for mediating this effect of the tumor microenvironment. Because lower levels of nuclear β-catenin predict a decreased chance of relapse-free survival in localized prostate cancer (31), this is one mechanism whereby overexpression of SFRP1 may contribute to prostate cancer progression.

Looking beyond SFRP1, a prominent gene expression feature of CAFs is a down-regulation of IFN-induced genes, including MX1, BST2, IFIT4, DDIT3, ISG15, IFIT1, OAS1, IFIT1, and KYN1 (Supplementary Table 1). Interestingly, down-regulation of IFN-induced genes has previously been reported for BPH1 cells relative to parental BPH1 cells, and stimulation of BPH1 cells with IFNs reduced the growth rate of the cells in vitro and in vivo (32). These data raise the possibility that CAFs may promote epithelial growth, in part, by producing one or more factors that cause a down-regulation of the IFN pathway in both the epithelial and stromal compartment of tumors.

A previous investigation of tumor stroma in prostate cancer identified changes in the extracellular matrix (ECM) within the tumor (3). In similar fashion, we identified expression differences for genes encoding proteins that indirectly regulate the structure of the ECM including a down-regulation of extracellular protease inhibitorsTIMP3 and SERPINB2 in CAFs relative to NAFs. Among these ECM-related genes, changes in biglycan are particularly interesting because of the potential relationship to transforming growth factor-β (TGF-β) signaling. Biglycan encodes a small extracellular proteoglycan that can bind to and negatively regulate TGF-β (33). TGF-β is a negative regulator of prostatic epithelial cell growth in cocultures (34). In addition, experiments in mice have shown that blocking the activity of the prostatic stroma to receive signals from TGFβ results in the formation of cancer in the adjacent epithelium (35). These observations suggest that the up-regulation of biglycan we observed in CAFs could promote tumor progression via inhibition of TGFβ signaling.

In summary, we have identified 119 genes that are differentially expressed between prostatic tumor stroma and normal stroma. Of these, 31 encode proteins that are secreted into the extracellular space where they may directly affect the behavior of adjacent epithelial cells. We confirmed that one of these genes, SFRP1, is both overexpressed by prostatic tumor stromal cells and overexpressed in an epithelial model of prostate cancer progression. SFRP1 is highly expressed during prostatic development, expressed at low levels in the normal adult prostate, and expressed in adenocarcinomas. Increasing the levels of SFRP1 in developing prostate increased growth. Increasing SFRP1 in prostatic epithelial cells caused increased proliferation, decreased apoptosis, and decreased signaling through the Wnt/β-catenin pathway in vivo. Increasing SFRP1 in prostatic epithelial cells also caused increased proliferation in vivo. These data suggest that SFRP1 is a growth-promoting protein during prostatic development that becomes reactivated as a growth-promoting stromal-to-epithelial paracrine factor during prostate cancer progression. This work also supports other studies that have implicated changes in β-catenin signaling as important for prostate cancer progression via a mechanism that is distinct from the mode of action of β-catenin in colon cancer (27–31).

The contrasting relationship of nuclear β-catenin level and tumor activity in prostate cancer compared with colorectal cancer reflects similar differences in the proposed role of SFRP1 in the etiology of these malignancies. Whereas the present study provides evidence that SFRP1 overexpression promotes prostate carcinogenesis, several reports have suggested that epigenetic suppression of SFRP1 gene expression by hypermethylation enhances the development of colorectal carcinoma (36, 37). Decreased expression of SFRP1 due to hypermethylation and/or other mechanisms also has been described in tumors from several other organs, supporting the idea that it functions in these contexts as a tumor suppressor (38–41). Alternatively, SFRP1 expression was found to be elevated in uterine leiomyomas, where it had antiapoptotic activity, as we observed in prostatic cells (42). With regard to regulation of apoptosis by SFRP1, again results vary with the cellular target, as SFRP1 was reported to promote apoptosis in the MCF7 mammary carcinoma line (43) but inhibit it in gingival fibroblasts (44). Taken together, this dichotomy of findings implies that SFRP1 has distinct roles in cell proliferation, survival, and tumorigenesis in different organs. This may be due to the presence of varying sets of Wnts and Wnt receptors in these locales, as well as the possibility of distinct Wnt-independent, SFRP interactions in different tissues (45).

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Identification of SFRP1 as a Candidate Mediator of Stromal-to-Epithelial Signaling in Prostate Cancer

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