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14. ABSTRACT
PURPOSE: The Sprouty gene family negatively regulates growth factor-induced receptor tyrosine kinase signaling in human prostate cancer (PCa). The purpose of this study was to investigate the expression of Sprouty1 in PCa, determine its biological function and elucidate the molecular mechanism(s) regulating its expression in PCa. RESULTS: Using immunohistochemical and quantitative RT-PCR analysis, I have shown that Sprouty1 is down-regulated in PCa tissues compared to matched normal prostate tissues. Transient forced expression of Sprouty1 significantly inhibited PCa proliferation, while stable over-expression of Sprouty1 was deleterious to PCa cell growth. I have shown by pyrosequencing and other methylation assays that DNA methylation of Sprouty1 promoter is a key mechanism for down-regulating Sprouty1 expression in PCa. Transcriptional studies have identified GATA and EGR transcription factors as key transcriptional repressors modulating Sprouty1 expression in PCa. CONCLUSION: I have demonstrated that the expression of Sprouty1, whose gene products encodes for a potential tumor suppressor activity is down-regulated in PCa. I have also shown DNA methylation and transcriptional repression to be key mechanisms for dys-regulating Sprouty1 expression in PCa.

15. SUBJECT TERMS
Sprouty1; DNA methylation; immunohistochemistry, RT-PCR, gene expression, transcriptional regulation; shRNA

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INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths in men in the United States. There is abundant evidence indicating that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (for review see [1]). Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development [2]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis [3-5]. While Drosophila has only one Sprouty gene, at least four Sprouty homologues (Sprouty1-4) have been found in humans and mice [2,6,7]. Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [8-15]. Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein, FRS2 [3,16] or the inhibition of Raf [11,17]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript [7] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [18]. Given that Sprouty proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

My research work has shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1, Sprouty2 and Sprouty4 are down-regulated in a subset of prostate cancers tissues when compared with normal prostate tissues [19,20]. This observation is supported by work carried out by McKie et al., [21] who observed that Sprouty2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). The decrease in Sprouty 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 FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

I and others have shown epigenetic inactivation to be a key mechanism for silencing Sprouty proteins in the prostate. For instance, I have observed promoter methylation at Sprouty4 CpG islands in prostate cancer. More than half of all prostate cancer tissue DNAs were methylated in this region and methylation significantly correlated with decreased Sprouty4 expression. Furthermore the treatment of prostate cancer cells with 5'Aza-2-deoxycytidine (5-aza-dC) a demethylation agent, reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 down-regulation [20]. Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated [21]. The suppressed Sprouty2 expression correlated with methylation of the CpG region in clinical samples indicating that methylation of the Sprouty2 promoter was the likely cause of its transcriptional inactivation in the prostate.

However, promoter methylation does not seem to explain Sprouty2 inactivation in breast cancer. Cultured breast cancer cell lines in the presence of 5-aza-dC, did not reactivate the expression of Sprouty2 and only minimal and patient specific methylation of the Sprouty2 CpG islands was found [22] indicating cancer-specific mechanisms of Sprouty down-regulation.

In studying the molecular mechanisms regulating Sprouty1 expression, I have observed strong transcriptional activity of *Sprouty1* promoter in prostate cancer cell lines even though Sprouty1 protein expression is down-regulated in these same cell lines. Treatment of cultured prostate cancer cell lines in the presence of 5-aza-dC reactivated the expression of Sprouty1 protein suggesting that epigenetic modification of the binding sites for transcription factors such as Sp1 may result in a refractory transcriptional response even in the presence of necessary trans-acting activities and/or transcriptional repression maybe regulating Sprouty1 expression in prostate cancer. I summarize my overall observations over the entire research period, describing the biological function and the regulation of Sprouty gene family in prostate cancer and I discuss how Sprouty proteins can be explored for therapeutic intervention in prostate cancer treatment.

BODY

As outlined in my Statement of work, I sort to accomplish 3 main tasks during the 3 years of funding. I have accomplished all these tasks within the proposed research period. I requested a 1 year no cost extension of the proposed research period because of a change in institutions from Baylor College of Medicine in Houston (Texas) to Howard University in Washington (D.C) in the final year of the research period in order to

complete my remaining studies. Details of the accomplished proposed tasks as outlined in my statement of work are given below as well as in the attached publications.

Task 1. Comprehensive evaluation of Sprouty1 gene inactivation in human prostate cancer (Months 1-36). The objective of this task was to investigate if Sprouty1 expression is down-regulated in prostate cancer tissues when compared to benign prostate tissues and to ascertain the molecular mechanisms underlying the inactivation of Sprouty1 expression in human prostate cancer.

A. Perform immunohistochemistry studies on prostate tissue samples. 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.

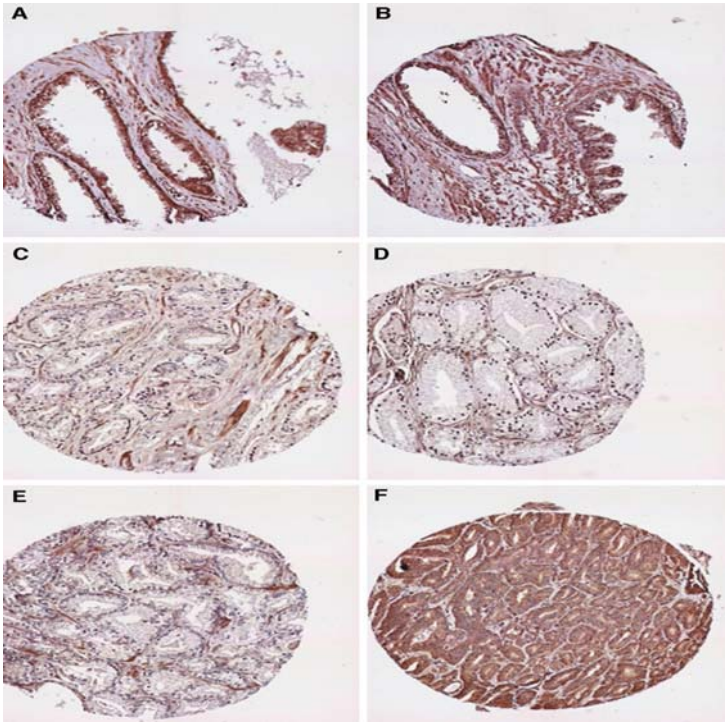

Fig. 1. Immunohistochemical analysis of Sprouty1 expression in tissue microarrays. Expression of Sprouty1 in normal prostate (*A* and *B*) and prostate cancer (*C*--*F*) was determined using tissue microarrays as described in Cancer Research 64; 4728-4735 (see Ref. 23 as an attachment)." *A* and *B*, 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.

B. Perform quantitative RT-PCR analysis using RNA samples extracted from matched benign and prostate cancer tissues. Using quantitative real-time PCR analysis, I found that Sprouty1 mRNA levels were significantly decreased in prostate cancer tissues in comparison with normal prostate tissue. In addition, I have also demonstrated that Sprouty2 and 4 mRNA were down-regulated in prostate cancer tissues when compared with normal prostate tissues (data not shown; see Ref. 24 as an attachment).

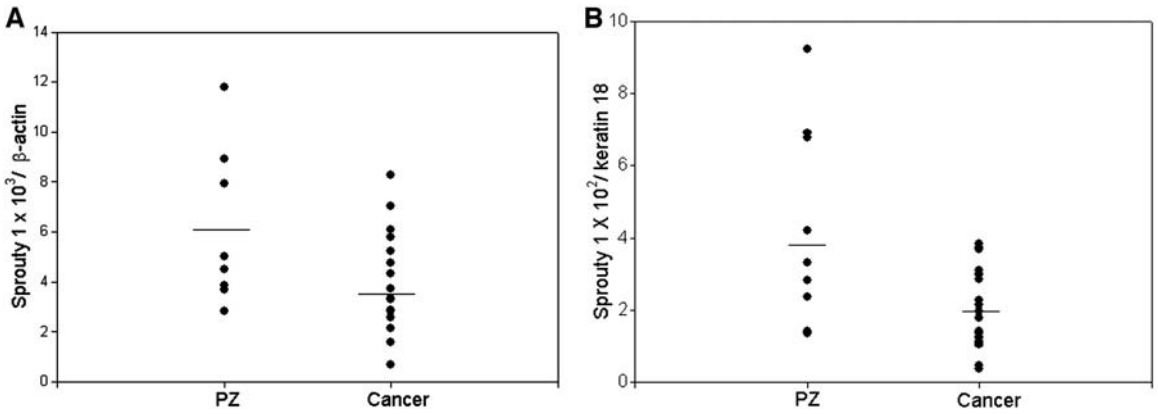

Fig. 2. Sprouty1 expression in normal prostate and prostate cancer as determined by quantitative reverse transcription-PCR. Sprouty1 expression in normal prostatic peripheral zone (*PZ*) and cancer tissues was assessed by quantitative reverse transcription-PCR using a real-time thermal cycler (iCycler; Bio-Rad). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts x 10^3 to ß-actin transcripts (*A*) or Sprouty1 transcripts x 10^2 to keratin 18 transcripts (*B*). The Sprouty1, ß-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 ß-actin and keratin 18 normalization.

C. Perform mutational analysis of the Sprouty1 coding sequence. To determine whether Sprouty1 is inactivated by mutation in prostate cancer, I analyzed DNAs isolated from 24 prostate cancers (23 clinically localized and one metastatic) of Sprouty1 coding sequence using overlapping sets of PCR primers designed to amplify the entire coding region. PCR products were then isolated and directly sequenced. 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 (data not shown).

D. Perform methylation analysis of the Sprouty1 promoter region in human normal prostate and prostate cancer. The human *Sprouty*1 transcript consists of two splice variants, 1a [23] and 1b [24] arising from two alternative promoters that map to human chromosome 4q27-28 and 4q25-28 respectively. Using the MethPrimer software package for CpG islands identification (http://www.urogene.org/methprimer/), I have identified 2 separate CpG islands: 1 spanning about 2 kbp of the *Sprouty*1a promoter region and the other spans about 110 bp of *Sprouty*1b promoter region (data not shown). Using a series of unidirectional PCR based deletion analysis followed by luciferase reporter assay, I have identified optimal promoter activity for Sproutyla Fwd5 and Sproutylb Fwd3 (described in task 2) and these are here after referred to as Sprouty1a and Sprouty1b promoter respectively. To investigate whether constitutively active *Sprouty*1a and/or *Sprouty*1b promoter activity was inhibited by the methylation of the promoter CpG island, I modified the promoter constructs with *Sss*I methylase treatment and examined the activity of the methylated promoter. When the *Sss*I methylated or non-methylated *Sprouty*1a and *Sprouty*1b promoter constructs were each transiently transfected into LNCaP cells the activity of the methylated Sprouty1a promoter was only 5 % of that of the unmethylated construct (Fig 3). On the other hand, CpG methylation of *Sprouty*1b

construct did not show significant effect on its activity when compared to the control unmethylated construct (Fig 3). This observation indicates that methylation of the *Sprouty*1a promoter may be involved in the control of *Sprouty*1 gene expression.

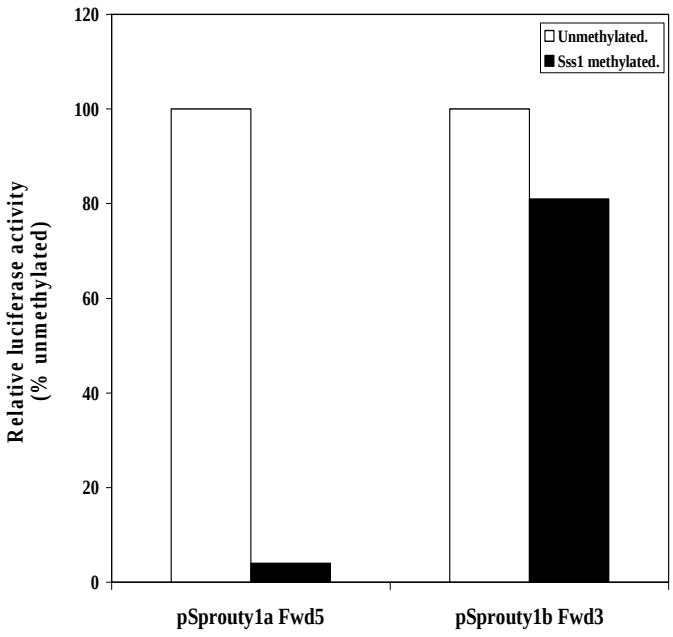

Fig. 3. Effect of *in vitro* methylation on the activity of the *Sprouty*1 promoter. *Sprouty*1a (pSprouty1a Fwd5) and 1b (pSprouty1b Fwd3) promoters Ire methylated *in vitro* by *Sss*I methylase. Methylated and unmethylated *Sprouty*1a and 1b promoters were transfected into LNCaP cells and assayed for luciferase activity. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Results are shown as percentages, with luciferase activity due to unmethylated promoter designated as 100%. Data represents the mean of triplicate experiments.

To further investigate whether DNA methylation plays a role in Sprouty1 expression, I tested the hypothesis that pharmacological modulation of methylation can reactivate gene expression. To achieve this, I treated pNT1A, DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (5-aza-dC). As shown in Fig 4, treatment of the prostate cancer cell lines, DU145, PC3 and LNCaP with 5-aza-dC (2 μM) led to a significant increase in Spry1 mRNA expression in the prostate cancer cell lines. Taken together, these data suggests that promoter methylation may play a role in down-regulating Spry1 expression in these cell lines and human prostate tumors.

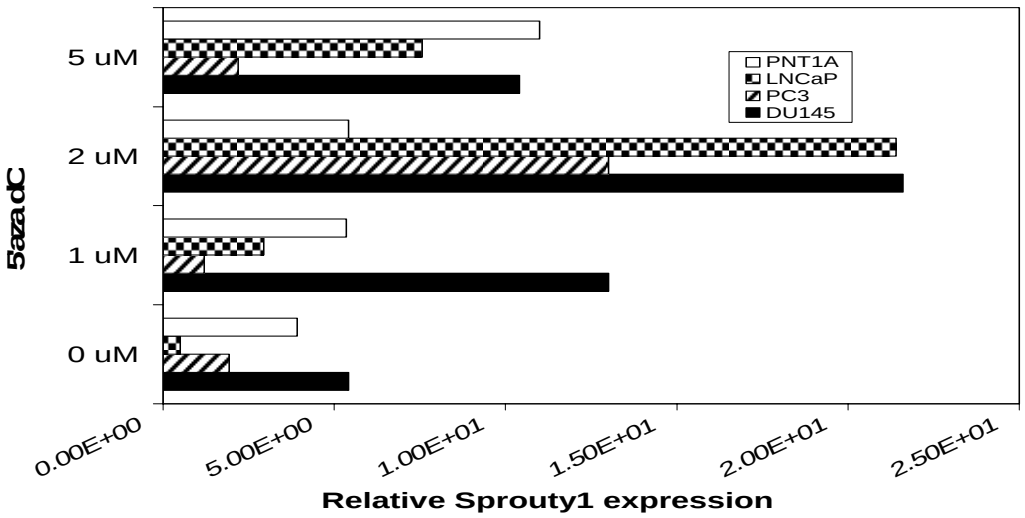

Fig. 4. Demethylation and Sprouty1 expression. Prostate cancer cell lines; LNCaP, PC3 and DU145, and immortalized primary prostatic epithelial cells; pNT1A were each treated with 5'-aza-2'-deoxycytidine (5'-aza-dC) at the indicated concentrations for 96 hours. Sprouty1 mRNA expression, expressed as relative Sprouty1 expression was determined by quantitative RT-PCR using iCycler and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

To verify that Sprouty1a promoter is methylated in human prostate tumors, I used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA of Sprouty1 3 CpG sites. Typical examples of bisulfite methylation profiles presented as pyrogram are shown for pNT1A and LNCaP cells (Fig 5). As shown in the pyrogram, the pNT1A cells demonstrated on average 5% methylation at each CpG site. On the other hand, LNCaP cells demonstrated an average of 30% methylation at each CpG site suggesting that Sprouty1 is hypermethylated in LNCaP cells but shows low methylation in pNT1A cells.

For 15 pairs of normal and matched prostate cancer tissue that I had both RNA and DNA samples, I measured the Sprouty1 expression using RT-PCR and compared it to the average percentage of methylation at the 3 Sprouty1 CpG sites for each patient sample (Fig. 6). The result demonstrates that Sprouty1 mRNA levels were significantly decreased in prostate cancer in comparison with normal prostate, whereas the %

methylation of the Sprouty1 promoter was significantly increased in prostate cancer tissues compared with normal prostate tissues. The inverse association between Sprouty1 mRNA expression and % DNA methylation level suggests that DNA methylation is an important mechanism for down-regulation of Sprouty1 in prostate cancer. This observation is supported by our published data showing that methylation of Sprouty4 significantly correlated with decrease in Sprouty4 expression in prostate cancer [20]. My data clearly demonstrate methylation as a key mechanism for the inactivation of Sprouty genes in human prostate cancer.

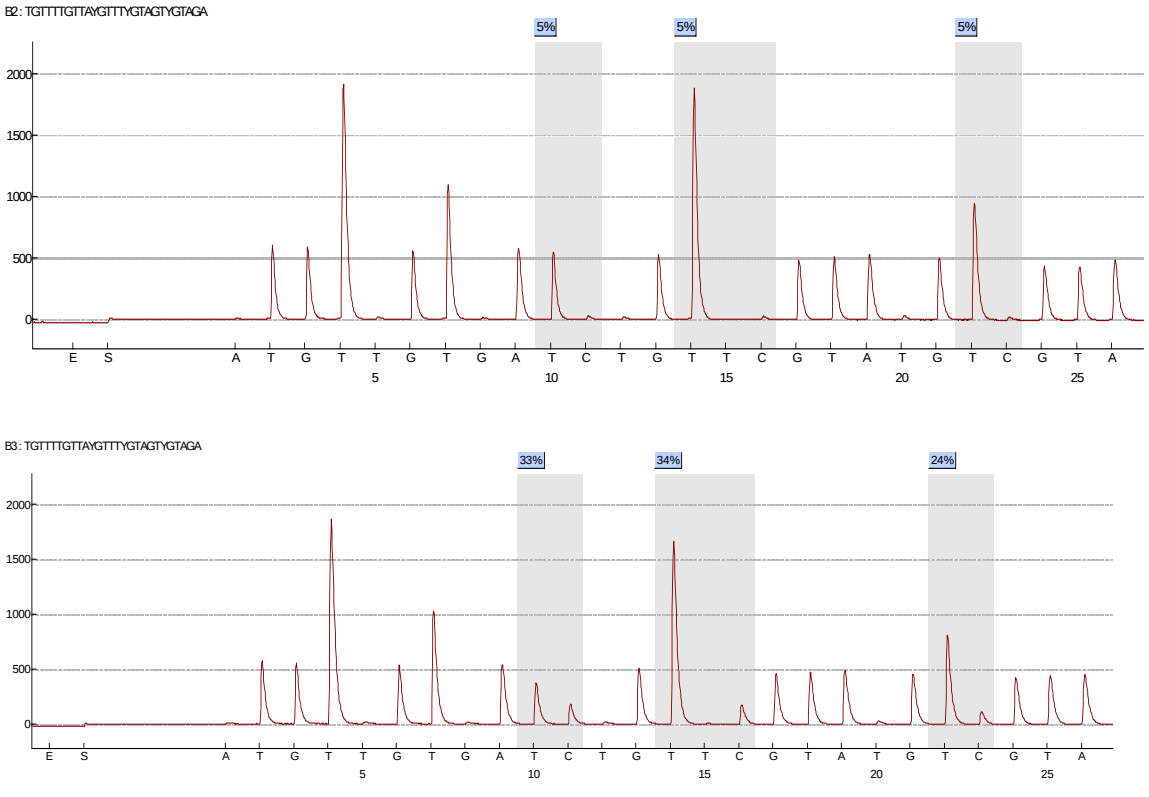

Fig. 5. Representative program traces for Sprouty1. Gray columns, regions of C to T polymorphic sites. Genomic DNA extracted from the immortalized normal prostate epithelial cell line pNT1A (top panel), shows low methylation, whereas genomic DNA extracted from prostate cancer cell line, LNCaP (bottom panel) shows a significant level of methylation at all three CpG sites. Top, percentage of methylation at each CpG site. Y-axis, signal peaks proportional to the number of nucleotide incorporated. X-axis, the nucleotide incorporated.

