

The Expression of Sprouty1, an Inhibitor of Fibroblast Growth Factor Signal Transduction, Is Decreased in Human Prostate Cancer

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ABSTRACT

A considerable body of evidence indicates that alterations of fibroblast growth factors (FGFs) and their receptors contribute to prostate cancer progression. Recently, a new family of regulators of FGF activity has been identified. The *Sprouty* gene family negatively regulates FGF signaling in a variety of systems and could potentially limit the biological activity of FGFs in prostate cancer. Immunohistochemical analysis of normal and neoplastic prostate tissues using tissue microarrays revealed that Sprouty1 protein is down-regulated in approximately 40% of prostate cancers when compared with matched normal prostate. By quantitative real-time PCR analysis, we found that Sprouty1 mRNA levels were significantly decreased in prostate cancers *in vivo* in comparison with normal prostate. In prostate cancer cell lines, there is loss of the normal up-regulation of Sprouty1 mRNA and protein in response to FGFs. The decrease in Sprouty1 expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGF receptor expression in prostate cancer.

INTRODUCTION

Prostate cancer is the most common visceral cancer in men and the second leading cause of cancer-related death. The lack of effective therapies for advanced prostate cancer reflects, in part, the lack of knowledge about the molecular mechanism involved in the development and progression of this disease (1). Normal prostate growth is controlled by a variety of polypeptide growth factors, including members of the fibroblast growth factor (FGF) gene family (2, 3). A considerable body of evidence indicates that alterations of these growth factors and their receptors contribute to prostate cancer progression. Yan *et al.* (4) have shown in the Dunning rat model system that as these transplantable tumors progress from a mixed stromal-epithelial phenotype to a stromal-independent phenotype, there are changes in the isoforms of FGF receptors (FGFRs) expressed, consistent with autocrine stimulation of growth. In humans, multiple FGFs are increased in prostate cancer. For example, FGF2 is significantly increased in prostate cancers when compared with uninvolved prostate (5). Expression of FGF6 by prostate cancer cells has been identified in 40% of human prostate cancers *in vivo* (6), and the majority of prostate cancers overexpress FGF8 (7–9). In addition, increased expression of FGFR-1 is present in poorly differentiated human prostate cancers *in vivo* (5, 10). Autocrine expression of FGFs and expression of FGFRs has been reported in all of the commonly used prostate cancer cell lines *i.e.*, PC-3, DU145, and LNCaP (11, 12), and these cell lines express appropriate receptors to respond individually to these FGFs (13–15).

Recently, a new family of regulators of FGF activity has been identified. Sprouty was originally identified as an antagonist of Breathless FGFR signaling during tracheal development in *Drosophila* (16). Subsequent studies have shown that Sprouty inhibits signaling mediated by the FGFR and the epidermal growth factor receptor during eye development and oogenesis in *Drosophila* (17–19). During *Drosophila* eye development, Sprouty seems to inhibit the activation of mitogen-activated protein kinase upstream of Ras function, whereas during wing development, it is reported to inhibit mitogen-activated protein kinase downstream of Ras function.

Four mammalian genes have been identified with sequence similarity to *Drosophila sprouty* (20). The mammalian *Sprouty* family members are expressed in highly restricted patterns in the embryo in early development, and their expression shows a close correlation with known sites of FGF signaling (21–23), which suggests that they may also function as negative regulators in FGF signaling during vertebrate embryonic development. All Sprouty proteins share a unique, highly conserved cysteine-rich domain at the COOH terminus, believed to be critical for targeting them to phosphatidylinositol (4,5-bisphosphate) in the plasma membrane, thus allowing their inhibitory role on the mitogen-activated protein kinase pathway (24, 25). The NH₂-terminal portion of the Sprouty proteins is less conserved because it exhibits only 25–37% identity among the different mouse family members. These sequence differences could be responsible for the functional divergence among the Sprouty proteins. *In vitro* studies have demonstrated that after growth factor stimulation, Sprouty1 and Sprouty2 translocate to the plasma membrane, become tyrosine-phosphorylated, and interact with components of the Ras/mitogen-activated protein kinase and Ras/Raf/Erk pathways, such as Grb2 (26, 27) and c-Cbl (28), but the precise molecular mechanism by which the signal is blocked remains unknown. Tyrosine phosphorylation appears to be necessary for the ability of Sprouty to inhibit receptor tyrosine kinase-dependent Ras/Erk signaling while c-Cbl regulates the stability and hence the activity of Sprouty protein (27). It is likely that Sprouty proteins can also act at additional stages of receptor tyrosine kinases signaling, because Sprouty2 has been shown to inhibit FGF-mediated extracellular-signal-regulated kinase activated at the level of Raf (29), whereas Sprouty4 inhibits vascular endothelial growth factor receptor signaling upstream of Ras (30). In contrast, epidermal growth factor receptor signaling is not reduced following expression of Sprouty2 or Sprouty4 (31). It is thus conceivable that Sprouty proteins control receptor tyrosine kinase activation at different stages, with some additional regulatory mechanisms still unknown.

A search of the Unigene database³ and the Cancer Genome Anatomy Project database⁴ indicates that Sprouty cDNAs are present in cDNA libraries from many human tissues including the prostate, with Sprouty1 being the most abundant human Sprouty homologue expressed in human prostate. However, the role of Sprouty1 in human prostate cancer is not known, and little is known about alterations of regulatory molecules that may down-regulate growth factor signals in

Received 12/2/03; revised 4/15/04; accepted 5/5/04.

Grant support: Department of Defense Prostate Cancer Research Program Grant DAMD 17-01-1-0021 and National Cancer Institute Grant P50CA058204 to the Baylor prostate cancer SPORC program.

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³ Internet address: www.ncbi.nlm.nih.gov/UniGene.

⁴ Internet address: www.ncbi.nlm.nih.gov/ncicgap.

Report Documentation Page

*Form Approved
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1. REPORT DATE JUL 2004	2. REPORT TYPE	3. DATES COVERED 00-00-2004 to 00-00-2004			
4. TITLE AND SUBTITLE The Expression of Sprouty1, an Inhibitor of Fibroblast Growth Factor Signal Transduction, Is Decreased in Human Prostate Cancer		5a. CONTRACT NUMBER			
		5b. GRANT NUMBER			
		5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S)		5d. PROJECT NUMBER			
		5e. TASK NUMBER			
		5f. WORK UNIT NUMBER			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine, Department of Pathology, Houston, TX, 77030		8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)			
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)			
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES The original document contains color images.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

prostate cancer cells. An important consideration is that if Sprouty proteins can be up-regulated in prostate cancer by FGF stimulation, this would tend to inhibit any effects of FGFR activation in the neoplastic cells and negate the effects of the increased FGF expression in cancer tissues. To address this issue, we have investigated the expression of Sprouty1 in normal and neoplastic prostate tissues. We have found that Sprouty1 protein is decreased in prostate cancer cells when compared with matched normal epithelium in approximately 40% of prostate cancers and that there is a similar decrease in Sprouty1 mRNA by quantitative reverse transcription-PCR. We have also found that in prostate cancer cells, there is loss of the normal up-regulation of Sprouty1 mRNA in response to FGFs. The marked decrease in Sprouty1 expression in the human prostate cancer implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer.

MATERIALS AND METHODS

Plasmid Construction. Plasmid pCDNA3.1 (Invitrogen, Carlsbad, CA) was used for the expression of full-length Sprouty1 cDNA. For the construction of pCDNA-Sprouty1, the coding sequence of Sprouty1 was amplified from pCMV-Sport6 plasmid containing the full-length Sprouty1 cDNA (ATCC 3461395) in a PCR reaction using primers designed against the published Sprouty1 sequence as follows: forward primer, 5'-AAGCTTAGACATG-GATC-CCCAAAATC-3'; and reverse primer, 5'-GAATTCTGATGGTTTAC-CCTGACCCC-3' (The underlined sequence indicates *Hind*III and *Eco*RI sites in the forward and reverse primers, respectively; the bold and underlined sequence shows the start and stop sites in the forward and reverse primers, respectively; the italicized and underlined G indicates an engineered G at position -3 to ensure proper initiation of translation). The PCR product was digested with *Eco*RI and *Hind*III and sub-cloned into pCDNA3.1.

Mutation Analysis of the Sprouty1 Coding Region. Total genomic DNA was extracted from 26 prostate tissue samples [including 23 radical prostatectomies (all 70% or more cancer), 1 metastasis, and 2 benign tissues from radical prostatectomies] as described previously (32). The DNAs were used in standard PCR reaction conditions with three sets of primers to amplify the entire Sprouty1 coding region. Set 1 primers (SF1): forward, 5'-ACGACACAGACACACAAG-3', and reverse, 5'-CAACCCACCTCCAAAAATCA-3'; set 2 primers (SF2): forward, 5'-CCTTCTTTGGATAGCCGTC-3', and reverse, 5'-CCCTCAAGTCATCCACAATC-3'; set 3 primers (SF3): forward, 5'-AGGACCCAGCATCATGTA-3', and reverse, 5'-GTGGCTGTGTGTCTGTGCT-3'. The nucleotide positions for the amplification products as given by the GenBank accession no. (XM_036349) are 137-628, 394-843, and 605-1317. The PCR products were purified and sequenced using the respective set of primers for each product.

Preparation, Quantification, and Dilution of DNA Standards. The Sprouty1 plasmid, keratin-18 plasmid (ATCC MGC-9348), and β -actin plasmid (ATCC MGC-10559) were prepared using the Qiagen Maxi-prep Kit (Qiagen, Valencia, CA). The FGF2 and FGF7 plasmids have been described previously (33). Quantification of plasmid was performed spectrophotometrically. The measurements of the plasmid concentration were done in duplicate and then converted to copy number. A dilution series of each plasmid (10^9 - 10^1 copies) was used as a DNA standard for real-time PCR.

Primer Design and Synthesis for Real-Time PCR. Oligonucleotide primers for Sprouty1 were forward, 5'-TGTCCGAAAAGGATTCAGATGC-3', and reverse, 5'-ACTGCCACTGCCA TGTTGAT-3'; for β -actin were forward, 5'-AGCACGGCATCGTCA CCAACT-3', and reverse 5'-TGGCTGGGGTGTGAAGGTCT-3'; for keratin 18 were forward, 5'-AGGGCTCAGATCT-TCGCAAAT-3', and reverse, 5'-GTCATCAATGACCTGCGGAG-3'; for FGF2 were forward, 5'-CCACTTCAAGGACCCCAAG-3', and reverse, 5'-ATAGCCAGGTAACGGTTAGC-3'; and for FGF7 were forward, 5'-CCTTCTGCTGTGATTTATGG-3', and reverse, 5'-GTTGCTGTGACGCTGTTG-3'. Primers were carefully designed to cross exon/intron regions and to avoid the formation of primer-dimers, hairpins, and self complementarity. The nucleotide positions for the amplification products as given per the GenBank accession nos. are 275-373, 458-622, 256-435, 539-721, and 26-209 for

Sprouty1 (XM_036349), Keratin 18 (BC020982), β -actin (BC004251), FGF2 (NM_0020006), and FGF7 (S81661), respectively.

cDNA Synthesis and Quantitative Real-Time PCR. Total RNA extracted from cells and tissues using TRIzol Reagent (Invitrogen) was used in first-strand DNA (cDNA) synthesis using Invitrogen SuperScript first-strand synthesis system for reverse transcription-PCR and according to the manufacturer's protocol. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Hercules, CA) as described previously (34) and incorporating the following optimized PCR reaction conditions. The amplification of Sprouty1 was carried out as follows: a 3 min hot start at 95°C; followed by 40 cycles of denaturation at 95°C for 15 s; and annealing at 61°C for 30 s. The amplification protocol for FGF2 was the same as for Sprouty1 except that annealing was done at 63.5°C. The amplification protocol for FGF7, β -actin, and keratin 18 was carried out as follows: a 3 min hot start at 95°C; followed by 40 cycles of denaturation at 95°C for 30 s; annealing at 56°C for 20 s; and a 72°C extension for 30 s. Each experiment was done in duplicate. The threshold cycle (C_t) values in log linear range representing the detection threshold values were used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

Northern Blot Analysis. A multiple tissue Northern blot (MTN Blot II) containing polyadenylated RNAs isolated from human adult tissues was obtained from Clontech (Palo Alto, CA). Northern hybridization was performed at 68°C in 10 ml of PerfectHyb Plus hybridization solution (Sigma, St. Louis, MO). The blot was prehybridized in the above buffer for 30 min. Hybridization was done for 1 h by adding 50 ng of full-length Sprouty1 or β -actin cDNA fragment that were radioactively labeled with [α - 32 P]dCTP using a RadPrime Labeling Kit (Invitrogen) and included at a concentration of 1×10^9 cpm/ml. Blots were washed according to the manufacturer's protocol, and signals were visualized by autoradiography.