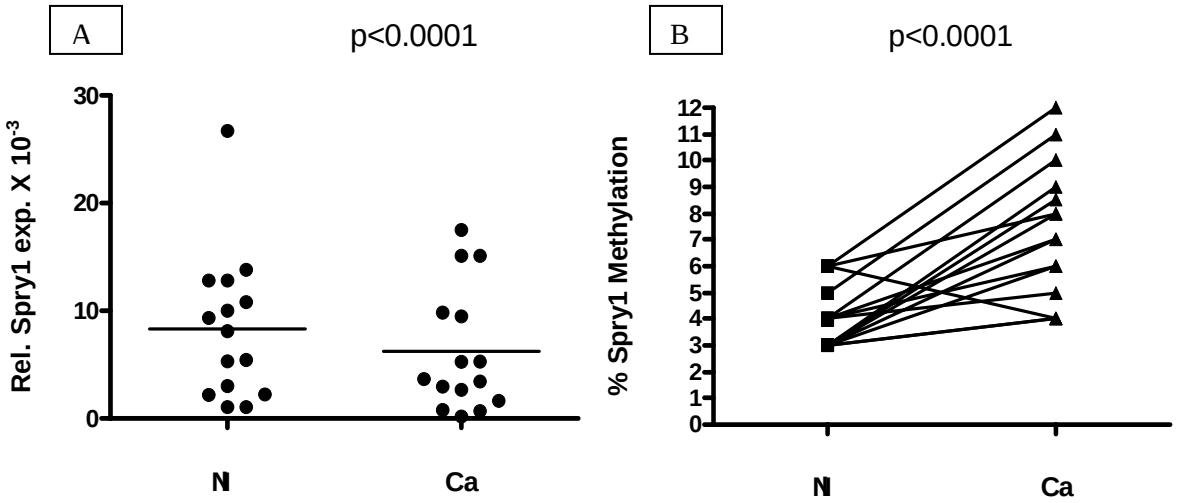

Fig. 6. A. Sprouty1 expression in normal prostate and prostate cancer as determined by quantitative reverse transcription-PCR. Sprouty1 expression in normal prostate tissues (Nl) and cancer tissues (Ca) was assessed by quantitative reverse transcription-PCR using a real-time thermal cycler (iCycler; Bio-Rad). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts x 10^3 to ß-actin transcripts (*A*). The % methylation level in match pair of normal prostate tissues (Nl) and cancer tissues (Ca) was analyzed using pyrosequencing (B).

E. Investigating the biological function of Sprouty1 expression in prostate cancer cell lines. 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. 7). 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, I 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. 8*A*), there was a profound decrease in proliferation in the cells transfected with the Sprouty1 expression construct (Fig. 8*B*). To confirm that the inhibition of growth is due to Sprouty1 expression, I repeated the transient transfection assay this time by cotransfecting the Sprouty1 plasmid with a vector containing GFP. Cells that Ire GFP positive Ire sorted and used in cell proliferation analysis. Fig. 8*C* 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.

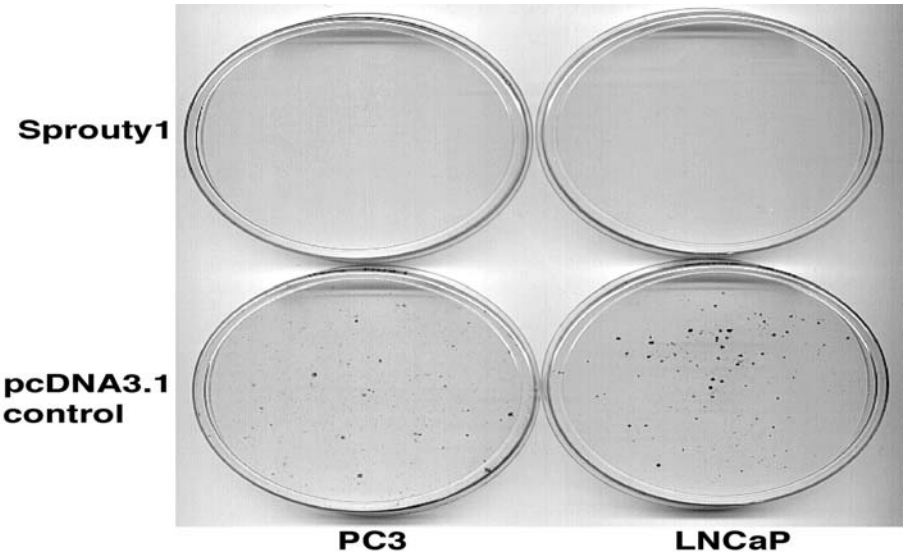

Fig. 7. Stable transfections of Sprouty1 plasmid into prostate cancer cells. Prostate cancer cell lines LNCaP and PC3 Ire each transfected with a Sprouty1 cDNA cloned into pcDNA3.1 or the pcDNA3.1 vector alone. After 2 Ieks of selection in Geneticin, cells Ire fixed and stained with crystal violet. Representative plates from each transfection are shown.

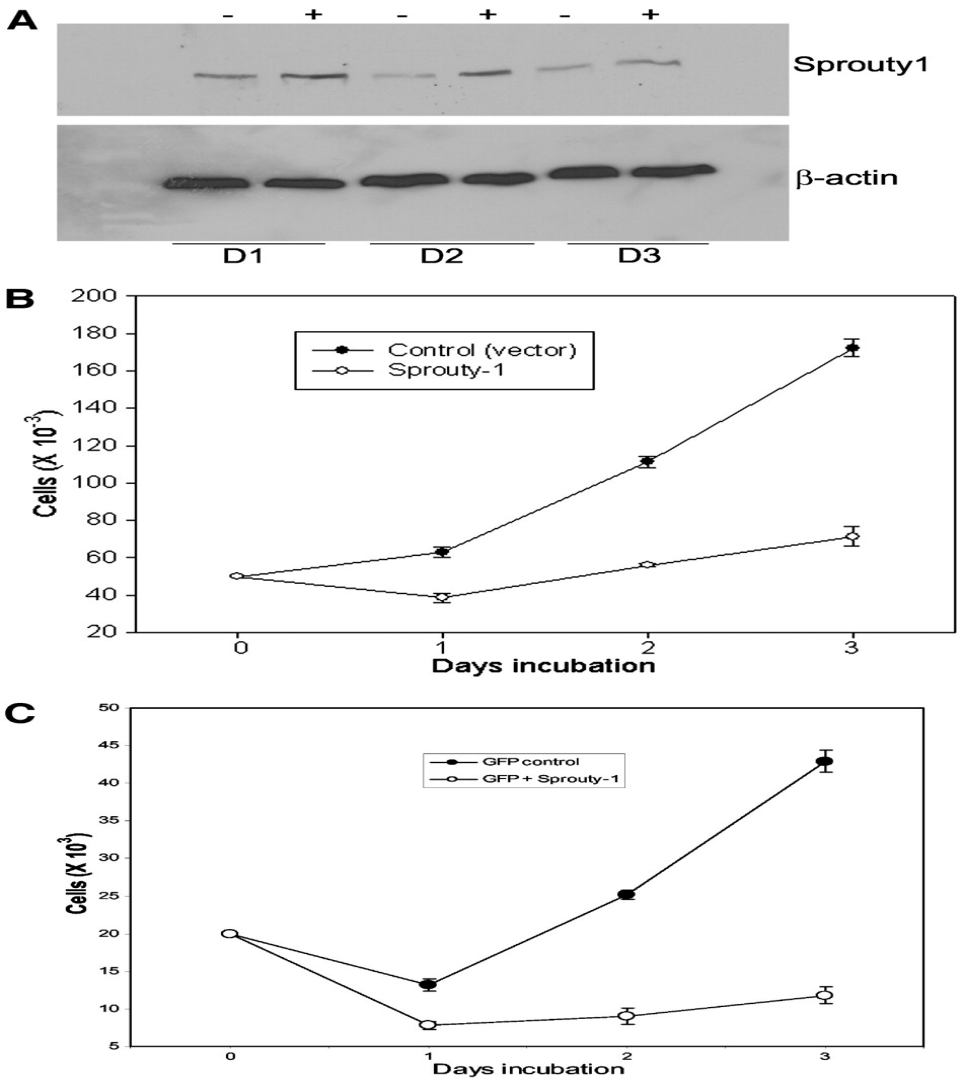

Fig. 8. Transient transfection of Sprouty1 in LNCaP prostate cancer cells. *A*, protein extracts were collected from LNCaP cells 1, 2, or 3 days after transfection with pcDNA3.1 (–) or Sprouty1 cDNA in pcDNA3.1 (+) and analyzed by western blotting with either anti-Spouty1 antibody or control anti-ß-actin 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.

Task2: Identifcation of the transcriptional elements that regulates human Sprouty1 expression (1-18 months). The objective of this task was to identify the putative transcriptional start site of the Sprouty1 promoter, map the promoter region in order to identify the transcription factor binding sites for the key transcription factors that regulate Sprouty1 expression.

A. Perform primer extension and 5'RACE analysis to identify Sprouty1 transcription start site. I used primer extension and 5' RACE analysis on commercially available human prostate mRNA using sequence information from the longest Sprouty1 EST clone and NCBI high throughput genomic sequence data (Fig. 9). I performed 5'-RACE analysis using poly $(A)^+$ RNA from fetal human lung and a *Sprouty*1 specific primer. I observed multiple bands after amplification, the largest of about 275 bp (Fig 9). Sequence analysis identified multiple transcription initiation sites within the region -315 to -305 nucleotides from the first ATG codon in a Kozak consensus sequence. The 5'-most start site found is located at nucleotide position 160026 of the published sequence (GenBank accession no. AC026402). Because this region corresponds to the 5'-UTR of Splice variant 1b, this may represent the corresponding promoter region. Using similar approach I identified the transcription start site for Splice variant 1a to be at nucleotide position 162754 in the same published sequence (GenBank accession no. AC026402).

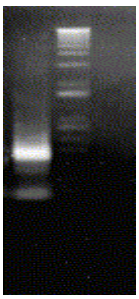

Fig. 9. 5' RACE analysis of poly $(A)^+$ RNA from fetal human lung using Sprouty1 specific primer. Left lane shows the PCR amplification product. Right lane shows 100bp marker.

B. Functional characterization of Sprouty1 promoter region. To localize the DNA elements that are important for promoter activity, I carried out a series of unidirectional deletion analyses of up to 2 kb and approximately 1 kb of the 5'-flanking region of *Sprouty*1a of *Sprouty*1b splice gene variants, respectively. Deletion fragments were generated by PCR and cloned into the promoterless pGL3-Basic, a luciferase reporter vector. Each resulting recombinant construct was then transiently transfected along with the internal control pSV β-galactosidase plasmid into prostate cancer cell lines; LNCaP, PC3 and DU145 and the immortalized normal prostate cell line pNT1A. After 48 h, cell extracts were prepared and luciferase activity was measured and normalized to β-galactosidase activity. As shown in Fig 10, the promoter activities demonstrated significant difference betIen Splice 1a (Fig. 10A) and 1b (Fig. 10B) variants. *Sprouty*1a promoter strength was between 2 to 5 fold above the basal level. Whereas *Sprouty*1b promoter activity was between 40 and 900 fold above basal level depending on the cell line. Furthermore, the reporter gene expression levels showed significant differences among the different prostate cell lines suggesting that cell-specific element(s) may be present in these sequences. Interestingly, the androgen-dependent cell line, LNCaP which expressed the least Sprouty1 protein level as determined by western blot analysis [19] showed the strongest promoter activity; expressing over 7 fold higher promoter activity than any of the other cell lines. The maximum promoter activity varied for each cell line: In LNCaP cells the maximum promoter activity was observed from the *Sprouty*1b Fwd3 (-175 to +50) construct. In the androgen independent prostate cancer cell lines PC3 and DU145, maximum promoter activity was observed from the *Sprouty*1b Fwd4 (-233 to + 50) and *Sprouty*1b Fwd6 (-530 to + 50) constructs respectively. In the immortalized

normal prostate cell line pNT1A, maximum promoter activity was seen with the *Sprouty*1b Fwd5 (-305 to + 50) construct. Because strong promoter activity was observed at the *Sprouty*1b promoter region, I believe this region has the transcriptional elements and enhancer sequence(s) necessary for *Sprouty*1 gene regulation. Therefore all subsequent promoter analysis was done at the *Sprouty*1b locus and is hereafter referred to as *Sprouty*1 promoter.

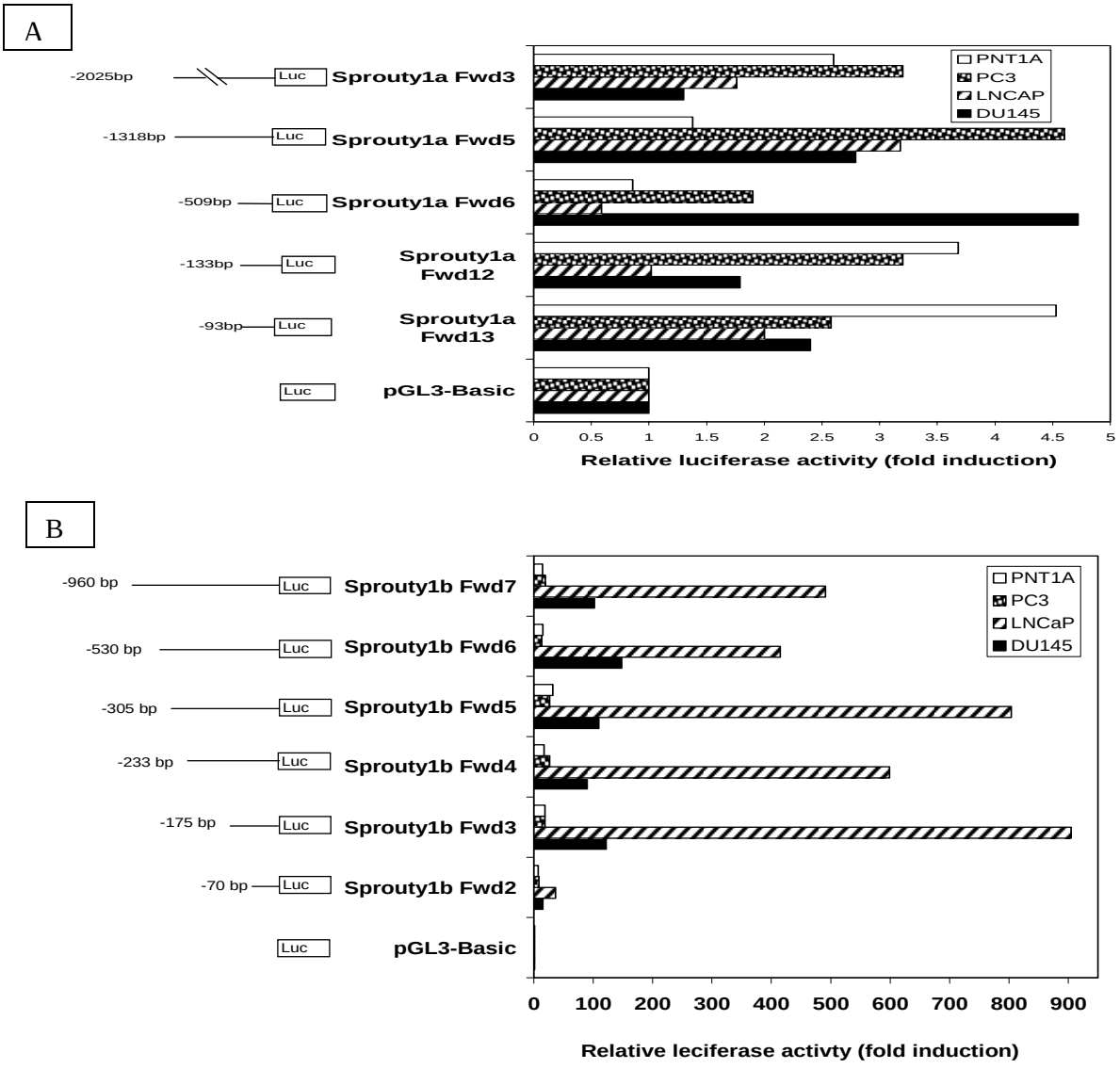

Fig. 10. Progressive deletion analysis of the 5'-flanking region of splice variant 1a and 1b of the human Sprouty1 gene. The schematic diagrams represent a series of *Sprouty*1a (**A**) and 1b (**B**) gene constructs with variable 5'-ends as indicated. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Data represents the mean of triplicate experiments.

C. Comparative sequence analysis of the Sprouty1 promoter locus. To further characterize the *Sprouty*1 promoter region, I searched for transcription factor binding sites using the MatInspector program [25]. I analyzed 2 kb of the genomic AC026402 sequence upstream of the *Sprouty*1b transcription start sites, using computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). I found potential binding sites for several TFs including GATA1 [26], EGR [27], ZBP [28], ETS [29], HIC [30] and FKHD [31] in the proximal promoter region. The human and murine [32] *Sprouty*1 5'-flanking region upstream of their transcription start sites Ire aligned for sequence comparison. Over the entire 5'-flanking region of the human *Sprouty*1 promoter, only a very short region in *Sprouty*1b promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Sprouty*1 promoter. As illustrated in Fig 11, Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3 [33], and WTE [34] are conserved between the two species. Interestingly, the nucleotide sequences immediately upstream from the EGR motif diverge in these species. Furthermore, I did not see any sequence homology between the human *Sprouty*1 promoter region and that of the published *Sprouty*2 [35] or *Sprouty*4 [36] promoters. The high sequence homology in the *Sprouty*1 promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Sprouty*1 gene regulation (Fig 11).

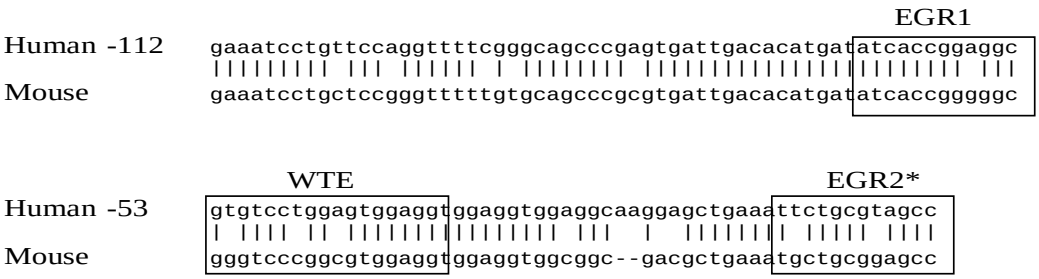

Fig. 11. Alignment of sequence in the 5'-flanking region of human and murine Sprouty1 gene. The nucleotide sequences surrounding the transcription start site and the 5'-flanking region Ire compared for human and mouse Sprouty1 gene. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. An asterics (*) indicate core similarity of 1.000 with human sequence.