Tissue Microarrays and Immunohistochemistry. The tissue microarrays used to study Sprouty1 expression in clinically localized prostate cancer have been described previously (35). In brief, three 0.6-mm cores of cancer and uninvolved prostate tissue were obtained from radical prostatectomy specimens and used to construct tissue microarrays. Patients received no adjuvant therapy such as radiation or hormonal therapy. Other patient characteristics were as described previously. A total of 511 of the original 640 cancers were evaluable, with some cases lost due to depletion of tumor or technical artifacts, and of these, 407 had matched evaluable normal tissue. Immunohistochemistry was performed as described previously (36). Antigen retrieval was performed for 30 min in a rice cooker in 10 mM citrate buffer (pH 6.0). Endogenous biotin and peroxidase were blocked using appropriate kits from Vector Laboratories (Burlingame, CA) according to the manufacturer's protocol. Rabbit polyclonal anti-Sprouty1 antibody (Upstate Biotechnology, Lake Placid, NY) was incubated with each tissue array section at 5 ng/ml at 4°C overnight followed by the avidin-biotin peroxidase complex procedure (Vector Laboratories) and counterstaining with hematoxylin as described previously. Slides were then scanned using a Bliss automated slide scanner system to produce high-resolution digital images. Staining was evaluated in the normal and prostate cancer epithelial cells as described previously. Staining intensity was graded as absent (0), weak (1+), intermediate (2+), or strong (3+). The extent of staining was estimated and scored as follows: no staining (0); 1-33% of cell stained (1+); 34-66% of cell stained (2+); or 67-100% of cells stained (3+). The staining index for each case was then calculated by multiplying the average intensity score for the three cores by the average percentage score for the three cores, yielding a 10-point tumor staining index ranging from 0 (no staining) to 9 (extensive, strong staining) for each case.

Western Blotting. Total protein was extracted from cells using protein lysis buffer as described previously. For Western blots, 30 mg of protein extract/lane were electrophoresed, transferred to nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Piscataway, NJ), and incubated overnight with a 1:100 dilution of anti-Sprouty1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:5000 dilution of anti- β -actin mouse monoclonal antibody (Sigma). Membranes were washed and treated with bovine antigoat IgG (1:5000; Santa Cruz Biotechnology) or rat antimouse IgG secondary antibody conjugated to horseradish peroxidase (1:2000 dilution; Southern Biotechnology Associates, Birmingham, AL) for Sprouty1 and β -actin, respectively. The antigen-antibody reaction was visualized using an enhanced chemiluminescence (ECL) assay (Amersham Biosciences) and ex-

posed to ECL film (Amersham Biosciences). Western blot signals were quantified using NucleoVision imaging station (Nucleotech, San Carlos, CA).

Cell Culture. The human prostate cancer cell lines PC3, DU145 and LNCaP and the immortalized normal prostate epithelial cell lines PNT1a and PNT2 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen). Primary epithelial and stromal cultures were established from normal peripheral zone tissue from radical prostatectomy specimens as described previously (33). For Western blotting studies, prostate cancer cell lines were incubated in primary epithelial growth medium for 24 h before collection.

Cell Transfection. For stable transfections, PC3 or LNCaP cells were seeded at 5×10^6 cells/100-mm dish and transfected with 10 μ g of Sprouty1 construct (pcDNA-Sprouty1) or vector only (pcDNA3.1) using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Two days after transfection, cells were selected in medium containing Geneticin (Sigma) at a final concentration of 400 and 200 μ g/ml for LNCaP and PC3 cells, respectively. After 14 days into the selection, individual Geneticin-resistant colonies were fixed with 10% formalin and stained with crystal violet, and the colonies visible to the naked eye were counted. For transient transfection, LNCaP cells were plated at 5×10^4 cells/60-mm dish and transfected with 2 μ g of Sprouty1 plasmid or vector only using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 24, 48, or 72 h, cells were trypsinized and counted using a Coulter counter. A second transfected plate was used to collect protein extract for Western blotting at the same time.

Cell Sorting of LNCaP Cells Transfected with GFP Constructs. LNCaP cells were plated at 5×10^6 cells/100-mm dish and transfected with either 24 μ g of pEGFP (BD Biosciences Clontech) alone or 6 μ g of pEGFP with 18 μ g of the Sprouty1 plasmid using LipofectAMINE 2000 transfection reagent (Invitrogen). After 24 h, GFP-positive cells were sorted by single color-flow cytometry using Epics-Altra flow cytometer (Beckman Coulter) and plated into complete growth medium. After 24, 48, or 72 h, cells were trypsinized and counted using a Coulter counter.

FGF2 Induction Studies. Primary epithelial, LNCaP, and PC3 cells were placed in serum-free medium for 24 h. Cells were refed with serum-free medium with 1% insulin, transferrin, and selenium (Sigma) with or without 25 ng/ml recombinant FGF-2 (R&D Systems, Minneapolis, MN) and incubated at 37°C for different time points. Cells were then harvested for either RNA or protein extraction. RNA extracted was used in real-time quantitative PCR, and protein extraction was used in Western blot analysis as described above.

RESULTS

Expression of Sprouty1 in Human Prostate Tissue and Cell Lines. Initial studies were carried out to investigate the expression of the human Sprouty1 homologue in adult human tissues. A multiple tissue Northern blot was hybridized to the full-length Sprouty1 cDNA. A single transcript (of approximately 3.5 kb) was present in all of the tissues analyzed (spleen, thymus, prostate, testis, ovary, small intestine, and colon) except peripheral blood leukocytes, with the strongest expression observed in the prostate and the testis. To evaluate Sprouty1 expression *in vitro*, total RNA samples derived from normal prostatic peripheral zone and two immortalized but nontumorigenic prostatic epithelial cell lines (PNT1a and PNT2) were analyzed for Sprouty1 expression by reverse transcription-PCR reaction. Sprouty1 was easily detectable in all three RNAs. We also compared the expression of Sprouty1 in primary cultures of prostatic epithelial and stromal cells using quantitative reverse transcription-PCR. Expression of Sprouty1 was 8-fold higher in the epithelial cells in comparison with the stromal cells (17.7 transcripts/ 10^3 β -actin transcripts for epithelial cells *versus* 2.2 transcripts/ 10^3 β -actin transcripts for stromal cells). Thus Sprouty1 is expressed in human prostate *in vivo* and in prostate epithelial cells and to a lesser extent, in stromal cells *in vitro*.