C. Identification of transcription factors binding to Sprouty1 promoter region using electrophoretic mobility shift assays. To verify the binding interaction of the Sprouty1 consensus sequence in vivo, I performed electrophoretic mobility shift assay (EMSA) using designed consensus radiolabelled oligonucleotide probes to recognize EGR1, PBX1, HNF-4 and SP1 and nuclear extracts prepared from either LNCaP, PC-3 or pNT1A. Since all three cell lines demonstrated a similar band-shift pattern with each probe, only results using nuclear extracts from LNCaP cells are shown in Fig 12. Three protein-DNA complexes (C1, C2 and C3) were formed with each of the oligonucleotide probes. These complexes represented sequence-specific interactions of proteins within this region, since the addition of 100-fold molar excess of the corresponding unlabelled oligonucleotide probe was able to compete away these complexes. To characterize these complexes further, supershift EMSA was conducted using specific antibodies. The result showed that although a supershift band was not clearly identified, addition of anti-SP1, clearly abrogated the formation of C2, whereas supershift with anti-PBX1 and anti-HNF4 reduced the signal intensity of the respective C2 complex suggesting that the C2 complex

is formed with SP1, PBX1 and HNF4 respectively. I did not see any significant effect of anti-EGR on the protein-DNA complexes. However, when the EGR1 consensus binding sequence was mutated (Mut EGR1), I observed a new complex migrating very close with complex C2. Cold competition assay with wild-type EGR1 oligonucleotide competed out complex C2 totally but only partially competed the new complex. Furthermore, supershift assay successfully competed C2. This indicates that EGR1 protein preferentially recognize and interact with the wild-type EGR1 consensus binding sequence (Fig. 12).

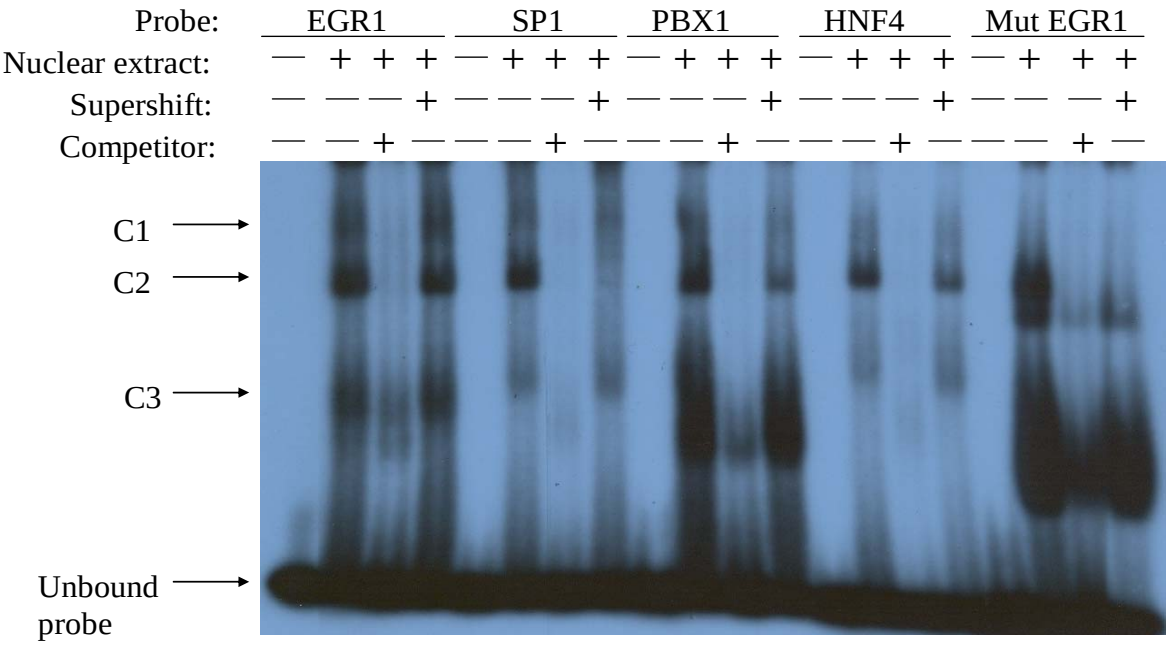

Fig. 12. Identification of transcription factors binding to the Sprouty1 promoter. The analysis includes EGR1, SP1, PBX1 and HNF4. Radiolabelled double-stranded DNA oligonucleotides (probes) Ire incubated with or without nuclear extracts from LNCaP cells. Protein-DNA complex is indicated (C1, C2, C3), free or unbound probe is indicated at the bottom. Specificity of DNA-protein complex was investigated using 100 fold molar excess of corresponding unlabelled probe shown as competitor or the corresponding antibody shown as supershift.

D. Identification of transcription factor binding activity for Sprouty1 promoter region usingTranSignal Protein/DNA Arrays. In order to assess the activities of the transcription factors regulating Sprouty1 expression, I employed a protein/DNA array technology. This array is a high-throughput, DNA-based system that facilitates profiling the activities of multiple TFs in one assay (see www.panomics.com/pdf/PD_Array_1_with_ap.pdf for the list of TFs binding sites on the array). To identify transcription factors whose activities might be altered in response to FGF2 stimulation, LNCaP cells were stimulated with or without recombinant FGF2 protein. As shown in Fig. 13. The array analysis detected increased activities of several TFs in the LNCaP cells stimulated with FGF2 (Fig. 13B) when compared with the unstimulated LNCaP cells (Fig. 13A). In particular, the activity of EGR, ETS, GATA, HNF-4, PBX1 and SPI which share consensus binding sites on Sprouty1 promoter were up-regulated in LNCaP cells stimulated with FGF2. Next, I compared the profile of the transcriptional activities of pNT1A, LNCaP and DU145 cells in response to FGF2 stimulation as shown in Fig. 14. The studies demonstrated differential activation of a number of transcription factors with consensus binding sites for Sprouty1 promoter in these cell lines. This includes AP ½, ARE, c-Myb, CREB, E2F1, EGR, ERE, GATA, Smad SBE, Stat 1-6, USF-1 and HSE following FGF2 treatment. The overall pattern of the response element occupancy indicates the activation of high number of transcription factors in the cancer cell lines (LNCaP and DU145) when compared to the normal pNT1A cell line. Of particular interest is the activation of transcriptional activator/repressor, GATA, specifically in the androgen dependent cell line LNCaP (indicated as boxed) which may be responsible for the low expression of Sprouty1 in

LNCaP cells when compared to pNT1A and DU145 as determined by western blotting (Fig. 15).

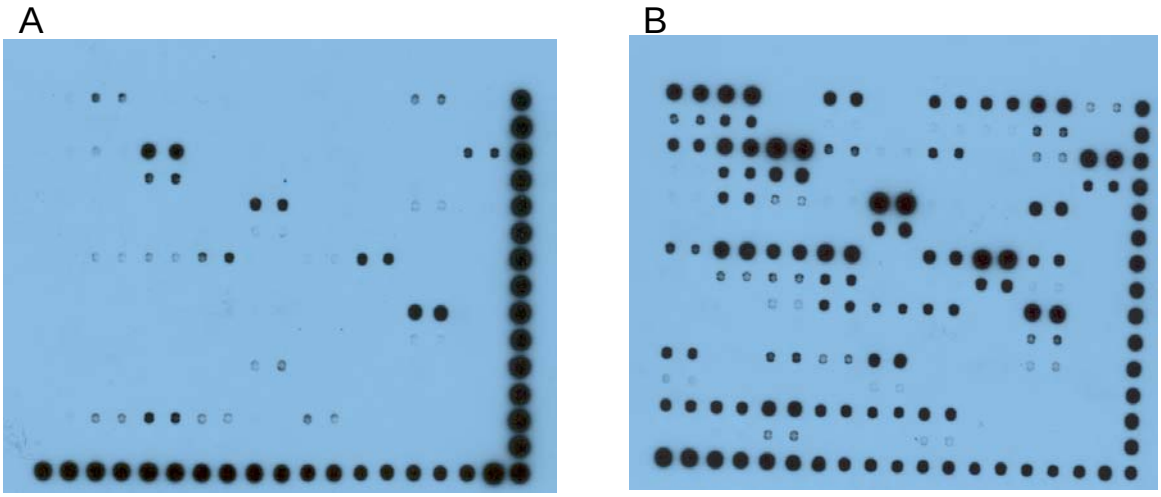

Fig. 13. Comparison of LNCaP cells stimulated with FGF2 only (A) or complete serum (B) in protein/DNA Array 1. The assay was performed using nuclear extracts from LNCaP cells treated with20ng/ml of FGF2 only (A) or treated with complete serum (B).

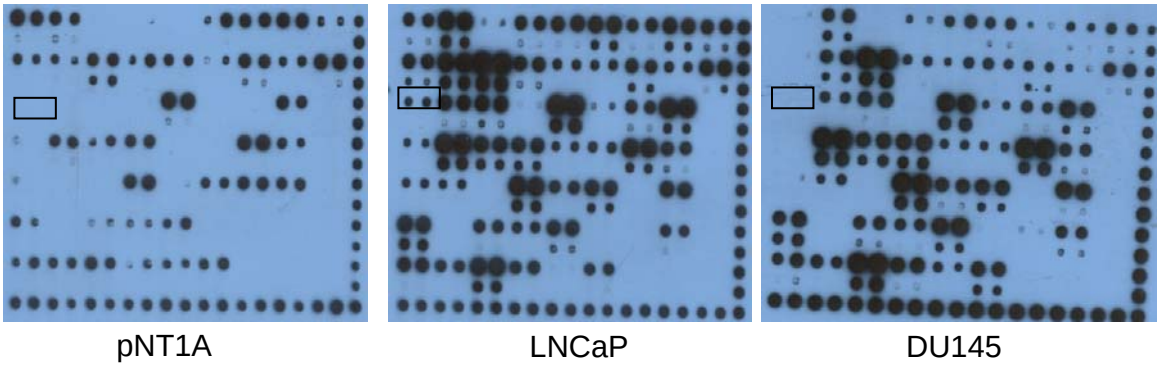

Fig. 14. Comparison of FGF2 stimulation in pNT1A, LNCaP and DU145 cells using protein/DNA array. The array was performed using nuclear extracts from pNT1A, LNCaP and DU145 cells grown in serum free medium and supplemented with FGF2 (20ng/ml). The boxed spots show different GATA signal intensities in the 3 cell lines.

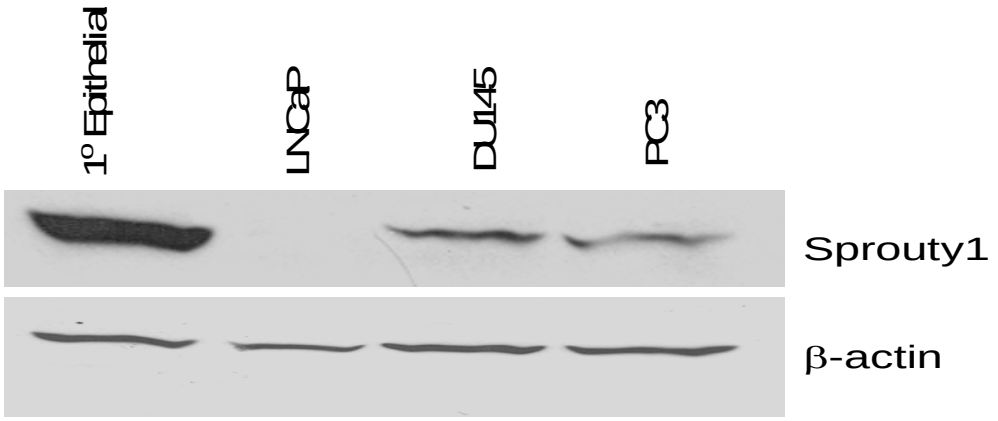

Fig. 15. Protein extracts from the prostate cancer cell line pNT1A Ire analyzed by western blotting with anti-Sprouty1 antibodies. In the LNCaP cells, the Sprouty1 protein expression is barely detectable. Loading control on the same filter with anti-β-actin antibody is shown in the lower panel.

Task 3: Characterization of transcription factor(s) responsible for interaction with Sprouty1 promoter (Months 18 – 36). The aim of this task was to identify the putative transcription factor(s) responsible for the regulation of Sprouty1 expression. First, the interaction between Sprouty1 promoter and TFs in vivo was established using chromatin immunoprecipitation (ChIP) assays and the involvement of specific TF(s) in Sprouty1 expression was verified by knockout studies.

A. Investigate in vivo molecular interaction of Transcription Factors and Sprouty1 promoter. I studied whether some of these putative TFs described above binds to the *Sprouty*1 promoter *in vivo* using ChIP assay. Figure 16 shows that indeed these TFs bound to *Sprouty*1 promoter *in vivo* as demonstrated by the same PCR product in the assay precipitation with different antibodies compared to the Anti-acetyl-Histone H4 antibody control (positive control). Conversely precipitation with normal goat IgG (negative control) did not show any binding. These studies clearly demonstrate that *Sprouty*1 proximal promoter region contain several sequence motifs (i.e., EGR, GATA, SP1, PBX1 and HNF4) which are specifically recognized by known as Ill as

uncharacterized transcription factors and are functionally important and likely to be responsible for driving the basal transcription of the *Sprouty*1 gene.

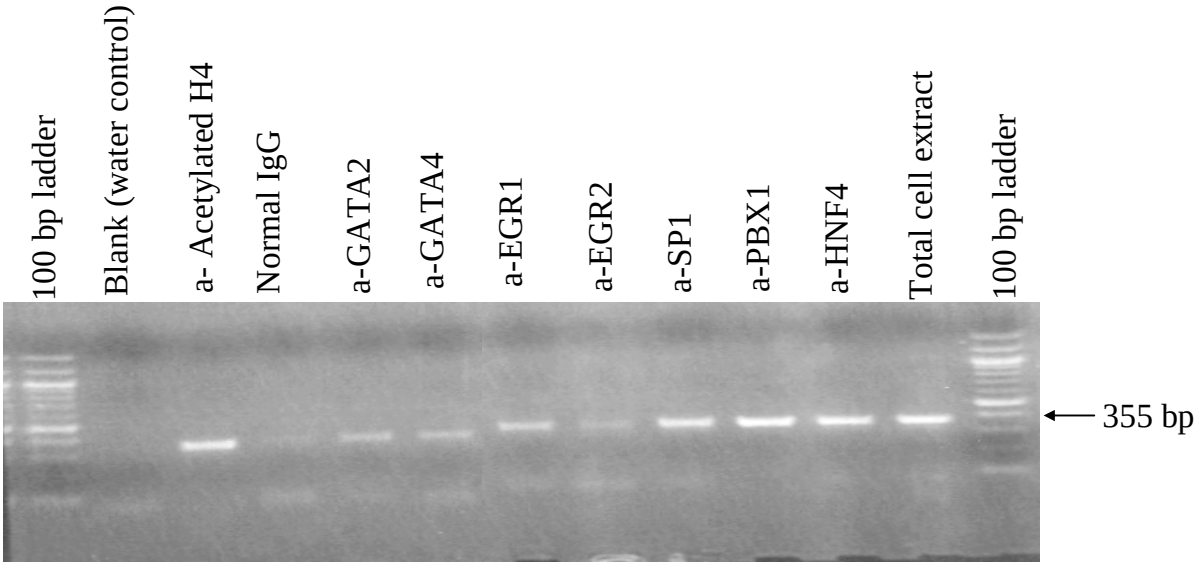

Fig. 16. Chromatin immunoprecipitation assays shows in vivo binding of different antibodies to the proximal *Sprouty*1b promoter. Anti-acetyl-Histone H4 antibody binding to DNA is used as a positive control (lane 3). Lanes 2 and 4 shows no amplification in the water and the normal IgG negative controls respectively.

B. Impact of EGR and GATA activity on Sprouty1 expression To verify the involvement of EGR and GATA transcriptional activity in regulating Sprouty1 expression, I transiently transfected LNCaP cells with siRNA duplexes corresponding to EGR1, EGR2, GATA2 and GATA4. Western blot analysis Ire performed using Sprouty1 antibody and total cell lysates in order to examine the silencing effect of the EGR-1, EGR-2, GATA-2 and GATA-4 siRNA transfections on Sprouty1 protein expression (see figure 17). The western blot signals were quantified and expressed relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; data not shown). Data indicated a modest increase in Sprouty1 protein expression in response to EGR (1 and 2) targeting. On the other hand, when LNCaP cells were transfected with GATA2 siRNA (100 mM) and GATA4 siRNA (100 mM), there were approximately 2 fold increases in

Sprouty1 protein expression. The observed Sprouty1 expression levels Ire in response to 49%, 47%, 52% and 58% reduction of EGR1, EGR2, GATA2 and GATA4 mRNA expression respectively as determined by quantitative RT-PCR (data not shown). The data indicates that the blockade of EGR1 and EGR2, GATA2 and 4 by small inhibitory RNA can increase Sprouty1 protein expression. The minimal increase in Sprouty1 expression due to ERG targeting could be explained by the low efficiency of EGR knockdown, suggesting that perhaps high dose knockdown of the EGR might demonstrate significant increase in Sprouty1 expression. To increase the knockdown efficiency, I am using lentiviral vector mediated expression of short hairpin RNA (shRNAs) against GATA and the EGR transcription factors. The lentivirus-delivered shRNAs has been demonstrated to be capable of specific, highly stable and functional silencing of gene expression in a variety of cell types and also in transgenic mice. This approach should therefore permit rapid and efficient analysis of EGR and GATA transcription factor knockdowns in prostate cancer cell lines.

I have made recombinant GATA (2 and 4) and EGR (1 and 2) shRNA constructs. Stable transfection of these recombinant vectors into the prostate cancer cell lines LNCaP and PC3 showed detrimental effect on cell growth after a week of cell growth under media selection (data not shown). My hypothesis is that knockdown of GATA and EGR should lead to increase in Sprouty1 protein expression, however our previous published data demonstrate that over-expression of Sprouty1 protein has deleterious effect on cell growth [19]. The challenge has been expressing the recombinant GATA and EGR shRNA long enough in the prostate cancer cell lines in order to observe biological effect and without killing the cells. I am currently in the process of optimizing the

experimentation to achieve high dose knockdown of GATA and EGR and at the same time obtain enough viable cells to analyze Sprouty1 protein expression.

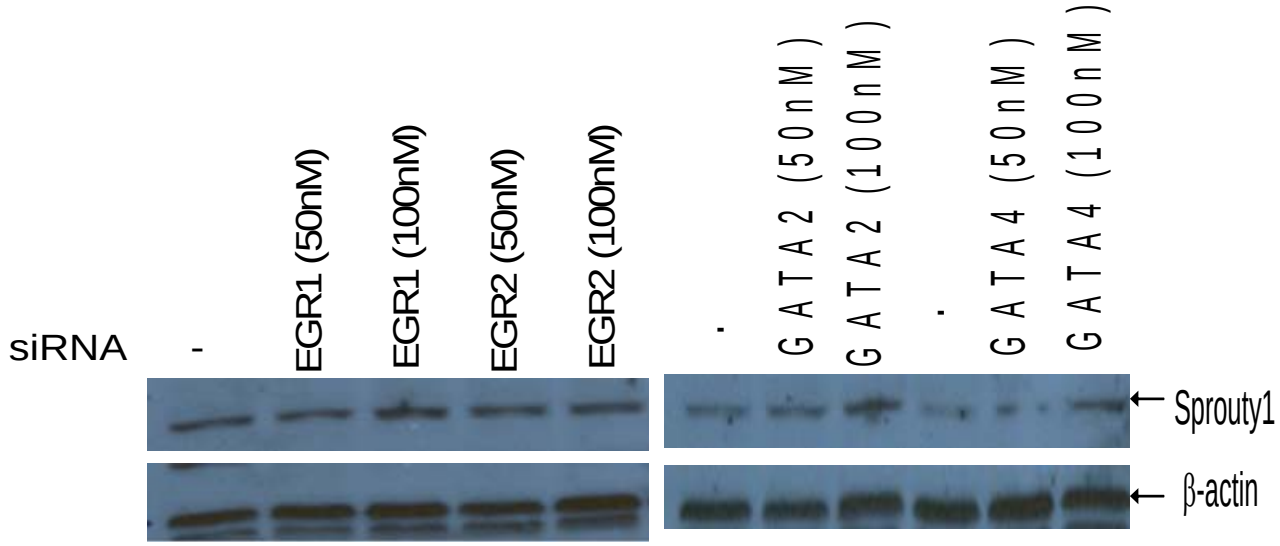

Figure 17. siRNA knock-down of EGR and GATA and Sprouty1 expression in LNCaP cells. The LNCaP cells Ire transiently transfected with either EGR1, EGR2, GATA2, or GATA4 siRNA duplexes for 72 hours. Total protein extracts from the transfected cells Ire used in Istern blotting with either anti-Sprouty1 or β-actin antibody.