Analysis of Sprouty1 Expression by Immunohistochemical Analysis of Tissue Microarrays. To determine whether Sprouty1 protein is decreased in clinically localized prostate cancers, we ana-

lyzed a total of 407 prostate cancers and matched normal peripheral zone tissues by immunohistochemistry of prostate cancer and prostate tissue microarrays. The tissue microarrays used were obtained from the Baylor prostate cancer SPORE and have been described previously (35). These arrays contain 0.6-mm tissue cores from cancers in triplicate as well as non-neoplastic peripheral zone tissue cores (also in triplicate) from patients undergoing radical prostatectomy. In normal prostate, the Sprouty1 protein is expressed in epithelial and smooth muscle cells, with some staining of stromal fibroblasts (Fig. 1, A and B). Sprouty1 expression in normal epithelium was variable. The vast majority of cases showed moderate to strong staining in the normal epithelium as shown in Fig. 1, although some tissues had only minimal staining. Prostate cancer epithelium also had quite variable expression. Some prostate cancer had minimal expression of Sprouty1 (Fig. 1, C--E). Other cancers had moderate to strong expression of Sprouty1 protein (Fig. 1F). To quantitatively compare the expression of Sprouty1 in normal and neoplastic prostatic epithelium, we quantitated the expression of Sprouty1 based on methodology described previously (35). In brief, stained slides were digitized, and staining was scored both for extent of staining (scale of 0–3) and intensity of staining (scale of 0–3). A staining index was calculated from the average extent of staining score for the three cores multiplied by the average staining intensity score. The mean staining index of Sprouty1 in normal epithelium was significantly higher than in the cancer cells ($P = 0.045$, Wilcoxon's signed ranks test). Perhaps more meaningfully, given the variability in Sprouty1 expression, we also compared the staining index in normal and cancer cells from the same patient. Overall 39% of cancer had a lower staining index than that of benign tissues from the same patient. However, it should be noted that a significant fraction of prostate cancers had higher expression of Sprouty1 in cancer cells than in normal epithelium. Therefore, although decreased Sprouty1 expression is seen in a substantial fraction of prostate cancers, loss of Sprouty1 expression is clearly not required in all prostate cancers.

Decreased Sprouty1 mRNA in Human Prostate Cancer Tissues. To determine whether Sprouty1 protein is decreased in human prostate cancers due to decreased mRNA and to confirm our observations on immunohistochemistry by an alternative technique, we carried out quantitative real-time PCR analysis. We quantitatively analyzed the expression level of Sprouty1 mRNA in a total of 29 prostate tissue samples including nine normal peripheral zone tissues and 20 clinically localized cancer (consisting of at least 70% cancer) by real-time PCR. We used β -actin as an endogenous mRNA control. The real-time data are presented as the ratios of Sprouty1 mRNA transcripts $\times 10^3/\beta$ -actin transcript for each group of samples analyzed (Fig. 2A). The expression of Sprouty1 in both normal prostate and cancer tissues was variable, presumably reflecting both random variability in tissue composition and variable expression per cell (as seen in the immunohistochemistry studies). Sprouty1 expression was about 70% higher on average in normal prostate tissues (6.60 ± 2.0 , SE) compared with prostate cancers (3.82 ± 1.1 , SE), despite the fact that FGFs are significantly up-regulated in the vast majority of prostate cancers. Of the 20 cancers analyzed, 16 had Sprouty1 mRNA levels lower than the mean Sprouty1 mRNA level in normal tissue. The difference in Sprouty1 mRNA level between normal and cancer tissue was statistically significant ($P < 0.035$, *t* test). Similar results were obtained when Sprouty1 expression was normalized using keratin 18 mRNA, which is expressed exclusively by epithelium (Fig. 2B). Expression of Sprouty1 normalized for epithelial content was almost 2-fold higher in normal tissues when compared with cancers (3.8 ± 0.9 *versus* 2.0 ± 0.2), and this difference was again statistically significant ($P < 0.02$, *t* test).

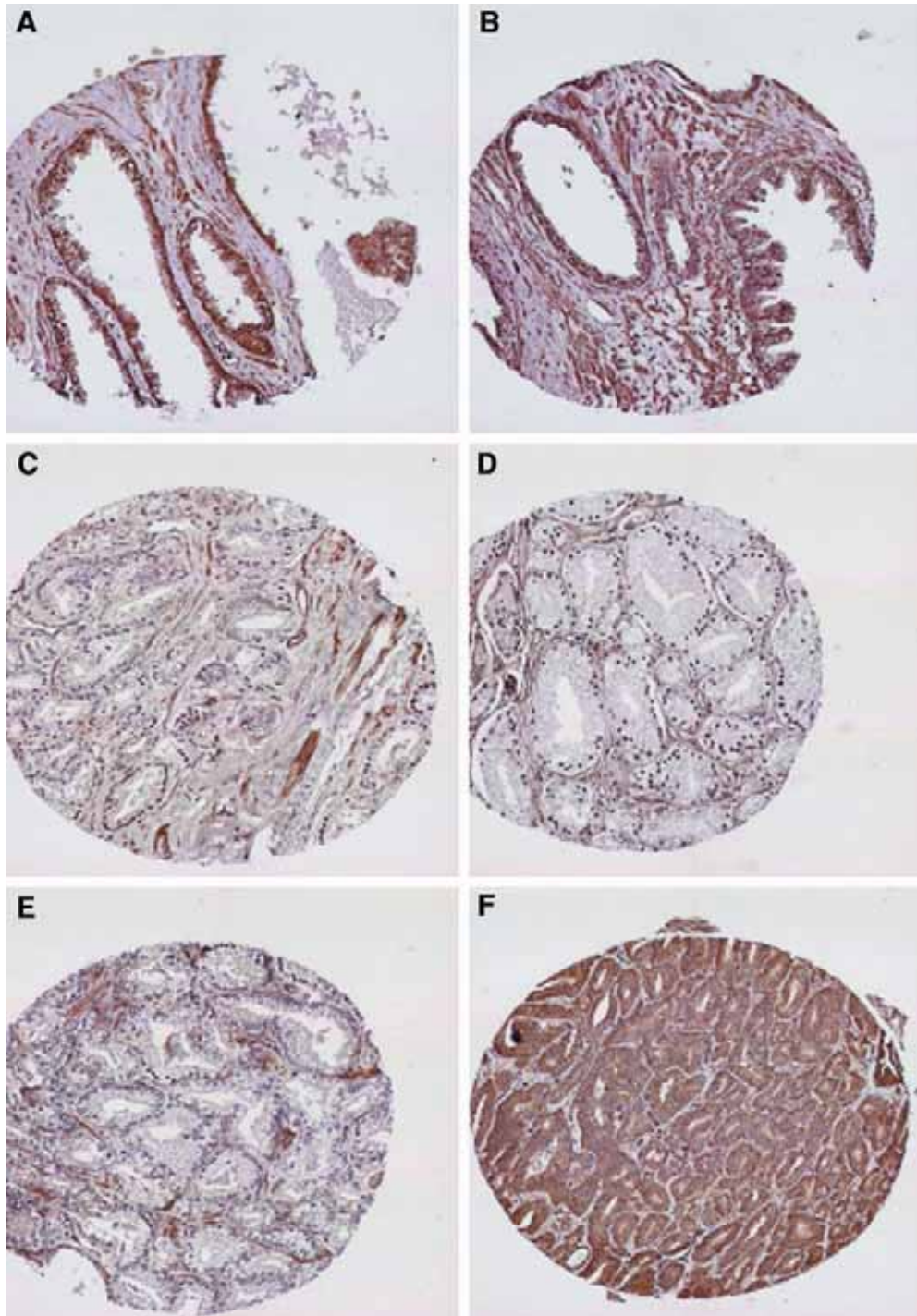


Fig. 1. Immunohistochemical analysis of Sprouty1 expression in tissue microarrays. Expression of Sprouty1 in normal prostate (A and B) and prostate cancer (C--F) was determined using tissue microarrays as described in "Materials and Methods." A and B, normal prostate peripheral zone tissue with expression of Sprouty1 in prostatic epithelial and smooth muscle cells. Some staining of fibroblastic cells is also present. C--E, prostate cancers with low Sprouty1 expression in prostate cancer cells. Note scattered staining of residual stromal smooth muscle cells. F, prostate cancer with strong Sprouty1 expression in neoplastic epithelial cells.