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. In this proposed study, I 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. I have also found by in situ hybridization and quantitative RT-PCR analysis that Sprouty2 and Sprouty4 were downregulated in the majority of human prostate cancers when compared to normal prostate tissue. In human prostate cancer tissues, there is up-regulation of FGFs when compared with uninvolved prostate. Previous studies has demonstrated 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 [37]. FGF9 is also present at equal levels in normal and neoplastic prostate tissue based on ELISA assay. In addition, FGF6 is expressed as an autocrine growth factor in 40% of prostate cancers [38] , and the majority of prostate cancers express FGF8 in a similar manner [39]. 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 [38,40] and/or decreased cell death [41] in prostate cancer and potentiate the effects of increased FGFs and FGFRs in prostate cancer. I 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.

I have found distinct differences in the functional roles for members of the Sprouty gene family. For instance, Sprouty1 and Sprouty4 have distinct biological roles in prostate cancer cell lines. Transient expression of Sprouty1 significantly inhibited prostate cancer proliferation while stable over-expression was markedly deleterious to prostate cancer cells [19]. In contrast, transient expression of Sprouty4 did not have any significant effect on prostate cancer cell proliferation while the stable over-expression of Sprouty4 inhibits prostate cancer cell migration [20]. It has been shown that in some tissues the expression patterns of the Sprouty family members do not overlap [7]. These data indicate that the different isoforms of Sprouty are not uniformly regulated and suggests that the different family members may not be functionally equivalent. Thus the individual Sprouty genes

may be regulated by specific combinations of factors to allow optimal control of signaling.

I have found that Sprouty1 expression in prostate cancer cells *in vitro* is no longer up-regulated by FGF2. This could be due to 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. Multiple genetic alterations can drive tumorigenesis and progression. The metastatic and drug/hormone-resistant phenotype of certain cancers such as prostate cancer may result from epigenetic events such as aberrant gene methylation. In my studies, I observed that Sprouty1 promoter was significantly methylated in a subset of tumor tissues compared with matched normal prostate tissues. The increased in methylation significantly correlated with the decreased Sprouty1 expression, as analyzed by quantitative RT-PCR, demonstrating that, in majority of cases, the down-regulation of Sprouty1 in a prostate cancer case is due to promoter methylation. I made similar observation of Sprouty4 inactivtion by promoter methylation. The data clearly demonstrates that methylation is a key mechanism for the down-regulation of Sprouty gene expression in prostate cancer. However, in some cases without detectable promoter methylation for low Sprouty1 expression, other mechanisms of gene inactivation, such as alteration in trans-acting factors could also affect Sprouty1 expression.

To investigate the transcriptional regulation of Sprouty1, I have cloned and functionally characterized the 5'-flanking region of the human *Sprouty*1 gene which is responsible for its transcriptional regulation in prostate cancer cell culture. I used a combination of luciferase reporter gene assays from transiently transfected cells and electrophoretic mobility shift assays to identify the cis-elements within the human *Sprouty*1 promoter region (including GATA1, EGR, ZBP, ETS, HIC and FKHD) that confers responsiveness to growth factor signaling. I have identified a highly conserved nucleotide binding site for the early growth response (EGR1) in the human and mouse *Sprouty*1 promoter region which underscore the importance of this motif in the regulation of the *Sprouty*1 promoter expression. In the human prostate, there is strong evidence to suggest that EGR1 overexpression is involved in prostate cancer progression [42]. For example, EGR1 expression levels are elevated in human prostate carcinomas in proportion to grade and stage. Whereas antisense oligonucleotides that block EGR1 function revert transformation of prostate cancer cells *in vitro* and delay prostate cancer progression *in vivo*. I have observed a modest induction of Sprouty1 expression in response to EGR1 and EGR2 knockdown. However, because the suppression by EGR1 and 2 was quite low, it is possible that the response to EGR1 and 2 might have been higher if the knockdown has been more substantial. On the other hand, even though similar knockdown levels were observed for GATA2 and 4, I detected much higher Sprouty1 expression in response to GATA2 and 4 knockdowns. A search of the cancer profiling database (www.oncomine.org) indicates that GATA2 expression is increased in human prostate cancer tissues when compared to normal prostate tissue suggesting that increased expression of GATA2 maybe involved in prostate cancer progression. The gene

knockdown studies has demonstrated transcriptional repression roles for EGR (1 and 2) and GATA (2 and 4) in regulating Sprouty1 expression suggesting that elevated EGR and GATA through its ability to repress Sprouty1 transcription, may contribute to prostate cancer progression.

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.

KEY RESEARCH ACCOMPLISHMENTS

- I have demonstrated using immunohistochemistry analysis of tissue array and quantitative RT-PCR analysis that Sprouty1 and Sprouty4 are down-regulated in prostate cancer tissues when compared to normal prostate tissues.
- I have found that Sprouty1 expression in prostate cancer cells *in vitro* is no longer up-regulated by FGF2. This could be due to 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. I did find mutation in the Sprouty1 coding sequence to explain for the loss of expression of Sprouty1 in human prostate cancer tissues

- I have demonstrated using pyrosequencing to quantitatively measure DNA methylation of the Sprouty1 promoter and have shown significantly higher Sprouty1 promoter methylation in DNAs from prostate cancer tissues when compared to match benign tissues. Systematic studies of the Sprouty1 promoter methylation correlated with gene expression in the prostate cancer. I have made similar observations for Sprouty4 gene in prostate cancer and my data clearly shows that DNA methylation changes is a key mechanism for down-regulating the expression of Sprouty1 and Sprouty4 genes in human prostate cancer.

- In order to elucidate the transcriptional machinery regulating Sprouty1 expression, I identified functional regions of the human *Sprouty*1 gene promoter, which are responsible for constitutive gene expression. I have shown that transcription repression as well as DNA methylation constitutes key mechanisms for the down-regulation of sprouty1 expression in prostate cancer. Gene knockdown studies has demonstrated transcriptional repression roles for EGR (1 and 2) and GATA (2 and 4) in regulating Sprouty1 expression suggesting that elevated EGR and GATA through its ability to repress Sprouty1 transcription, may contribute to prostate cancer progression.

REPORTABLE OUTCOMES

Abstract and oral presentations

- AACR Annual Meeting- Age-Related DNA Methylation Changes in Normal Human Prostate. (2007) Los Angeles CA.
- AACR Annual Meeting- Profiling the transcriptional regulation of Sprouty1, a negative regulator of growth factor signaling in androgen dependent and independent human prostate cancer cells. **Kwabi-Addo B**, Ren C, Ittmann M. (2006) Washington D.C.
- AACR Annual Meeting- Elucidating the functional regulation of Sprouty4, a growth inhibitor in prostate cancer. Kwabi-Addo et al., (2005) Anaheim, CA (Abstract).
- AACR Annual Meeting- Sprouty1, an inhibitor of fibroblast growth factor signaling is down-regulated in prostate cancer. **Kwabi-Addo B** (2004) Orlando, FL (Oral; *mini symposium*).
- AACR/NCI/EORTC- Sprouty1, an antagonist of Fibroblast growth factor signaling is down-regulated in prostate cancer. **Kwabi-Addo B** *et al*., (2003) Boston, MA (Abstract).

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- **Kwabi-Addo B**, Ozen M, Ittmann M. (2004). The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr Relat Cancer*. Dec; 11(4): 709-24.
- **Kwabi-Addo B**, Wang J, Erdem H, Vaid A, Castro P, Ayala G, Ittmann M.. (2004). Sprouty1, an inhibitor of Fibroblast growth factors is down-regulated in prostate cancer. *Cancer Res*. Jul 15; 64(14) 4728-35.

Manuscript submitted to Epigenetics

- **Bernard Kwabi-Addo**, Chengxi Ren and Michael Ittmann (2008). DNA Methylation and aberrant expression of Sprouty1 in Human Prostate Cancer. (Submitted to the Epigenetics journal)**.**

Manuscript in preparation

- **Bernard Kwabi-Addo**, Songping Wang and Michael Ittmann (2008). Transcriptional regulation of Sprouty1 expression in human prostate cancer cells.

Funding applied for based on work supported by this award

- In studying the molecular mechanisms regulating Sprouty1 expression, I came to appreciate the role of aberrant DNA epigenetic changes as an important mechanism in regulating gene expression. Based on some preliminary DNA methylation studies published in Clinical Cancer Research (*Clin. Cancer Res*. Jul 1:13(13): 3796-3802), I have been successful in obtaining a DOD Idea Award to

investigate endogenous and exogenous factors involved in *de novo* DNA methylation changes in prostate cancer.

Research opportunity based on experience/training supported by this award

- Through the continuous grant support from DOD, I got employed in March 2007, as a faculty member of Howard University Cancer Center at the level of a Research Assistant Professor with my own laboratory to pursue my research goals.

CONCLUSION

I have carried out ground breaking studies of the role of Sprouty1 in prostate cancer. My manuscript in Cancer Research (*Cancer Research* 64: 4728-4735) was the first to report the loss of expression of this tumor suppressor gene in any malignancy. The Sprouty gene family functions as negative regulators of receptor tyrosine kinase signaling. I have identified functional regions of the human *Sprouty*1 gene promoter, which are responsible for constitutive gene expression. I have shown that transcription repression and DNA methylation changes in the Sprouty1 promoter region constitutes key mechanisms for the down-regulation of sprouty1 expression in prostate cancer. I have observed strong transcriptional activity in the prostate cancer cell lines even though Sprouty1 expression is down-regulated, suggesting that epigenetic modification of the binding sites for transcription factors such as Sp1 may also result in a refractory transcriptional response even in the presence of necessary trans-acting activities. Complete understanding of the molecular mechanisms controlling Sprouty1 expression may prove useful in elucidating the regulation of growth factor signals in prostate cancer which may in turn provide an attractive new target approach for therapeutic intervention

that may modulate a large number of potential growth promoting stimuli, including multiple growth factors and their receptors.

"So what section"

The importance of Sprouty proteins in the control of signal transduction pathway in a variety of cell types is well established. Nearly a decade has passed since the Sprouty gene family was first identified as antagonists of the FGF-induced signaling pathway. Since then, their biological role has expanded to encompass many more signaling pathways and biological processes mostly involving an inhibitory function in RTK-induced MAPK signaling. While there are many open questions regarding Sprouty's mechanisms of action in individual signaling pathways, it is now clear that Sprouty proteins are multi-functional and they are highly regulated in their expression and function. Continuing studies will shed more light on the complexity of Sprouty function in controlling the multiple outputs of mitogen-induced cascade in physiological and pathological processes in general and in prostate cancer in particular. Novel insights about their molecular function may set the stage for the development of innovative therapeutic approaches to interfere with RTK-mediated pathological processes including prostate cancer.

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Age-Related DNA Methylation Changes in Normal Human Prostate Tissues

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Abstract

Purpose: Prostate cancer is a leading cause of cancer death among the aging male population but the mechanism underlying this association is unclear. Aberrant methylation of promoter CpG islands is associated with silencing of genes and age-dependent methylation of several genes has been proposed as a risk factor for sporadic cancer. We examined the extent of gene methylation in pathologically normal human prostate as a function of age.

Experimental Design: We used pyrosequencing to quantitatively analyze the methylation status of nine CpG islands in normal prostate tissue DNA from 45 organ donors and 45 patients who had undergone cystoprostatectomy for bladder cancer. We also analyzed 12 pairs of matched benign and prostate cancer tissue DNA from patients with prostate cancer.

Results: Linear regression analysis revealed a significant increase in promoter methylation levels correlating with age for CpG islands at RARβ2 ($r = 0.4$; $P < 0.0001$), RASSF1A ($r = 0.27$; $P = 0.01$), GSTP1 ($r = 0.59$; $P < 0.0001$), NKX2-5 ($r = 0.27$; $P = 0.008$), and ESR1 ($r = 0.244$; $P = 0.023$) in the normal prostate tissue samples studied. A calculated average methylation (z score) at all nine CpG loci analyzed in the normal prostate tissues showed a strong correlation with age ($r = 0.6$; $P < 0.001$). Comparison of the methylation level for the matched benign and prostate cancer tissues from individual patients with prostate cancer showed significantly higher methylation in the prostate cancer tissue samples for RARβ2 ($P < 0.001$), RASSF1A ($P = 0.005$), GSTP1 ($P < 0.001$), NKX2-5 ($P = 0.003$), ESR1 ($P = 0.016$), and CLSTN1 ($P = 0.01$).

Conclusions: Our findings show aberrant hypermethylation as a function of age in the normal prostate tissues. Such age-related methylation may precede and predispose to full-blown malignancy.

Prostate cancer is a common malignancy and a leading cause of cancer death among men in the United States. The molecular mechanisms underlying its development and progression remain poorly understood. There are three well-established risk factors for prostate cancer: age, race, and family history (1). Growing evidence suggests that epigenetic mechanisms, such as DNA methylation, affect gene expression in an age-dependent and tissue-specific manner (2, 3). Age-dependent DNA methylation alters cell physiology and may predispose cells to neoplastic transformation (3). Various studies have emphasized DNA hypermethylation as an important mechanism for the inactivation of key regulatory genes in prostate cancers [reviewed by Li et al. (4)].

Hypermethylation of the pi-class glutathione *S*-transferase gene (GSTP1) promoter sequences constitutes the most common genomic alteration described for human prostate cancer (5). The loss of GSTP1 expression through hypermethylation occurs even in the earliest stage of tumorigenesis, prostate intraepithelial neoplasia (6). DNA hypermethylation has also been shown to inactivate tumor suppressor genes in prostate cancers. For example, the ras association domain family protein 1, isoform A (RASSF1A) gene promoter is frequently hypermethylated in prostate carcinomas (7), and promoter hypermethylation of the retinoic acid receptor β2 (RARβ2) is found in the vast majority of prostate adenocarcinomas, high-grade prostatic intraepithelial neoplasia, and a significant number of benign prostatic hyperplasia lesions (8).

Age-related methylation has been proposed as a risk factor in colon cancer (9). However, little information is available about methylation in the normal aging prostate and whether such a phenomenon might contribute to neoplastic transformation and prostate disease. Identification of genes that undergo age-related methylation in the prostate would be potentially useful for several reasons: first, it would help in quantifying methylation in the prostate as a function of age, and to study

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whether such events in genes may predispose aging cells to neoplastic transformation. Second, such a methylation profile would be useful to distinguish benign prostate from cancerous prostate, and provide correlations between methylation and prostate tissue pathologic features such as stage, grade, and recurrence. Finally, differences in gene methylation profiles from individuals of the same age differing in the race or ethnicity may help in understanding how genetic factors or environmental exposures (or both) contribute to prostate cancer. In the present study, we used pyrosequencing to quantify the methylation status of nine CpG islands as a function of age in normal and cancerous human prostate tissues.

Materials and Methods

Human prostate tissue samples. We collected a total of 90 normal prostate (disease free) peripheral zone tissue samples; of which 45 were from organ donors (age range, 17-68 years) and 45 samples from patients who underwent cystoprostatectomy for bladder cancer (age range, 39-84 years). In addition, we collected 12 pairs of matched normal and prostate cancer tissue samples from patients with prostate cancer (age range, 52-73 years). All samples were obtained from the Baylor Specialized Programs of Research Excellence (Baylor College of Medicine) Prostate Cancer Tissue Bank in accordance with institutional policies. Organ donor samples from accidental death victims were removed after obtaining consent from next of kin. Normal prostate tissues from bladder cancer patients without any evidence of prostate cancer were obtained at the time of cystoprostatectomy. Matched normal and prostate cancer tissues from patients with prostate cancer were obtained from the Baylor Specialized Programs of Research Excellence at the time of radical prostatectomy. In all cases, the normal tissues were free from prostate intraepithelial neoplasia and cancer on histologic examination.

Bisulfite DNA modification. High molecular weight genomic DNA extracted from prostate tissues and WBC were modified by sodium bisulfite treatment (10). Briefly, genomic DNA (2 μg) was denatured in 0.3 mol/L of NaOH at 37°C for 15 min, sodium bisulfite and hydroquinone were added to final concentrations of 3.1 mol/L and 0.5 mmol/L, respectively. The reaction was incubated at 50°C for 16 h, and desalted using Wizard DNA purification resin (Promega) according to the instruction of the manufacturer. Bisulfite modification was completed by DNA desulfonation in 0.3 mol/L NaOH treatment at 37°C for 15 min. Modified DNA was precipitated with ethanol, washed in 70% ethanol, dried and resuspended in 50 μL of TE buffer.

Pyrosequencing. Bisulfite PCR primers were designed based on bisulfite-converted sequences from specific CpG island of various target genes ensuring that the bisulfite-PCR primers avoid CpG sites and that they are designed as close to the transcription start site as possible. Either one-step or two-step PCR reactions were carried out using 2 μL of bisulfite-converted genomic DNA and either one or two sets of different bisulfite PCR primers in a standard PCR reaction mix containing oligonucleotide inhibitor of Taq polymerase (11). One of the primers (reverse primer) in the first or second step PCR reaction was biotinylated in order to create a ssDNA template for the pyrosequencing reaction. Where indicated, we used a previously described amplification protocol (12) based on a universal primer approach. Briefly, the biotinylated reverse primer was substituted with a 5′ tailed unlabeled reverse primer, and a biotinylated universal primer at a ratio of 1:9 in the PCR reaction. The integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The PCR product was immobilized on streptavidin-sepharose beads (Amersham), washed, denatured and the biotinylated strands released into an annealing buffer containing the sequencing primer. Pyrosequencing was done using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Biotage). PCR primer sequences and sequencing primer sequences are listed in Table 1. Bisulfite-converted WBC DNA from normal volunteers and blank reactions, with water substituted for DNA, served as negative control and bisulfite-converted *Sss*I methylase-treated WBC DNA served as a positive control. Each pyrosequencing reaction was done at least twice.

Table 1. Primer sequences used in the pyrosequencing analysis

Gene	Primers
GSTP1	First step PCR: forward AAGGAGGTTAGGGGTAAAAGTTATA reverse CCAAAACCTCCCCAATAC Second step PCR: forward GAGTTAGGGGGAGGATGT reverse biotin-CCAAAACCTCCCCAATAC sequencing (F) GGGGGAGGATGTTAAG
AR	First step PCR: forward TAGGAAGTAGGGGTTTTTTAGGGTTAG reverse ACCCAACCCACCTCCTTACCT Second step PCR: forward GTAGGGGTTTTTTAGGGTTAGAGTTAG reverse GGGACACCGCTGATCGTTTACCTCCCCTTTCCTTTTCTCC reverse biotin-UNIVR sequencing (F) TTGTTTTTTTAAAGTTATTAGGTA
RASSF1A	Single-step PCR: forward GGGGGAGTTTGAGTTTATTGA reverse biotin-CTACCCCTTAACTACCCCTTCC sequencing GGGTAGTATTAGGTTGGAG
MYOD1	First step: forward AATTAGGGGATAGAGGAGTATTGAAAG reverse ACAACCCTAAACRACTACACTTAACTC reverse universal UNIVR-ACAACCCTAAACRACTACACTTAACTC Second step: forward GAAAGTTAGTTTAGAGGTGA reverse biotin-UNIVR sequencing GAGGTTTGGAAAGGG
p16	Single-step PCR: forward GGTTGTTTTCGGTTGGTGTTTT reverse biotin-ACCCTATCCCTCAAATCCTCTAAAA sequencing TTTTGTTTGGAAAGAT
RARβ2	First step: forward AGTTGGGTTATTTGAAGGTTA reverse TACCCAAACAAACCCTACTC reverse universal UNIVR-CCCAAACAAACCCTACTC Second step: forward AAGTAGTAGGAAGTGAGTTGTTTAGA reverse biotin-UNIVR sequence GGGACACCGCTGATCGTTTA
ESR1	One-step PCR: forward TGTGTTTTTTTTTTAGGTGG reverse biotin AACCATCCCAAATACTTTAATA sequencing GGATACGGTTTGTATTTTG
CLSTN1	First step PCR: forward GAATTTAGGGGTTTTAGTTTTTTTAGTAGAG reverse TACTACCCCACCCTTAACTATTTACCA Second step PCR: forward AGGGGTTTTAGTTTTTTTAGTAGAGGTGTT reverse biotin-GGGACACCGCTGATCGTT sequencing GAAAGGGGTTTAGGA
NKX2-5	First step PCR: forward GAGAGTAGGGTTGGGGAATATG reverse AACCCCTAACCCAATAACAAACT Second step PCR: forward TAAGGTTTTTGGTAGTTTTTTTGTATGG reverse UNIVR-CAATAACAAACTAAATCCCCCTCCTCTA biotinylated universal primer sequencing GGTAGTTTTTTTGTATGGTG

NOTE: The underlined sequence is the universal primer sequence also shown as 5′-UNIVR.

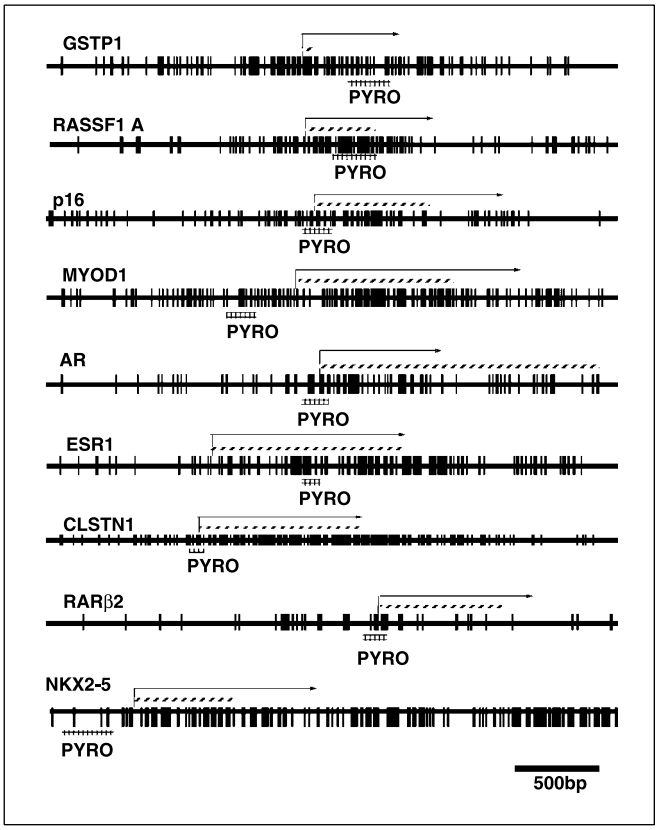

Fig. 1. Schematic representation of CpG islands analyzed. Maps represent CpG islands of 3 kb sequence (*horizontal black bar*) around exon 1 (*hatched box*) of the nine genes analyzed in the study. Short vertical bars, the CpG sites for each gene. Arrows, known or presumed transcriptional start sites. Grids, regions analyzed by pyrosequencing (*pyro*).

Data analysis. The methylation index (MtI) at each gene promoter and for each sample was calculated as the average value of $^{m}C/(^{m}C + C)$ for all examined CpG sites in the gene and expressed as the percentage of methylation. The Spearman test was used to determine correlations, with significance set at $P < 0.05$. r represents the measure of the relationship between two variables, and varies from -1 to +1.

Results

To investigate DNA methylation as a function of age in normal human prostate tissues, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite-modified genomic DNA. We examined a total of nine CpG islands including GSTP1, RARβ2, the RASSF1A tumor suppressor gene, androgen receptor (AR), and *p16*, which has been previously shown to be hypermethylated in human prostate cancers (4), and myoblast determination protein 1 (MYOD1) and estrogen receptor 1 (ESR1), which has been shown to be affected by age-related methylation (9, 13). In addition, we analyzed two novel genes, i.e., calsyntenin-1 (CLSTN1) and a member of the homeobox gene family of transcription factors (NKX2-5) which we have identified to be differentially methylated in prostate cancer.[4] Figure 1 shows CpG maps of the genes analyzed, along with the locations of the regions amplified. We investigated the methylation status of these CpG islands in normal prostate tissue DNA obtained from 45 organ donors (age range, 17-68 years) and 45 patients who underwent cystoprostatectomy for bladder cancer (age range, 39-84 years). In addition, we analyzed the methylation status of these CpG islands in DNA samples from 12 pairs of matched benign and prostate cancer tissues from patients with prostate cancer (age range, 52-73 years). Typical examples of bisulfite methylation profiles presented as pyrogram are shown for GSTP1 (Fig. 2). As shown in the pyrogram, the internal control (shown as a hatched box) in the pyrosequencing reaction checks for the adequacy of bisulfite treatment, that is, methylation of non-CpG cytosines, suggesting that 100% of the DNA samples used for the GSTP1 analysis were satisfactorily converted by bisulfite treatment. Samples with failed results were repeated, where possible, with freshly prepared bisulfite-modified genomic DNA from the original tissue samples, and the pyrosequencing reaction was repeated for all samples.

For each CpG island studied, the percentage of methylation at a specific promoter region was expressed as a function of age (Fig. 3). There was considerable variation in the percentage of CpG island methylation in the individual patient samples studied, presumably reflecting both random variability in tissue composition and variable methylation level per cell. The variable range of methylation could also reflect differences in genetic susceptibility to methylation, lifestyle or exposure factors (including diet), and the random nature of the methylation event. The methylation level was not significantly different (Mann-Whitney rank sum test; Fig. 4A) between the samples from organ donors and those from patients with bladder cancer for the same age group (age range, 38-68 years); therefore, we grouped these samples together and these are hereafter referred to as normal prostate tissues. Based on this criteria, we found a significant increase

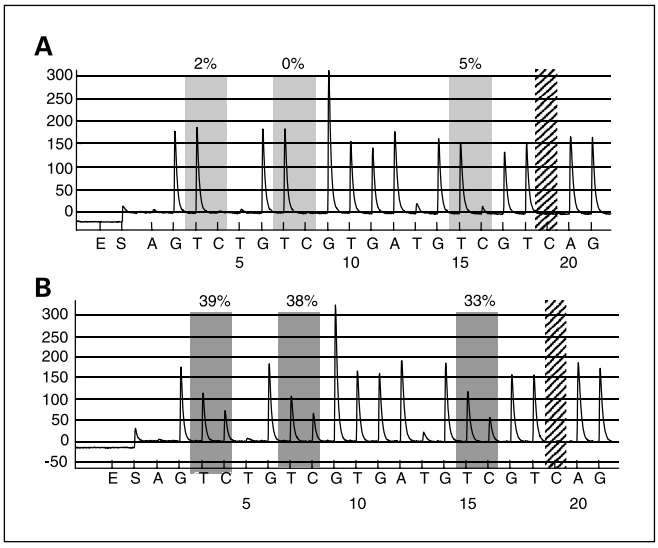

Fig. 2. Representative pyrogram traces for GSTP1. Gray columns, regions of C to T polymorphic sites. Hatched columns, control, non-CpG cytosine residues showing complete conversion of cytosine to uracil by bisulfite treatment (*right*). Blood sample from a 52-y-old normal male volunteer (*top*) shows no methylation, whereas normal prostate tissue sample (*bottom*) from a 58-y-old male organ donor shows a significant level of methylation at all three CpG sites. Top, percentage of methylation at each CpG site. *Y*-axis, signal peaks proportional to the number of nucleotides incorporated. *X*-axis, the nucleotides incorporated.

[4] Unpublished observation.

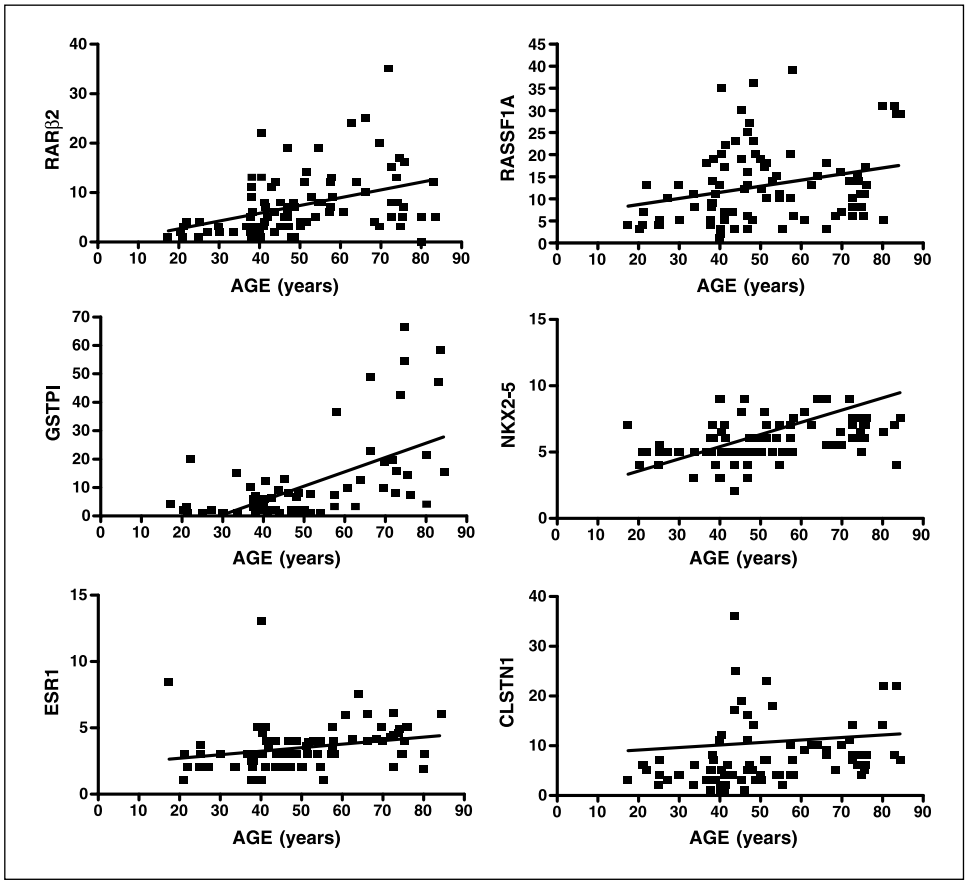

Fig. 3. Age-related methylation analysis in normal prostate tissues. CpG islands for RARβ2, RASSF1A, GSTP1, NKX2-5, and ESR1 in 90 bisulfite-modified genomic DNA extracted from normal prostate tissues [consisting of 45 organ donor samples and 45 cystoprostatectomy for bladder cancer patient samples (age range, 17-84 y old)]. *Y*-axis, the percentages of methylated cytosines in the samples as obtained from pyrosequencing. Each CpG island has a different scale range. *X*-axis, ages in years.

in promoter methylation to correlate with age for the CpG islands at RARβ2 ($r = 0.4$; $P < 0.0001$), RASSF1A ($r = 0.27$; $P = 0.01$), GSTP1 ($r = 0.59$; $P < 0.0001$), NKX2-5 ($r = 0.27$; $P = 0.008$), and ESR1 ($r = 0.244$; $P = 0.023$). We also observed methylation of the CLSTN1 gene but this did not significantly correlate with age. The MYOD1, AR, and p16 CpG islands were entirely unmethylated in the normal prostate tissue samples (data not shown). An unbiased analysis of all the data by *z* score normalization for the nine genes showed a strong correlation with age ($r = 0.6$; $P < 0.001$) in the normal prostate tissues (Fig. 4B). These results indicate that DNA methylation increases with age in normal human prostate.

Having established age-related methylation in the prostate, we next wanted to compare the level of the methylation in normal and prostate cancer tissues from men 50 years and older. We compared the methylation levels in normal prostate tissues from three different sources, i.e., normal prostate tissues from organ donors, cystoprostatectomy for patients with bladder cancer, and normal as well as prostate cancer tissues from patients with prostate cancer (Fig. 5A). Compared with the normal prostate tissues, we observed more extensive methylation in prostate cancer tissues. For the RARβ2 gene, the average methylation seen in cancer samples was at least 2.7-fold higher when compared with the normal prostate tissues, but there was no significant difference between the normal prostate tissues from different sources. Similarly, for the RASSF1A gene, the average methylation level in prostate cancer tissues was at least 2-fold higher compared with that in normal prostate tissues. Furthermore, the methylation level in the benign tissues from patients with prostate cancer was significantly higher (~2-fold; $P < 0.001$, *t* test) when compared with the normal prostate tissues from organ donors and the cystoprostatectomy for bladder cancer patient. Because RASSF1A methylation levels are low in normal prostate tissues and elevated in the prostate cancer and the surrounding benign tissues, quantitation of RASSF1A CpG islands could also be useful for distinguishing between normal and prostate cancer tissues. The methylation level of GSTPI showed at least 5-fold higher methylation in the cancer samples when compared with normal tissues from organ donors and benign prostate tissues from patients with prostate cancer. However, the methylation level in the cancer samples was only 1.5-fold higher compared with that of the normal tissues from cystoprostatectomy for patients with bladder cancer. This result can be explained by the high methylation levels observed in normal aging prostate tissues. The average methylation level of NKX2-5 in prostate cancer samples was ~3-fold higher than in the normal samples, however, the methylation levels were virtually identical among the three different sources of normal prostate tissues analyzed, suggesting that NKX2-5 methylation levels could also be a good candidate for distinguishing between normal and prostate cancer tissues. Overall, the ESR1 and CLSTN1 CpG methylation levels were lower, however, the average methylation level of ESR1 and Clstn1 were each ~2-fold higher in the cancer tissues when compared with the normal prostate tissues.

Finally, we compared the methylation levels for benign and matched pair cancer tissue for each prostate cancer patient

(Fig. 5B). The results showed significant hypermethylation for RARβ2 ($P < 0.001$, t test), RASSF1A ($P = 0.005$, t test), GSTP1 ($P < 0.001$, t test), NKX2-5 ($P = 0.003$, t test), ESR1 ($P = 0.016$, t test), and CLSTN1 ($P = 0.01$, t test) in the prostate cancer tissue samples when compared with the matched benign tissues. However, the frequency of hypermethylation in the prostate cancer tissues was low for the ESR1 gene. There was no methylation in the prostate cancer tissues for MYOD1, AR, and p16 CpG islands (data not shown) just as observed in the normal and benign prostate tissues. Because of the high frequency of hypermethylation of these five genes, i.e., *RARβ2*, *RASSF1A*, *GSTP1*, *NKX2-5*, and *CLSTN1* in the prostate tumors, promoter methylation of these five genes could theoretically serve as useful tools to distinguish between benign and prostate cancer tissues. Also, these data suggest that hypermethylation occurring in normal prostate tissues is a prerequisite for hypermethylation in prostate cancer.

Discussion

In the present study, we used pyrosequencing to examine the methylation profile of nine CpG islands in 90 normal prostate tissue samples and 12 pairs of matched benign and prostate cancer tissues from patients with prostate cancer. Pyrosequencing offers a semiquantitative, high-throughput, and reliable method with an inbuilt internal control for adequacy of bisulfite treatment (12, 14).

DNA methylation is a common event in cancer, and in several genes, promoter methylation has been reported. For example, aberrant methylation of the GSTP1 gene is perhaps the most common genomic alteration in human prostate cancer and occurs in the earliest stages of prostate carcinogenesis (15). The RARβ2 gene is hypermethylated in the vast majority of prostate adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and in a number of benign prostate hyperplasia lesions (8). RASSF1A promoter hypermethylation occurs at a high frequency in prostate tumor samples and is also detected in some nonmalignant prostate tissue samples (16), and a large percentage of prostatic intraepithelial neoplasia samples also exhibit RASSF1A promoter methylation (17). We have observed a significant increase in promoter methylation correlating with age in normal prostate tissues for the CpG islands at RARβ2, RASSF1A, and GSTP1. This observation indicates that not only are the RARβ2, RASSF1A, and GSTP1 genes hypermethylated in prostate cancer but promoter methylation of these genes actually starts in the normal prostate as a function of age, which markedly increases in cancer.

The use of epigenetic changes such as DNA methylation as noninvasive diagnostic tools in cancer has implications for the identification of high-risk subjects, patients with preinvasive or early stage lesions, and for monitoring residual disease. One such gene that holds promise as a diagnostic tool in patients with suspected prostate malignancy and a negative biopsy is

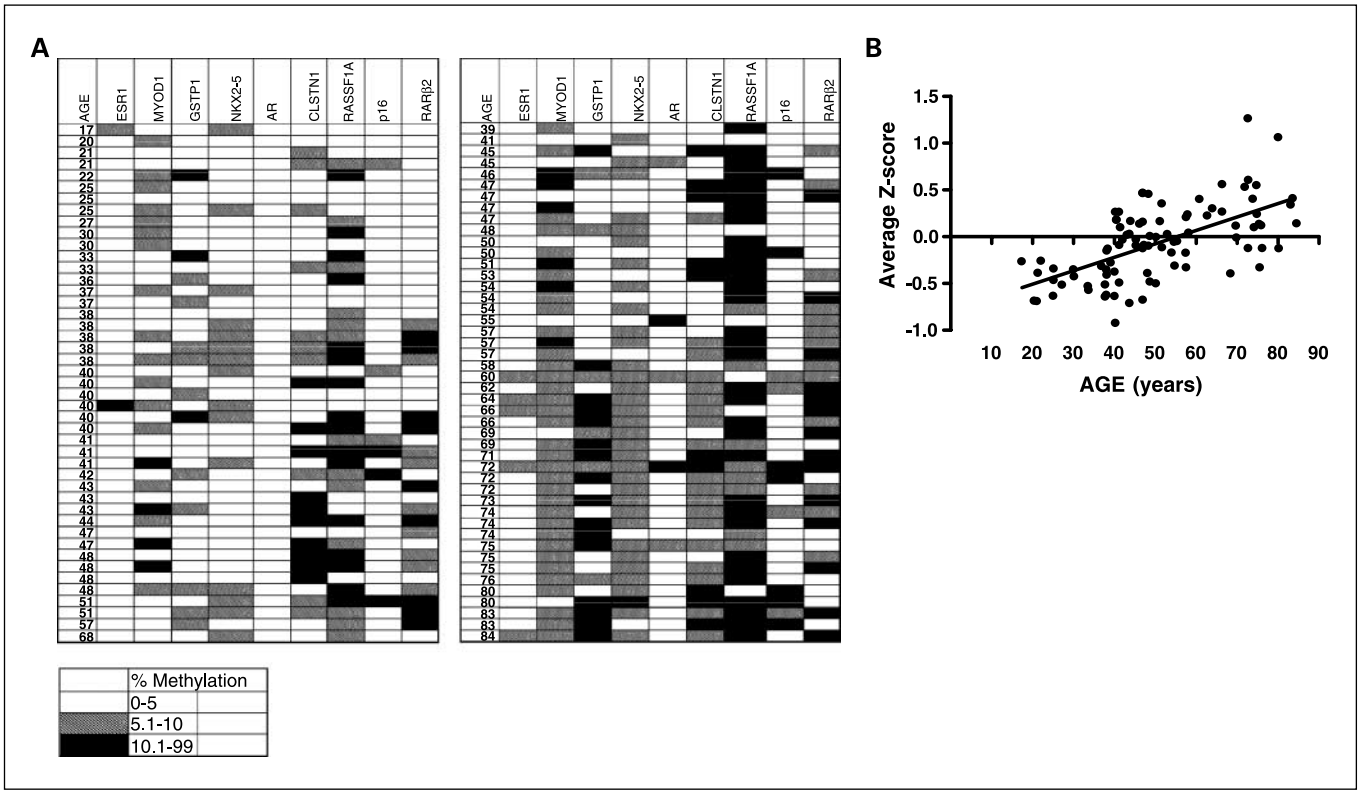

Fig. 4. The age distribution and methylation levels of nine CpG islands in normal human prostate tissues. *A,* percentage of methylation at nine CpG islands for 90 normal prostate tissues consisting of 45 organ donor samples (*left*) and 45 samples from patients who underwent cystoprostatectomy for bladder cancer (*right*) are shown with the ages of the individual patients. *B,* normalized average methylation levels (z score; $z = [(\text{methylation} - \text{mean}) / \text{SD}]$) at CpG island were calculated for all the patient samples analyzed and expressed as a function of age. *Y*-axis, the average z scores of nine CpG islands. *X*-axis, ages in years. The correlation coefficient (r) of 0.6 shows a significant increase in methylation as a function of age ($P < 0.001$).