One possible explanation for the decreased amounts of Sprouty1 mRNA in cancer tissues could be that Sprouty1 mRNA is expressed at higher levels in stroma compared with epithelium *in vivo* and that in cancer the stroma is replaced by neoplastic epithelium. If this were the case, one would expect that there would be an inverse correlation between the level of keratin 18 mRNA and Sprouty1 mRNA content in benign tissue samples. There was a 5-fold variation among the benign prostate tissues in keratin 18 mRNA content (relative to β -actin) due to variation in the percentage of epithelium in the tissue

as a result of sampling variability. However, there was no correlation (inverse or positive) between keratin 18 mRNA levels and Sprouty1 mRNA levels. This indicates that there is expression of Sprouty1 mRNA in both the normal epithelial and the stromal compartments *in vivo*. In addition, based on immunohistochemistry with anti-Sprouty1 antibodies, the majority of Sprouty1 protein in normal prostate is in epithelial cells, with significant amounts in prostatic smooth muscle cells. Therefore, the decreased Sprouty1 mRNA observed in the prostate cancer tissues is almost certainly due to lower levels of

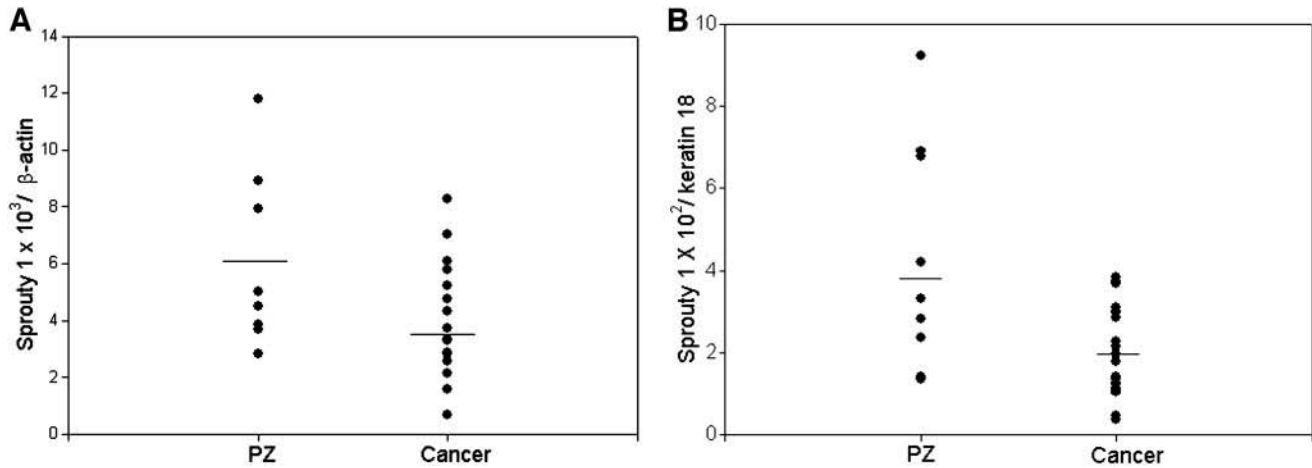


Fig. 2. Sprouty1 expression in normal prostate and prostate cancer as determined by quantitative reverse transcription-PCR. Sprouty1 expression in normal prostatic peripheral zone (PZ) and cancer tissues was assessed by quantitative reverse transcription-PCR using a real-time thermal cycler (iCycler; Bio-Rad). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts $\times 10^3$ to β -actin transcripts (A) or Sprouty1 transcripts $\times 10^2$ to keratin 18 transcripts (B). The Sprouty1, β -actin, and keratin 18 values were calculated from standard curves. The data are a representative of duplicate experiments. The mean expression level is indicated. The Sprouty1 expression value from cancer tissues is significantly different from the PZ tissues; $P < 0.05$ (t test) for both β -actin and keratin 18 normalization.

Sprouty1 mRNA in the prostate cancer cells compared with normal epithelium.

Another possible explanation for decreased expression of Sprouty1 mRNA in the cancer tissues is decreased expression of FGFs in a subset of the prostate cancers. Our laboratory has shown previously that FGF2 and FGF7 are expressed in the stromal cells of cancer tissues and that FGF2 protein is approximately 2.5-fold higher in prostate cancer tissues, whereas FGF7 protein levels are similar in normal and prostate cancer tissues (5). We therefore compared the expression of FGF2 and FGF7 mRNA by quantitative reverse transcription-PCR in cancer tissues with Sprouty1 expression below the mean value for all cancers (average 1.2 Sprouty1 transcripts/ 10^2 K18 transcript) to those with Sprouty1 expression above the mean (average 3.22 Sprouty1 transcripts/ 10^2 K18 transcript). There was no significant difference in expression of FGF2 between these two groups (2.31 ± 0.8 versus 2.61 ± 0.9 FGF2 transcripts/ 10^3 β -actin transcripts; mean \pm SE, $n = 10$). It should be noted that both groups have higher FGF2 mRNA transcript levels than a set of nine normal peripheral zone tissues analyzed at the same time (1.3 FGF2 transcripts/ 10^3 β -actin transcripts). Similarly, FGF7 transcript levels were not significantly different between the two groups (0.77 ± 0.3 versus 0.50 ± 0.2 transcripts/ 10^3 β -actin transcripts; mean \pm SE, $n = 10$). Thus it is unlikely that the decreased Sprouty1 mRNA expression in many of the cancer tissues *in vivo* reflects decreases in FGF ligand concentration in the cancer tissues.