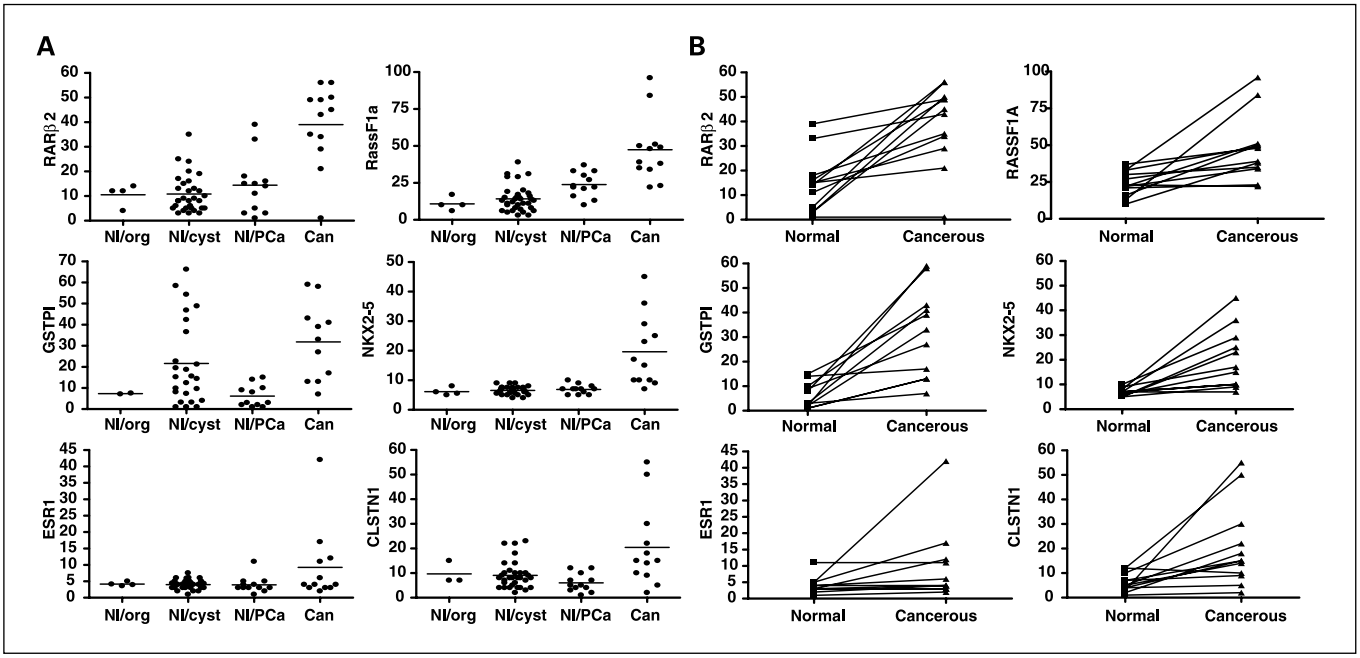

Fig. 5. Comparison of percentage of methylation levels. *A,* the methylation levels for RARβ2, RASSF1A, GSTP1, NKX2-5, ESR1, and CLSTN1 genes were compared between prostate samples as follows: normal prostate tissues from organ donors (*NI/org*), normal cystoprostatectomy tissues from patients with bladder cancer (*NI/cyst*), benign prostate tissues from patients with prostate cancer (*NI/PCa*), and prostate cancer (*Can*) tissue samples (*X*-axis). *Y*-axis, the percentages of methylated cytosine in each patient sample as obtained from pyrosequencing. Each CpG island has a different scale range. Horizontal bars, average methylation levels. *B,* the methylation levels were compared between matched benign and prostate cancer tissues from 12 patients with prostate cancer. *Y*-axis, the percentages of methylated cytosine in each patient sample as obtained from pyrosequencing. Each CpG island has a different scale range. *X*-axis, benign (normal) or prostate cancer (cancerous) tissues.

GSTP1. Abnormal GSTP1 methylation found postbiopsy may be helpful for the identification of patients at risk for harboring malignancy despite a negative biopsy, and to determine whether or not a repeat biopsy in the event of negative initial result is necessary (18). However, our data indicates that not only is GSTP1 hypermethylated in prostate cancer but that it is also methylated in an age-related manner, suggesting that the use of GSTP1 as a diagnostic tool must be carried out with caution especially when using it as a tool for analyzing samples from older patients.

In contrast with RARβ2, RASSF1A, and GSTP1, we found NKX2-5 to be specifically hypermethylated in the prostate tumors when compared with the normal prostate tissues. Furthermore, NKX2-5 does not show age-related methylation. NKX2-5 belongs to a family of homeobox genes that encodes a class of transcription factors regulating the expression of target genes in a time- and spatial-dependent manner. Loss of one member of this gene family, NKX3.1, has been shown to be an early event in the initiation of prostate cancer (19). NKX2-5 seems to be a novel frequent cancer-associated hypermethylated CpG island in prostate cancer, the hypermethylation of which is associated with neoplastic transformation unlike RARβ2, RASSF1A, and GSTP1 which are hypermethylated in premalignant prostate tissues in an age-dependent manner. Therefore, the inclusion of NKX2-5 as a new marker in a panel of hypermethylated genes in prostate cancer can potentially increase the sensitivity and specificity of prostate cancer detection.

The incidence of methylation in the human prostate seems to be promoter-specific, because we did not detect methylation at CpG islands for AR, MYOD1, p16, and CLSTN1. Methylation-mediated inactivation for AR has been shown in primary prostate cancer tissues (20, 21) and in androgen-independent cancers (22). However, data on AR methylation remains discrepant. The MYOD1 has been shown to be hypermethylated in cervical cancer (23) and undergoes age-related methylation in colon cancer (9). We did not observe age-related methylation of MYOD1 in the human prostate, suggesting that MYOD1 methylation may be tissue-specific. The *p16* gene has been reported to undergo methylation in primary prostate cancer and metastatic tumors, however, only partial methylation of the *p16* gene was observed in this study (24–26). We did not see methylation of this gene in the normal or prostate cancer tissues examined in our study. On the other hand, aberrant methylation of p16 seems to occur more commonly in other solid tumors, including breast carcinomas, gliomas, and colorectal carcinomas (27, 28). The CLSTN1 gene has a cadherin domain and a calcium ion–binding domain, and it may be related to cell adhesion molecules (29). We have observed aberrant methylation of CLSTN1 in prostate cancer tissues as well as in normal prostate tissues, however, the methylation of CLSTN1 does not seem to be age-related.

In conclusion, we have used pyrosequencing technology to show the epigenetic profile of normal prostate tissues. Our data indicates that several genes which are hypermethylated in prostate cancer tissue may undergo methylation in normal prostate tissues in an age-dependent manner. To our knowledge, this is the first study to directly examine the relationship between methylation and age in human prostate tissues. Our studies clearly show that methylation starts in normal prostate tissues as a function of age and markedly increases in cancer.

A larger prostate cancer population study is needed to confirm our observations and to determine if methylation status can provide reliable information for the detection of prostate cancer.

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Sprouty4, a Suppressor of Tumor Cell Motility, is Downregulated by DNA Methylation in Human Prostate Cancer

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PURPOSE. Alterations of fibroblast growth factors (FGFs) and their receptors contribute to prostate cancer progression by enhancing cellular proliferation, survival, and motility. The Sprouty gene family negatively regulates FGF signaling and may limit the ability of FGFs to enhance tumor progression. Sprouty1 is downregulated in human prostate cancers and Sprouty1 expression can markedly inhibit prostate cancer proliferation in vitro. Sprouty4 has been shown to negatively regulate both proliferation and cell migration in other systems. We therefore examined whether Sprouty4 expression was altered in prostate cancer.
EXPERIMENTAL DESIGN. Expression of Sprouty4 was examined by in situ hybridization and quantitative RT-PCR. Methylation of the Sprouty4 gene promoter was assessed using bisulfite modification and sequencing. The effect of Sprouty4 expression on cell migration was determined using an in vitro wounding assay.
RESULTS. By in situ hybridization Sprouty4 is expressed in normal prostatic epithelial cells and is decreased in a subset of prostate cancers. Quantitative RT-PCR confirms that Sprouty4 expression is decreased in approximately one half of prostate cancers. Analysis of the 5′-regulatory region revealed a CpG island approximately 1 kb upstream of the transcription initiation site, the proximal portion of which was preferentially methylated in prostate cancer tissues. More than one half of all prostate cancer DNAs were methylated in this region and methylation was significantly correlated with decreased Sprouty4 expression as determined by quantitative RT-PCR. When overexpressed in prostate cancer cell lines, Sprouty4 did not inhibit cell proliferation but did inhibit cell migration.
CONCLUSIONS. Sprouty4 expression is downregulated in human prostate cancer by DNA methylation and this decreased expression may contribute to increased cell migration. *Prostate 66: 613–624, 2006.*

KEY WORDS: **Sprouty; prostate cancer; methylation; cell migration; growth factor**

INTRODUCTION

Prostate cancer is the most common malignancy in US men and the second leading cause of cancer deaths. There is abundant evidence that increased fibroblast growth factor receptor signaling plays a critical role in the initiation and progression of prostate cancer (for review see Kwabi-Addo et al. [1]). Fibroblast growth factors (FGFs) have multiple biological activities in prostate cancer including increasing proliferation [2], angiogenesis [3], and cell motility [4] and as well as inhibiting cell death [5], all of which can promote tumor

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progression. In normal tissues, FGF signaling can be controlled by a number of mechanisms. Sprouty was originally identified in *Drosophila* as a negative regulator of fibroblast growth factor signaling during tracheal development [6]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in *Drosophila* development and organogenesis [7–9]. While *Drosophila* has only one Sprouty gene, at least four Sprouty homologues (Sprouty 1–4) have been cloned in human as well as mouse [10,11]. Mammalian Sprouty proteins inhibit growth factor induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [12–19]. Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with FRS2 or Shp2 [7,12] or the inhibition of Raf [16,17]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors increase the level of Sprouty transcript [11] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [20]. Furthermore, growth factors control Sprouty activity through the rapid and reversible tyrosine phosphorylation [21]. Importantly, each Sprouty family member is selectively tyrosine phosphorylated by a unique cohort of growth factors and with different kinetics, suggesting non-redundant functions for the Sprouty proteins [21]. Recently, Sprouty4 was shown to inhibit the kinase activity of the testicular protein kinase 1, TESK1 by binding to it through the C-terminal cysteine-rich region [22,23]. TESK1 is a serine/threonine kinase that phosphorylates cofilin and plays a role in integrin-mediated actin cytoskeletal reorganization and cell spreading [24–27]. Although tyrosine phosphorylation is required for the inhibitory activity of Sprouty4 on a Ras/MAP kinase pathway, mutation of the corresponding tyrosine (Tyr-75 in human Sprouty4) to alanine had no apparent effect on its inhibitory actions on TESK1 activity and cell spreading, suggesting a novel cellular function of Sprouty4 to regulate the actin cytoskeleton, independent of it's inhibitory activity on the Ras/MAP kinase signaling.

Given that Sprouty proteins can inhibit FGF signal transduction, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression. We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 is downregulated in approximately 40% of prostate cancers when compared with normal prostate [28]. Downregulation of Sprouty2 mRNA has also been reported in breast cancer and a number of other common malignancies [29]. In the present study, we demonstrate that Sprouty4 is also downregulated in prostate cancer. The downregulation of these Sprouty isoforms in human prostate cancer implies a loss of an important regulatory mechanism in prostate cancers that may potentiate the effects of increased FGFs and FGF receptor expression in prostate cancer. In this report, we demonstrate the extensive methylation of a Sprouty4 CpG island in the majority of cancer cases when compared to normal peripheral tissue samples, which correlated with decreased Sprouty4 expression. In addition, treatment of the LNCaP prostate cancer cell line, in which this CpG island is methylated, with 5′-aza 2′-deoxycytidine restored Sprouty4 gene expression, confirming that methylation caused the gene downregulation. Furthermore we demonstrate that Sprouty4, unlike Sprouty1, does not cause cell growth inhibition but rather inhibits cell migration, suggesting that Sprouty1 and 4 perform different functions in prostate cancer. These observations support the idea that different Sprouty isoforms have distinct functions as tumor suppressors in prostate cancer and that expression of one or more Sprouty genes is decreased in most prostate cancers.

MATERIALS AND METHODS

Plasmid Construction

Plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) was used for the expression of full length Sprouty4 cDNA. For the construction of pcDNA-Sprouty4, the full coding sequence of Sprouty4 was amplified from EGFP-tagged hspry4 cDNA [23] (a gift from Onno C. Leeksma) in a PCR reaction using primers designed against the published Sprouty4 sequence as follows: forward primer 5′-AAGCTTA*G*AC**ATG**CTC AGCCCCC TCC-3′ and a reverse primer 5′-*GAATTC***CTA**GAAAGGCTTGTCGGG-3′ (the underlined sequence indicates *Hind* III and *Eco*RI sites in the forward and reverse primers, respectively; bold and underlined shows the start and stop sites in the forward and reverse primers respectively; 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. The pcDNA-Sprouty4 construct was verified by restriction digestion analysis and sequencing.

In Situ Hybridization of Sprouty4 in Prostate Tissues

The full-length Sprouty4 cDNA was cut from pcDNA-Sprouty4 (described above) and cloned as an *Eco*RI and *Hind* III fragment into pCMV-tag2B vector (Invitrogen). The universal primer T3 and T7 sequences in pCMV-tag2B were used to generate sense and

anti-sense RNA probes. Digoxigenin-labeled (Dig-labeled) anti-sense and sense RNA probes were synthesized using MAXIscript™ in vitro RNA transcription kit (Ambion, Inc., Austin, TX) with the linearized Sprouty4 plasmid as a template. A tissue microarray slide containing 14 prostate tissues was used for the in situ hybridization. Briefly, prostate tissues were dewaxed in xylene for 10 min (three times) and hydrated. Tissue sections were digested with 40 μg/ml proteinase K for 7 min at room temperature and then fixed in 4% paraformaldehyde for 20 min. The Dig-labeled probe (1 μg) was then added to 1 ml hybridization buffer (50% formamide, 10% SSC). Hybridization was performed at 70°C overnight, after which slides were sequentially washed by Dig Wash and Block Buffer Set (Roche Diagnostics, Indianapolis, IN) according to the manufacture's instruction. Antibody (1:2,000) against digoxigenin was used to detect the signal and NBT/BCIP was used as substrate for color development (Boehringer Mannheim, Germany). The staining in tissues was scored as no staining, weak staining, moderate, or strong staining.

Human ProstateTissue Samples

All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank [30]. Fresh frozen tissue punches of normal and tumor tissue were obtained at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70%–100% with Gleason scores of 6–8. Formalin-fixed, paraffin-embedded specimens were also obtained from the Baylor SPORE prostate cancer tissue bank.

Bisulfite Modification and Sequencing

The methylation status of the Sprouty4 gene 5′-flanking CpG islands was analyzed by bisulfite treatment, which converts unmethylated cytosines to uridines (then thymidines) while retaining methylated cytosines as unchanged nucleotides, followed by PCR amplification, cloning, and sequencing. Briefly, DNA samples prepared from prostate tissues were modified by sodium bisulfite treatment using MethylEasy kit (Human Genetic Signatures, Sydney, AUS) according to the manufacturer's protocol. The modified DNA samples were used in PCR analysis with primer pairs designed using MethPrimer software package for methylated and unmethylated CpG islands identification (http://www.urogene.org/methprimer/). Primers used for the analysis of the proximal CpG island of the promoter were forward 5′-GTTTTTGGTGGAGTTT-GAGTTAGTT-3′ and reverse 5′-CCACTACCTAAA-AAAA TAAC TTTTT-3′; for the analysis of the distal CpG island of the promoter were forward 5′-GGTTT-TATTTATTTATTTGGTTAGTTTT-3′ and reverse 5′-TAAATATCCTTT CTCTATCCCAATC-3′. The PCR amplification step was as follows: 95°C for 3 min, then denature at 95°C for 30 sec, anneal at 58°C (proximal CpG island) or 60.8°C (distal CpG island) for 30 sec, extension at 72°C for 30 sec for 35 cycles, and a final 10 min extension at 72°C. The PCR product was subsequently cloned into pCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instruction and the recombinants were sequenced using M13 reverse and T7 universal primers.

Cell Culture

The human prostate cancer cell lines, PC3, DU145, and LNCaP were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen). The human vascular endothelial cells (HUVEC) were cultured in complete EGM-2 medium (Cambrex Bioscience; Walkersville, MD).

Cell Transfection

For stable transfection, DU145 cells were seeded at 5×10^6 cells per 100 mm dish and transfected with 2.4 μg of Sprouty4 construct (pcDNA-Sprouty4) or vector only (pcDNA3.1) using Lipofectamine 2000 transfection reagent (Invitrogen) and according to the manufacturer's protocol. Two days after transfection, cells were selected in Geneticin (Sigma, St. Louis, MO) containing medium at a final concentration of 250 μg/ml. After 14 days into the selection Geneticin resistant clones were pooled together and propagated. For transient transfection, LNCaP, DU145, PC-3, and HUVEC were plated at 5×10^4 cells per 60 mm dish and transfected with 2 μg of Sprouty4 plasmid or vector only using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturers protocol. After 24, 48, or 72 hr 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.

Preparation, Quantification, and Dilution of DNA Standards

The Sprouty4 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 Spred2 plasmid was constructed by amplifying the Spred2 coding sequence from LNCaP cDNA in a standard PCR reaction using primers designed as forward 5′-AGACGATGACAGC

TATATTGTGCGT-3′ and reverse 5′-TCTCGTCGCT-AGTATCGCACG-3′. The PCR product was cloned into pCR 2.1 TOPO vector using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instruction. The FGF2 plasmid have been previously described [31]. 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 to 10^1 copies) was used as a DNA standard for real-time PCR.

Primer Design and Synthesis for Real-Time PCR

Oligonucleotide primers for Sprouty4 were forward 5′-TGACCAACGGCTCTTAGAC-3′; and reverse 5′-GCATTTACACTTCCCACAGG-3′; for keratin 18 were forward 5′-AGGGCTCAGATCTTCGCAAAT-3′ and reverse 5′-GTCATCAATGACCTTGCGGAG-3′; for β-actin were forward 5′-AGCACGGCATCGTCACCA-ACT-3′ and reverse 5′-TGGCTGGGGTGTTGAAGGT CT-3′; for Spred2 were forward 5′-TGAGCTTG-GCGATGATGAC-3′ and reverse 5′-CGAGGTGA-TAGTGGTCTGTG-3′ and for FGF2 were forward 5′-CCACTTCAAGG ACCCCAAG-3′; and reverse 5′ATAGCCAGGTAACGGTTAGC-3′. Primers were carefully designed to cross exon/intron regions, avoid the formation of primer-dimer, hair pin, and self complementarity. The nucleotide positions for the amplification products as given per the Genbank accession numbers are 577–757, 256–435, 458–622, 607–779, and 539–721 for Sprouty4 (AF227516), β-actin (BC004251), keratin 18 (BC020982), Spred2 (NM_181784), and FGF2, (NM_002006) 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 Super-Script™ first strand synthesis system for RT-PCR and according to the manufacturer's protocol. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Bio-Rad, Hercules, CA) as described previously [32] and incorporating the following optimized PCR reaction conditions: The amplification of Sprouty4 or Spred2 was carried out as follows: a 3 min hot start at 95°C followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing at 61°C for 30 sec. The amplification protocol for FGF2 was the same as Sprouty4 except annealing was done at 63.5°C. The amplification protocol for β-actin or 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 sec, annealing at 56°C for 20 sec, and a 72°C extension for 30 sec. Each experiment was done in duplicate. The 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.