Effect of FGF2 on Sprouty1 Expression in Normal and Neoplastic Prostate Epithelial Cells. We next examined the expression of Sprouty1 protein *in vitro* in normal primary prostatic epithelial cells and prostate cancer cell lines. Consistent with our *in vivo* data, the three commonly used prostate cancer cell lines (LNCaP, DU145, and PC3) all expressed lower levels of Sprouty1 protein, as determined by Western blotting, than did the normal epithelial cells (Fig. 3). It should be noted that these three cell lines express FGFs in an autocrine fashion and so would be expected to express higher levels of Sprouty1 than normal epithelial cells if FGFs are inducing Sprouty1 expression as in other systems. To investigate whether regulation of Sprouty1 expression in response to FGF stimulation is altered in the prostate cancer cells, we examined the effect of FGF2 stimulation on Sprouty1 mRNA expression *in vitro*. Fig. 4A shows that when primary epithelial cells were stimulated with FGF2, there was a 5-fold increase in Sprouty1 expression

within 30 min. However, this expression was rapidly down-regulated to below basal level in 1 h. There was a subsequent increase in Sprouty1 expression after 2 h that was sustained, again peaking within 24 h, suggesting that there is a biphasic increase in expression of Sprouty1 in response to FGF2 stimulation. In contrast to primary epithelial cells, LNCaP and PC3 cells did not show induction of Sprouty1 expression in response to FGF2 stimulation. In fact, there was a slight down-regulation of Sprouty1 at 30 min or 2 h after FGF stimulation in PC3 and LNCaP cells, respectively. This result indicates that, unlike normal prostate cells, Sprouty1 expression in prostatic cancer cells is no longer up-regulated by FGF2. To determine whether Sprouty1 mRNA correlates with protein level, we investigated the effects of FGF2 stimulation on Sprouty1 protein expression *in vitro*. Because basal sprouty1 expression in PC3 cells is much lower than primary cells (Fig. 3), we have normalized expression in each cell line to basal expression in that cell type in normal growth medium to facilitate comparison of changes in expression, but absolute expression of Sprouty1 in PC3 cells is far lower than in the primary epithelial cells. Fig. 4B shows that when primary cells were stimulated with FGF2, there was a gradual and a rather sustained increase in Sprouty1 protein expression that was 3-fold higher than basal Sprouty1 expression in normal growth medium by 24 h. In contrast, with the primary cells,

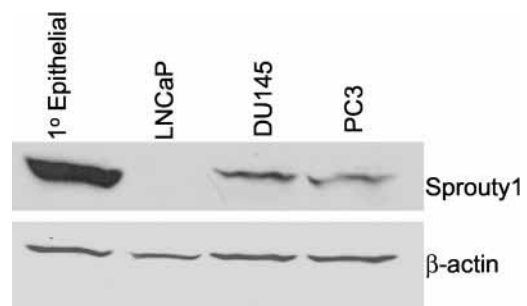


Fig. 3. Expression of Sprouty1 protein in primary prostatic epithelial cells and prostate cancer cells lines. Protein extracts of primary cultures of prostatic epithelial cells and the prostate cancer cell lines LNCaP, PC3, and DU145 were analyzed by Western blotting with anti-Sprouty1 antibodies as described in "Materials and Methods." LNCaP had a faint Sprouty1 band on longer exposures in which the primary epithelial cell lane is overexposed (not shown). Loading control on the same filter with anti- β -actin antibody is shown in the bottom panel.

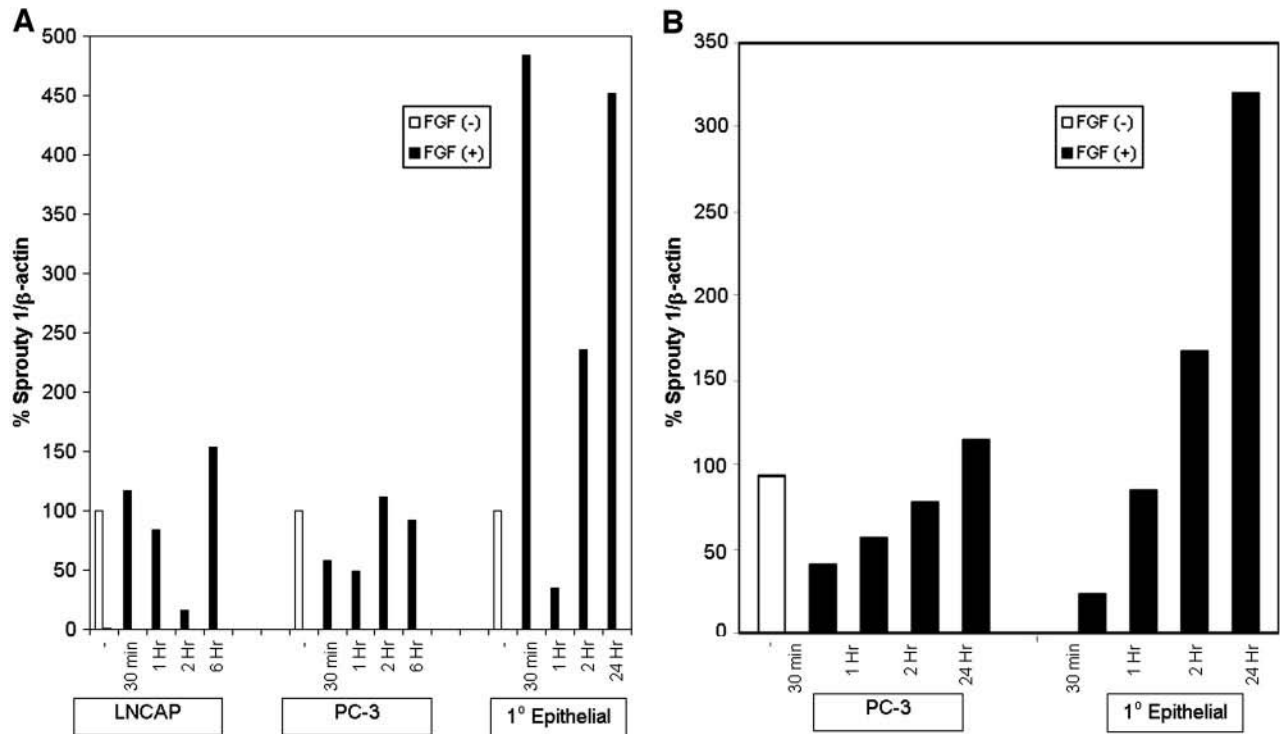


Fig. 4. Sprouty1 expression in response to FGF2 stimulation of normal or neoplastic prostate epithelial cells. *A*, prostatic primary epithelial cells, LNCaP, and PC3 cells were grown in serum-free medium for 24 h. Cells were stimulated with serum-free medium with or without 25 ng/ml recombinant FGF2. At different time points, the Sprouty1 expression in the cells were analyzed by quantitative reverse transcription-PCR using total RNA. Sprouty1 expression levels are displayed as the ratio of Sprouty1 to β -actin to correct for variation in the amounts of reverse-transcribed RNA, with the ratio before FGF2 treatment set as 100% for each cell line. The Sprouty1 and β -actin values were calculated from Sprouty1 and β -actin standard curves, respectively. The data are a representative of duplicate experiments. Values are the mean \pm SD. *B*, prostatic primary epithelial cells and PC3 cells were grown in serum-free medium for 24 h. Cells were stimulated with serum-free medium with or without 25 ng/ml recombinant FGF2. At different time points, protein extracts were collected from the cells and analyzed by Western blotting with either anti-Sprouty1 antibody or control anti- β -actin antibody. The Western blot signals were quantified using NucleoVision imaging workstation and calculated as the ratio of Sprouty1 protein to β -actin protein. Expression in normal growth medium was substantially lower in PC3 cells than in the primary cells, so the ratio from each cell type grown in complete medium and collected at the same time was set at 100% for each cell type, and data for that cell type are expressed relative this control. Sprouty1 protein was undetectable in primary epithelial cells after 24 h of incubation in serum-free medium.