Western Blotting

Total protein was extracted from cells using protein lysis buffer as described previously [33]. For Western blots, 30 μg of protein extract/lane were electrophoresed, transferred to nitrocellulose membrane (Hybond™ ECL™, Amersham Pharmacia Biotech, Picataway, NJ) and incubated overnight with a 1:2,000 dilution of anti-Sprouty4 rabbit polyclonal antibody (Upstate Biotech, Waltham, MA) or a 1:5,000 dilution of anti-β-actin mouse monoclonal antibody (Sigma). Membranes were washed and treated with goat anti-rabbit IgG (1:5,000; Bio-Rad) or rat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1:2,000 dilution; Southern Biotechnology Associates, Birmingham, AL) for Sprouty4 and β-actin, respectively. The antigen-antibody reaction was visualized using an enhanced chemiluminesence (ECL) assay (Amersham) and exposed to ECL film (Amersham).

Induction of Sprouty4 Expression by 5′-Aza-2′-Deoxycytidine (5′-aza-dC)

LNCaP cells were seeded at 5×10^5 cells/100-mm tissue culture dish. After 24 hr of incubation, the culture media was changed to media containing 5′-aza-dC for 96 hr. Cells were then harvested for RNA extraction and the extracted RNA used in real-time quantitative PCR as described above.

Wounding Assay of Scatter/Migration

Prostate cancer cells were seeded at 2×10^6 in 60-mm diameter culture dished and grown to confluence in complete medium and analyzed using a classical scratch wound method. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS. Complete medium was added and cells were allowed to scatter/migrate into the area of clearing for a total of 48 hr and photomicrographs taken at 0, 24, and 48 hr time points.

RESULTS

Expression of Sprouty4 in Normal and Neoplastic Human ProstateTissue

We initially investigated the expression of the Sprouty4 gene in human prostate tissues. To achieve this, we used in situ hybridization for the detection of Sprouty4 in 14 prostate tissues samples, since currently

available antibodies are not suitable for immunohistochemistry (unpublished observation). In normal prostate tissues we observed Sprouty4 expression in the epithelium (Fig. 1a) with minimal expression in prostatic stromal cells. Sprouty4 expression was variable; some normal tissues showed moderate expression (Fig. 1a) while others showed strong expression (Fig. 1b,c). Prostate cancer cells also had quite variable expression. Many prostate cancer tissues showed weak expression of Sprouty4 (Fig. 1b–d). Other cancers had moderate or even strong expression of Sprouty4 (Fig. 1e). Interestingly in some samples where we observed moderate or strong expression of Sprouty4 expression in the normal cells, the adjacent cancer cells showed less Sprouty4 expression (Fig. 1b,c). In situ hybridization with sense probe gave no detectable signal (Fig. 1f). To quantitatively compare the expression of Sprouty4 in normal and neoplastic prostatic epithelium, we used quantitative real-time PCR analysis to determine the expression level of Sprouty4 mRNA in a total of 25 pairs of matched normal and tumor prostate tissue samples. We used β-actin as an endogenous mRNA control. The real-time data is presented as the ratio of Sprouty4 mRNA transcripts X 10^3/β-actin transcript for the samples analyzed (Fig. 2a). The expression of Sprouty4 in both normal prostate and cancer tissues was variable, presumably reflecting both random variability in tissue composition (i.e., epithelial content) and variable expression per cell. However, Sprouty4 expression was about fivefold higher on average in the normal prostate tissues (14.5 ± 12.5, SEM) compared to prostate cancers (3.10 ± 0.962, SEM). Examination of paired normal versus cancer tissues revealed decreased Sprouty4 expression in 11 out of 25 cancer cases (44%) relative to matched normal tissues. This is similar to the decrease in Sprouty1 expression in normal versus cancer cells as determined by immunohistochemistry of tissue microarrays [28]. Since Sprouty4 is expressed almost exclusively in the epithelium, we also normalized Sprouty4 expression

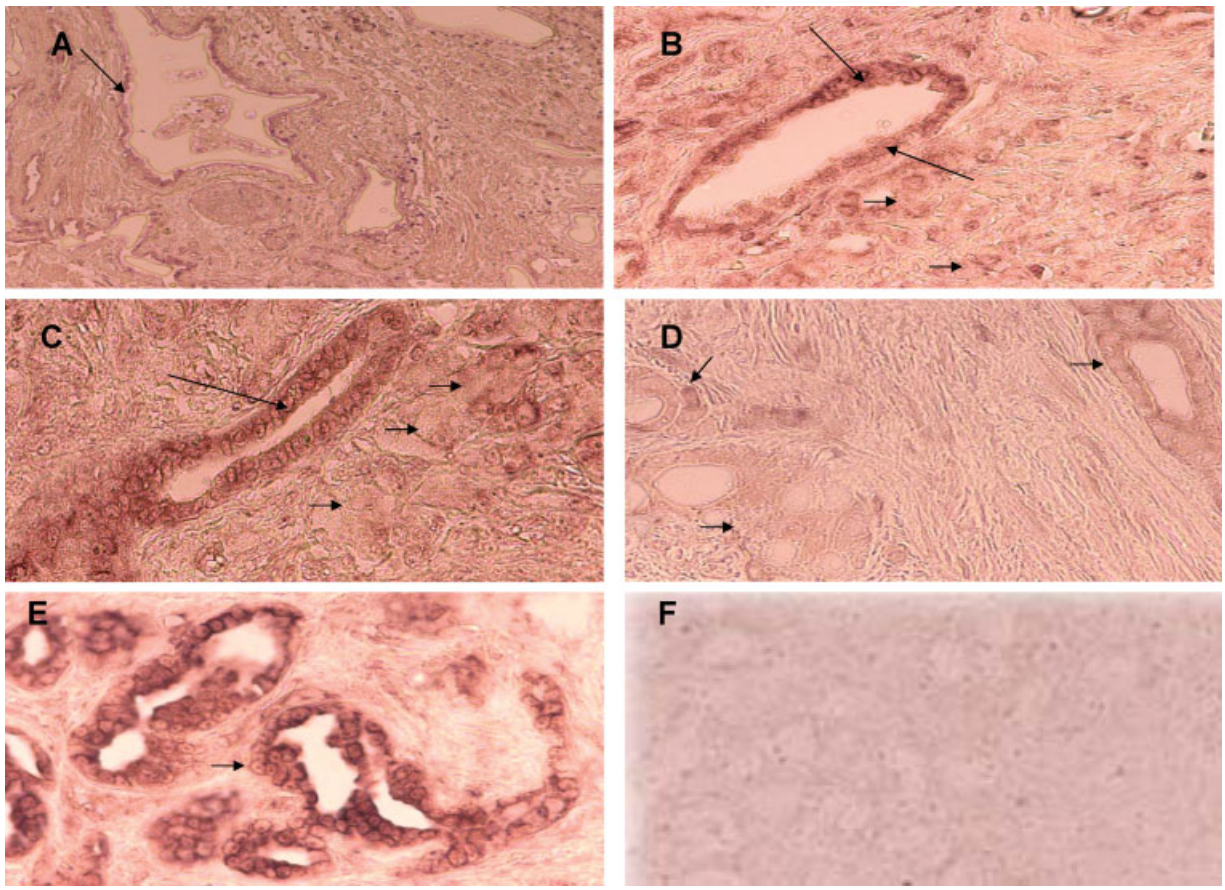

Fig. 1. In situ hybridization analysis of Sprouty4 expression in prostate tissues. Expression of Sprouty4 in normal prostate (**A**) and prostate cancer (**B–F**) was determined using in situ hybridization as described in Materials and Methods. A: Normal prostate peripheral zone tissue with expression of Sprouty4 in prostatic epithelial cells (long arrows). B–C: Normal prostate with strong expression of Sprouty4 (long arrows) with weak expression of Sprouty4 in diffusely infiltrating cancer cells surrounding normal tissue (some of which are indicated by short arrows). D: Prostate cancer glands with low Sprouty4 expression (short arrows). E: Prostate cancer with strong Sprouty4 expression in cancer glands (short arrow). F: Prostate tissue hybridized with sense probe.

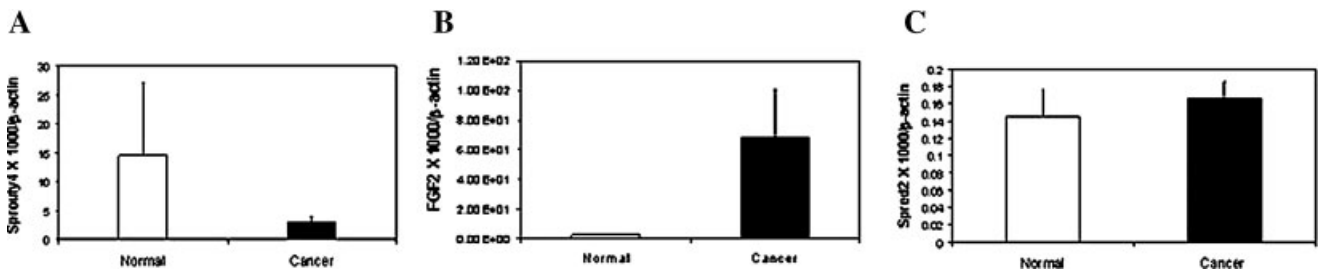

Fig. 2. Quantitative RT-PCR to determine the mRNA expression of Sprouty4, FGF2 and Spred2 in matched normal and cancer prostate tissue samples. Gene expression in normal prostatic tissues and cancer tissues was assessed by quantitative RT-PCR. Gene expression levels are displayed as a ratio of transcripts $\times 10^3$ to β-actin transcripts. The specific gene and β-actin values were calculated from standard curves. The data is a representative of duplicate experiments. The mean expression level (±SEM) is indicated. **A**: Sprouty4 expression (**B**) FGF2 expression (**C**) Spred2 expression.

using keratin 18 mRNA, which is expressed only by epithelium, and as such may be a more relevant gene for normalization. Out of a total of 25 pairs of matched normal and tumor prostate tissue samples, 16 cancer samples revealed decreased Sprouty4 expression (64%) relative to the matched normal tissue (data not shown). One possible explanation for decreased expression of Sprouty4 mRNA in the cancer tissues is decreased expression of FGFs in a subset of the prostate cancers. We have previously shown 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, while FGF7 protein levels are similar in normal and prostate cancer tissues [33]. We therefore investigated FGF2 mRNA expression in the 25 pairs of matched normal and tumor prostate tissue samples using quantitative RT-PCR (Fig. 2B). We found that the FGF2 mRNA expression level was more than 20-fold higher on average in this set of prostate cancer samples (67.8 ± 6.4, SEM) compared to normal prostate tissues (2.57 ± 0.49, SEM; Fig. 2B). This data clearly demonstrates that the decrease in Sprouty4 expression in the prostate cancer tissues does not reflect decrease of FGF ligands. Finally, we examined the expression of Spred2 mRNA in prostate cancer and normal prostate tissue. The Spred genes are related to Sprouty and also inhibit Ras/Raf signaling and activation of MAP kinases [34]. As shown in Figure 2c, there was no significant downregulation of Spred2 mRNA in prostate cancer. Thus, the downregulation of Sprouty4 mRNA appears to be specific, since not all negative regulators of FGF signaling are decreased in prostate cancer.

Epigenetic Analysis of Sprouty4 Gene 5′-Flanking Region

The human Sprouty4 gene maps to the long arm of chromosome 5 and is approximately 14.5 kb in length interrupted by two introns. Exon1 only encodes the 5′-UTR of the cDNA, whereas exon 2 contains the translation initiation codon. The remainder of the open-reading frame for the protein as well as the entire 3′-UTR is encoded by the third exon. Multiple transcription start sites have been identified by 5′-RACE analysis [35]. The 5′-flanking region of the human Sprouty4 gene lacks a canonical TATA box or CAAT sequence within the expected proximity of the transcription start site. Transient transfection studies by this group revealed the presence of the maximal basal promoter activity within the 1,182-bp 5′-flanking region upstream from the transcription initiation sites [35]. As further evidence demonstrating the importance of this core promoter region, a comparison of human and murine Sprouty4 proximal promoter sequences showed significant homology with each other. We therefore investigated whether aberrant gene methylation of Sprouty4 in this region is a mechanism of downregulation in of Sprouty4 gene expression in prostate cancer. Using the MethPrimer software package for CpG islands identification (http://www.urogene.org/methprimer/) we have identified 3 CpG islands spanning from 1,100 nt upstream of the putative transcription start through the first exon into the intronic sequence (Fig. 3A). This led us to investigate the possibility of epigenetic inactivation at this locus. We performed methylation analysis on 7 pairs of matched normal and tumor prostate tissue samples and an additional 15 prostate cancer tissue samples. Initial analysis of CpG islands in the 5′-flanking region of the Sprouty4 of genomic DNA samples from the five pairs of matched normal and tumor prostate tissues showed methylation in the CpG island approximately 1 kb upstream of the putative transcription start site, hereafter referred to as the 5′-flanking CpG island (labeled A-I in Fig. 3A). No methylation was observed in the CpG island in the proximal promoter region,

therefore all subsequent methylation analysis was carried out on the 5′-flanking CpG island. Examples of the methylation analysis of individual normal and cancer DNA samples are illustrated in Figure 3B. The mean frequency of methylation at individual CpG dinucleotides in the DNA samples is summarized in Figure 3C. Genomic DNA samples from tumor tissues had a higher frequency of methylation, with the CpG dinucleotides labeled E–I having a greater than fivefold higher frequency of methylation. Because CpG dinucleotides E–I exhibited about the same frequency of methylation, we also analyzed the average frequency of methylation in these five CpG dinucleotides for each individual patient (Fig. 3D). The results show that the number of methylations of CpG dinucleotides E–I is significantly higher in tumor samples ($P \leq 0.001$, Mann–Whitney rank sum test). To determine if methylation was correlated with decreased Sprouty4 expression, we determined the level of Sprouty4 transcripts in the same matched normal and cancer tissue samples used in the methylation analysis by quantitative RT-PCR (Fig. 3E). To examine the relationship between Sprouty4 promoter methylation and Sprouty4 expression, we compared the proportion of cases with Sprouty4 promoter methylation that have Sprouty4 expression that is lower than matched normal tissue (11 of 14 cases) to the proportion of cases without promoter methylation that have low Sprouty4 expression (2 of 8 cases). This difference is statistically significant by Fisher exact test ($P = 0.026$). However, it should be noted that in two cases with low Sprouty4 expression (cases 9 and 29) there was no detectable methylation at these sites.

Treatment of LNCaP with 5-Aza-2′Deoxycytidine can Restore Sprouty4 Expression

We initially measured Sprouty4 expression in the three commonly used prostate cancer cell lines LNCaP, DU145, and PC3. LNCaP cells had quite low expression (2.9×10^{-4} transcripts/β-actin transcript) with DU145 having slightly higher expression (4.04×10^{-4} transcripts/β-actin transcript) and PC3 having high expression (2.0×10^{-2} transcripts/β-actin transcript). These relative RNA levels correlate with protein expression as determined by Western blotting in which LNCaP had essentially undetectable expression of Sprouty4 (see Fig. 5, below). Analysis of LNCaP cells revealed methylation of the Sprouty4 promoter, which was not seen in DU145 or PC3 cells (data not shown). To test the hypothesis that pharmacological modulation of methylation can reactivate gene expression [36], we treated LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2′deoxycytidine (Aza dCR). Figure 4 indicates that treatment of LNCaP cells with Aza dCR (2.5 μM) led to over twofold increase in Sprouty4 mRNA expression. Overall, the in vivo and in vitro data demonstrate that methylation in the Sprouty4 gene is responsible for its downregulation in prostate cancer.

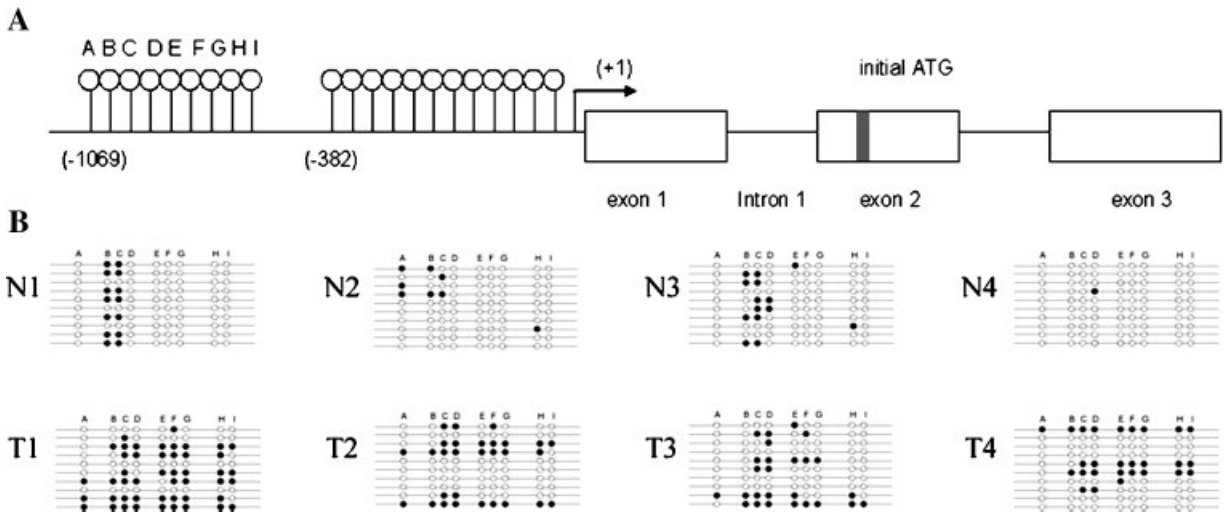

Fig. 3. Methylation analysis of the Sprouty4 gene. **A**: A schematic representation of the Sprouty4 gene. Distal CpG island is shown as 9 CpG dinucleotides (sites A–I). Transcriptional start (+1) site is shown as bar with an arrow-head. Exons are shown as open-boxes and translational start site shown as thick black bar. **B**: Analysis of CpG methylation in bisulfite-converted genomic DNA derived from four normal prostate tissue samples (N1–N4) and four cancer tissue samples (T1–T4). For each sample, 10 PCR clones were analyzed by sequencing. CpG methylated (black circle), CpG unmethylated (white circle). **C**: Frequency of methylation for each individual CpG dinucleotide from seven normal tissues (□) and 22 cancer tissues (■). **D**: Mean of methylation frequency of five CpG dinucleotides (sites E–I) in each individual patient. **E**: Ratio of Sprouty4 mRNA expression in cancer versus normal prostate tissue from each patient. Horizontal bar indicates equal expression in normal and cancer tissue samples.