PC3 cells did not show induction of Sprouty1 protein expression in response to FGF2 stimulation. Rather, there was a decrease in Sprouty1 protein expression within 30 min before returning to basal level at 24 h. The initial decrease in Sprouty1 protein could be a direct result of proteosomal degradation mediated by c-Cbl after growth factor signaling, as described for Sprouty proteins in other systems (27, 37–39).

Mutational Analysis of Sprouty1 in Human Prostate Cancers.

To determine whether Sprouty1 is inactivated by mutation in prostate cancer, we analyzed DNAs isolated from 24 prostate cancers (23 clinically localized and one metastatic). Our initial analysis of the human genome database revealed that the entire coding region (and 3'-untranslated region) is present on a single exon. We therefore designed PCR primers to amplify the entire coding region as overlapping PCR products that were then isolated and directly sequenced. All tumor specimens were at least 80% carcinoma, and we have detected regions of loss of heterozygosity in all of these specimens using PCR-based approaches (40). A single base pair alteration (T to C) was detected in one clinically localized prostate cancer at bp 1250 that would lead to an amino acid change from tyrosine to histidine at amino acid residue 304 of the Sprouty1 protein. Analysis of DNA from benign tissue from the same patient revealed the exact same alteration. Therefore, this sequence variation represents either a germline mutation or a relatively uncommon polymorphism. No evidence of mutation was seen in the Sprouty1 coding region in any other sample.

The Effect of Sprouty1 Expression in Human Prostate Cancer Cells. To ascertain the biological effect of Sprouty1 expression in human prostate cancer cells, pcDNA-Sprouty1 (encoding the full

length of Sprouty1 sequence) was transfected into the human prostate cancer cell lines LNCaP and PC3, and transfected cells were selected in Geneticin. Only rare colonies were observed in both the LNCaP and PC3 cells transfected with the Sprouty1 plasmid, whereas numerous colonies were observed when PC3 and LNCaP were transfected with the vector only plasmid (Fig. 5). The inhibition of colony formation by Sprouty1 was more than 99%, suggesting that sustained overexpression of Sprouty1 has a markedly deleterious effect on prostate cancer cells proliferation and/or survival. To determine whether more modest, transient expression of Sprouty1 could also inhibit prostate cancer cell proliferation, we analyzed proliferation of LNCaP cells after transient transfection of a Sprouty1 expression plasmid. Sprouty1 expression levels were analyzed on the same cells by Western blotting. Despite the modest increase in Sprouty1 expression under these conditions (Fig. 6A), there was a profound decrease in proliferation in the cells transfected with the Sprouty1 expression construct (Fig. 6B). To confirm that the inhibition of growth is due to Sprouty1 expression, we repeated the transient transfection assay this time by cotransfecting the Sprouty1 plasmid with a vector containing GFP. Cells that were GFP positive were sorted and used in cell proliferation analysis. Fig. 6C shows that LNCaP cells transfected with the Sprouty1 plasmid had a profound decrease in proliferation when compared with the GFP only transfection, which is consistent with our initial observation. Thus in prostate cancer cells, either sustained or transiently increased Sprouty1 expression markedly inhibits proliferation, which is similar to observations made in other systems (30, 41, 42).

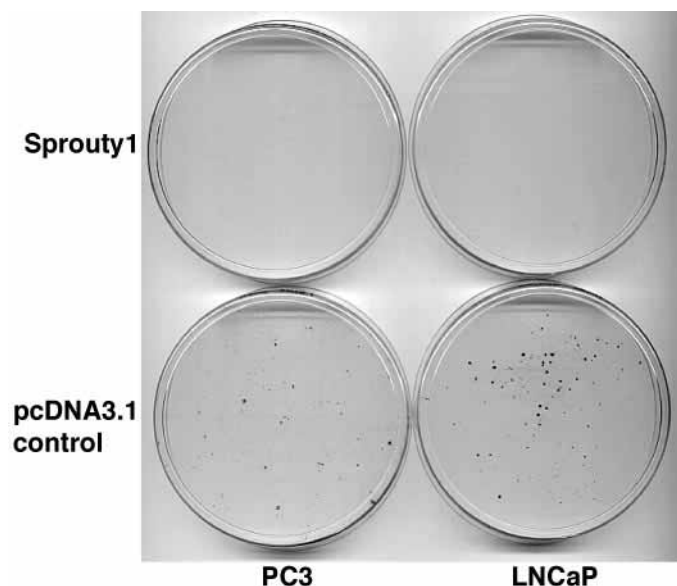


Fig. 5. Stable transfections of Sprouty1 plasmid into prostate cancer cells. Prostate cancer cell lines LNCaP and PC3 were each transfected with a Sprouty1 cDNA cloned into pcDNA3.1 or the pcDNA3.1 vector alone. After 2 weeks of selection in Geneticin, cells were fixed and stained with crystal violet. Representative plates from each transfection are shown.

DISCUSSION

One important way that cancers can grow in an uncontrolled way is by expressing increased amounts of growth factors and/or having increased activity of growth factor receptors. Cancers may also exhibit loss of regulatory factors that control the activity of growth factor receptors. For example, the PTEN gene, which is a negative regulator of the phosphatidylinositol 3-kinase pathway, is inactivated in a variety of human malignancies, including prostate cancer (40). In the present study, we have found that one protein that may have an important role in controlling growth signals, Sprouty1, is decreased in almost 40% of human prostate cancer tissues when compared with normal prostate tissue in the same patient. It is not surprising that only a fraction of prostate cancers show decreased Sprouty1 expression. All epithelial malignancies have a variety of genetic and epigenetic alterations, and in general, only a fraction of cases of a given tumor type have a specific alteration. For example, only a subset of prostate cancers have alterations of the PTEN tumor suppressor gene (40). There are exceptions to this rule, for example, the very high prevalence of RAS mutations in pancreatic cancer, but such instances are distinctly uncommon in epithelial malignancies.

In human prostate cancer tissues, there is up-regulation of FGFs when compared with uninvolved prostate. We have shown previously that the tissue content of FGF2 is increased more than 2-fold in prostate cancer tissue compared with control prostate, whereas FGF7 is present at essentially equal levels (5). FGF9 is also present at equal levels in normal and neoplastic prostate tissue based on ELISA assay.⁵ Finally, FGF6 is expressed as an autocrine growth factor in 40% of prostate cancers (6), and the majority of prostate cancers express FGF8 in a similar manner (7–9). Thus, the decreased Sprouty1 expression seen in 40% of cancers cannot be due to loss of FGF ligands in these cases. Loss of Sprouty1 expression may give rise to unrestrained signal transduction by FGFs that could result in increased proliferation (2, 6) and/or decreased cell death (43) in prostate cancer and potentiate the effects of increased FGFs and FGFRs in prostate

cancer. We have also seen that some prostate cancers have increased Sprouty1 expression. These cancers must have other alterations that allow them to resist the negative growth regulatory effects of Sprouty1 that were seen in LNCaP cells, which have very low basal Sprouty1 expression. The nature of these alterations is currently under investigation.

We have found that Sprouty1 expression in prostate cancer cells *in vitro* is no longer up-regulated by FGF2. This could be due to

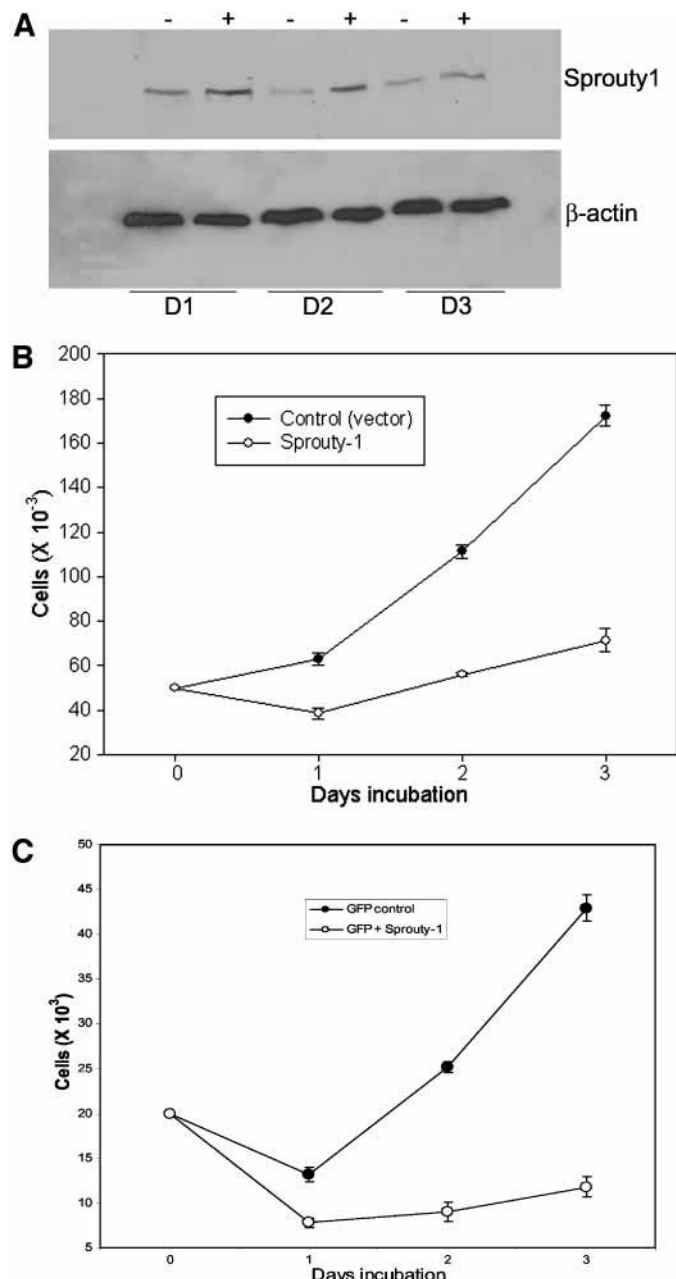


Fig. 6. Transient transfection of Sprouty1 in LNCaP prostate cancer cells. A, protein extracts were collected from LNCaP cells 1, 2, or 3 days after transfection with pcDNA3.1 (–) or Sprouty1 cDNA in pcDNA3.1 (+) and analyzed by Western blotting with either anti-Sprouty1 antibody or control anti- β -actin antibody. B, the LNCaP prostate cancer cell line was transfected with a Sprouty1 cDNA cloned in the mammalian expression vector pcDNA3.1 or the pcDNA3.1 vector only. At the indicated times after transfection, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate, and the SD is shown. C, the LNCaP cells were either transfected with pEGFP alone or cotransfected with pcDNA3.1-Sprouty1. The GFP-positive cells were sorted using flow cytometry analysis and replated. At the indicated times after cell sorting, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate, and the SD is shown.

⁵ M. Ittman, unpublished data.

decreased transcription, for example, secondary to alterations of *trans*-acting factors, such as loss of essential transcription factors or up-regulation of negative regulatory factors, or it could be a consequence of increased mRNA degradation. The loss of expression of Sprouty1 in prostate cancer *in vivo* could also be due to alterations in the gene itself, such as deletion or methylation. The *Sprouty1* gene maps to chromosome 4q27.⁶ Comparative genomic hybridization shows loss of this region in 23% of prostate cancers examined (44), so it is possible that in some cases, decreased Sprouty1 expression could be due to hemi- or homozygous deletion of the Sprouty1 locus. Methylation has been shown to be involved in loss of gene expression in prostate cancer (45). Systematic studies of Sprouty1 promoter methylation and correlation with gene expression in prostate cancers *in vivo* will need to be carried out to exclude this possibility. Additional work is currently under way seeking to understand the molecular mechanisms that lead to decreased Sprouty1 mRNA in prostate cancer.

In summary, there is considerable evidence showing up-regulation of FGFs in prostate cancer based on studies in animal models, human tissues, and human prostate cancer cell lines. Sprouty1, an inhibitor of FGF signal transduction, is decreased in approximately 40% of clinically localized prostate cancers and may lead to the unrestrained signal transduction by FGFs and hence tumor progression. Because Sprouty1 may inhibit the transduction of many growth factor signals, it could be an attractive target to explore for drug intervention or gene therapies of prostate cancer.

ACKNOWLEDGMENTS

The assistance of Anna Frolov with the statistical analysis and Terry Timme with use of the NucleoVision imaging workstation is gratefully acknowledged.

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