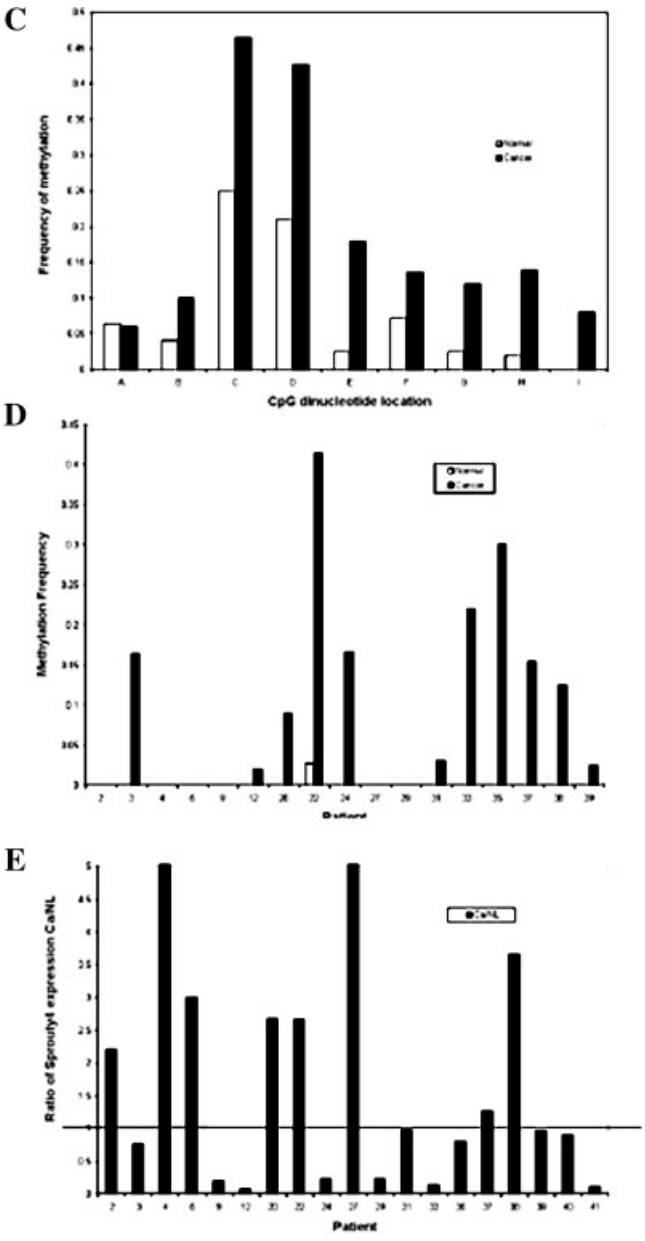

Fig. 3. (*Continued*)

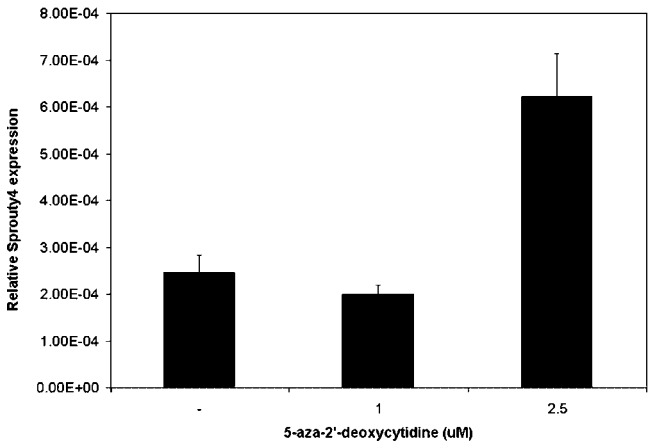

Fig. 4. Demethylation and Sprouty4 expression. Prostate cancer cell line LNCaP was treated with 5′-aza-2′-deoxycytidine (5′-aza-dC) at the indicated concentrations for 96 hr. Sprouty4 mRNA expression was determined by quantitative RT-PCR and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

The Biological Effect of Sprouty4 Over-Expression in Human Prostate Cancer Cells

To ascertain the biological effect of Sprouty4 expression in human prostate cancer cells, we transfected pcDNA-Sprouty4 (encoding the full length of Sprouty4 sequence) into human prostate cancer cell lines LNCaP, DU145, and PC3 and monitored cell proliferation. Transient expression of Sprouty4 in the prostate cancer cells did not seem to affect cell proliferation when compared the vector only control (Fig. 5A). To confirm the presence of Sprouty4 protein, these same cells were analyzed by Western blotting (Fig. 5B). The results shows an increase in Sprouty4 protein in both LNCaP and DU145 cells transfected with the Sprouty4 plasmid, however, we did not detect any significant increase of Sprouty4 protein in the PC3 which already has high basal Sprouty4 protein compared to the other two cell lines. We further verified the biological activity of Sprouty4 by transiently transfecting Sprouty4 plasmid into HUVEC. Transient transfection of HUVEC with Sprouty4 plasmid inhibited cell proliferation by 40% over 3 days when compared to cells transfected with the vector only control (data not shown). The result suggests that Sprouty4 protein mediated growth inhibition maybe cell-type dependent.

Increased cell migration and invasion is one of the characteristics associated with highly malignant phenotype of prostate cancer. To determine whether Sprouty4 protein inhibits prostate cancer cell migration, we over-expressed Sprouty4 in the DU145 prostate cell line, which shows modest basal Sprouty4 expression level (see Fig. 5B). The Du145 cells were transfected with Sprouty4 plasmid or vector only plasmid and several G418-resistant clones were selected and pooled together. We validated G418-resistant clones over-expressing Sprouty4 by Western blot analysis (Fig. 5C). In agreement with our transient transfection analysis, the stable over-expression of Sprouty4 in Du145 cells did not have any significant effect on cell proliferation (data not shown). To evaluate the effect of over-expressing Sprouty4 on Du145 cell migration, we utilized the scratch wound assay by assessing the rate of wound closure after scraping cells from an area of monolayer cultures.

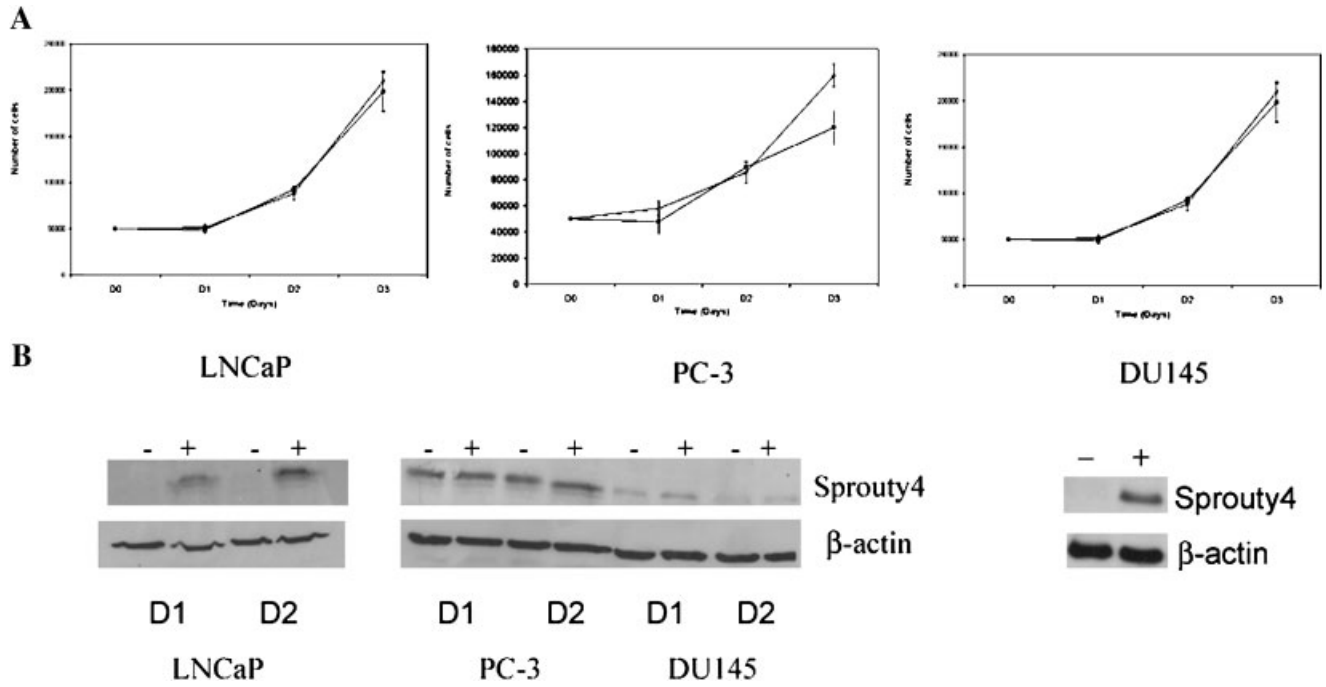

Fig. 5. Over-expression of Sprouty4 in prostate cancer cells. **A**: LNCaP, DUI45, and PC-3 prostate cancer cell lines were each transfected with a Sprouty4 cDNA cloned in the mammalian expression vector pcDNA3.I (■) or the pcDNA3.I vector only (Δ). At the indicated times after transfection, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate and the standard deviation is shown. **B**: Protein extracts were collected from LNCaP, DUI45, and PC-3 cells I and 2 days after transfection with pcDNA3.I (−) or Sprouty4 cDNA in pcDNA3.I (+) and analyzed by Western blotting with either anti-Sprouty4 antibody or control anti-β-actin antibody. **C**: DUI45 prostate cancer cell line was stably transfected with pcDNA3.I vector only (−) or pcDNA-Sprouty4 (+). After 2 weeks of selection in Geneticin, stable clones were selected, pooled together, and analyzed by Western blotting with either anti-Sprouty4 antibody or control anti-β-actin antibody.

Confluent DU145 cells were scraped and cells were allowed to migrate for 48 hr. As shown in Figure 6, control cells which were G418-resistant but not over-expressing Sprouty4 (vector control transfection) demonstrated higher rates of migration or wound closure when compared to cells over-expressing Sprouty4, which showed an obvious slower closer rate at the 24 and 48 hr time points. This experiment was replicated a total of four times with identical results.

DISCUSSION

In the present study, we were interested in understanding the role of Sprouty4 in prostate cancer and to elucidate the molecular mechanism regulating its expression. We have found by in situ hybridization and quantitative RT-PCR analysis that Sprouty4 is downregulated in the majority of human prostate cancers when compared to normal prostate tissue. Previously, we have shown that Sprouty1 is down-regulated in prostate cancer tissues [28] and that Sprouty1 can markedly decrease prostate cancer cell proliferation. Our data indicates distinct differences in the functional roles for Sprouty1 and Sprouty4 in prostate cancer cell lines. Transient expression of Sprouty1 significantly inhibited prostate cancer cell proliferation while stable over-expression was markedly deleterious to prostate cancer cells [28]. In contrast, transient expression of Sprouty4 did not have any significant effect on prostate cancer cell proliferation while stable over-expression of Sprouty4 inhibits prostate cancer cell migration. It has been shown that in some tissues the expression patterns of the Sprouty family members do not overlap [11]. These data indicate that the different isoforms of Sprouty are not uniformly regulated and suggests that the different family members may not be functionally equivalent. Thus the individual Sprouty genes may be regulated by specific combinations of factors to allow optimal control of signaling. It is likely that Sprouty4 mediates inhibition of cell migration in prostate cancer, at least in part, by repressing the kinase activity of TESK1. Sprouty4 has been shown to regulate the actin cytoskeletal reorganization by modulating the level of cofilin activity through TESK1 inactivation [22]. We have evaluated the expression of TESK1 by quantitita-tive RT-PCR and TESK1 is expressed at similar levels in normal and neoplastic prostate tissues, as well as in

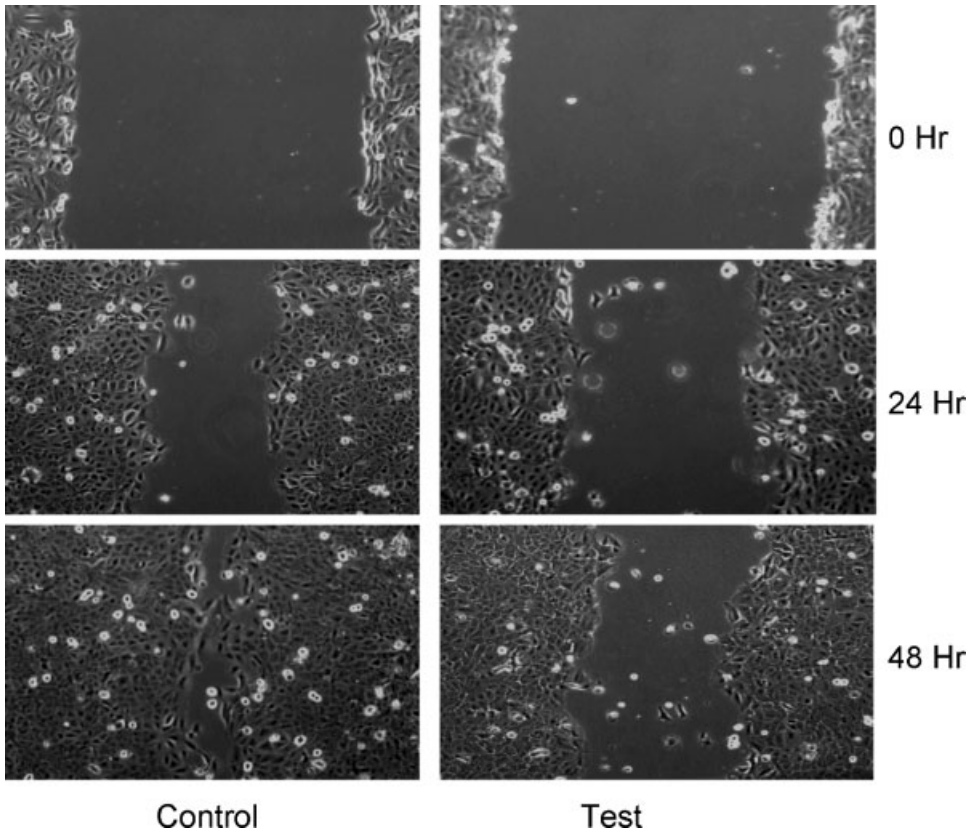

Fig. 6. Wounding assay of scatter/migration. DU145 cells stably transfected with Sprouty4 plasmid (test) or vector only plasmid (control), were used in a scratch wound assay as described in Materials and Methods. The cells were permitted to migrate into the area of clearing for a total of 48 hr, and photomicrographs taken at 0, 24, and 48 hr. Results shown are typical of four separate experiments.

primary prostatic epithelial cells, immortalized normal prostatic epithelium (PNT1a), and all prostate cancer cell lines tested, namely PC3, DU145, LNCaP, and LAPC4 (data not shown). Thus TESK1 is present in prostate cancer cells and can be inactivated by Sprouty4 in the absence of Sprouty4 methylation. It should be noted that while the majority of prostate cancers have decreased Sprouty4 expression, some have robust expression, based on both in situ hybridization and quantitative RT-PCR. Given that FGFs can induce Sprouty gene expression in normal tissues and that multiple FGFs are expressed at increased levels in prostate cancer tissues [1] this would be the expected pattern in the absence of specific alterations in cancer cells affecting Sprouty4 expression. Further investigations are needed to determine whether Sprouty4 expression inhibits disease progression in this subset of prostate cancers.

Multiple genetic alterations can drive tumorigenesis and progression. The metastatic and drug/hormone-resistant phenotype of certain cancers such as prostate cancer may result from epigenetic events such as aberrant gene methylation [37–39]. Generally, aberrant gene methylation occurs at proximal promoter CpG islands [40]. However, the methylation of CpG islands several kilobases away from promoter region, typically in gene enhancer region, can also lead to aberrant gene expression as seen in genetic imprinting and also in cancer (for review see Bird [40]). In our studies we did not observe CpG methylation in the Sprouty4 promoter CpG island in five pairs of matched normal and tumor prostate tissue samples or in the LNCaP prostate cancer cell line. On the other hand, we observed that Sprouty4 was extensively methylated at the 5′-flanking CpG island (approximately 1 kb upstream of the putative transcription start site) in a subset of tumor tissues compared with matched normal prostate. Studies by Ding et al. [35] showed significant enhancement of Sprouty4 promoter activity in transient transfection assays when the region from 979 to 1,182-bp upstream from the transcription initiation site was included in the promoter constructs, suggesting the presence of an enhancer activity upstream of the core promoter region in the region containing the methylated CpG island that we have identified. The increase in methylation significantly correlated with the decreased Sprouty4 expression, as analyzed by quantitative real-time PCR, demonstrating that, in majority of cases, the down-

regulation of Sprouty4 in a prostate cancer cases is due to promoter methylation. However, two cases without detectable promoter methylation had low Sprouty4 expression. It is possible that failure to detect methylation reflects the well-known problem that the bisulfite modification technique followed by PCR, cloning, and sequencing, while considered the ''gold standard'' for quantitative analysis of methylation, is not 100% efficient in detecting methylation due to a number of potential problems, which may be gene specific, that limit its efficiency (for discussion see Dahl and Guldberg [41]). Other mechanisms of gene inactivation, such as alterations in trans-acting factors and heterozygous or homozygous deletion could also affect Sprouty4 expression and remain to be explored. Of note are the recent studies by McKie et al. [42] that have identified methylation of the Sprouty2 promoter in high-grade prostate cancer, which is correlated with decreased Sprouty2 expression. Thus more than one Sprouty gene is inactivated by methylation in human prostate cancer. Whether the Sprouty1 promoter is also methylated in prostate cancer is being actively investigated by our laboratory. However, the diverse biological roles of different Sprouty family members in prostate cancer suggests that Sprouty signaling may provide an attractive new target approach for therapeutic intervention that may modulate a large number of potential growth promoting stimuli, including multiple growth factors and their receptors.

CONCLUSIONS

Sprouty4 expression is downregulated in human prostate cancer by DNA methylation and this decreased expression may contribute to increased cell migration.

ACKNOWLEDGMENTS

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Endocrine-Related Cancer (2004) **11** 709–724

REVIEW

The role of fibroblast growth factors and their receptors in prostate cancer

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Abstract

Prostate cancer is the most common malignancy in men in the USA and the second leading cause of cancer deaths. Fibroblast growth factors (FGFs), including FGF1 (acidic FGF), FGF2 (basic FGF), FGF6 and FGF8 are all expressed at increased levels in prostate cancer as paracrine and/or autocrine growth factors for the prostate cancer cells. In addition, increased mobilization of FGFs from the extracellular matrix in cancer tissues can increase the availability of FGFs to cancer cells. Prostate cancer epithelial cells express all four types of FGF receptors (FGFR-1 to -4) at variable frequencies. Expression of FGFR-1 and FGFR-4 is most closely linked to prostate cancer progression, while the role of FGFR-2 remains controversial. Activation of FGF receptors can activate multiple signal transduction pathways including the phospholipase Cγ, phosphatidyl inositol 3-kinase, mitogen-activated protein kinase and signal transducers and activators of transcription (STAT) pathways, all of which play a role in prostate cancer progression. Sprouty proteins can negatively regulate FGF signal transduction, potentially limiting the impact of FGF signaling in prostate cancer, but in a significant fraction of prostate cancers there is decreased expression of Sprouty1 mRNA and protein. The effects of increased FGF receptor signaling are wide ranging and involve both the cancer cells and surrounding stroma, including the vasculature. The net result of increased FGF signaling includes enhanced proliferation, resistance to cell death, increased motility and invasiveness, increased angiogenesis, enhanced metastasis, resistance to chemotherapy and radiation and androgen independence, all of which can enhance tumor progression and clinical aggressiveness. For this reason, the FGF signaling system it is an attractive therapeutic target, particularly since therapies targeting FGF receptors and/or FGF signaling can affect both the tumor cells directly and tumor angiogenesis. A number of approaches that could target FGF receptors and/or FGF receptor signaling in prostate cancer are currently being developed.

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Introduction

Prostate cancer is the most common visceral malignancy in men in the USA and the second leading cause of cancer deaths in this population. There is a large body of literature linking alterations of the fibroblast growth factor (FGF) system to initiation and progression of a wide variety of malignancies, including prostate cancer. There have been a number of excellent reviews of both the biology of FGFs and FGF receptors (Basilico & Moscatelli 1992, Johnson & Williams 1993, Dow & deVere White 2000, Powers *et al.* 2000, Ornitz & Itoh 2001) and their role in neoplastic transformation (Cronauer *et al.* 2003, Munro & Knowles 2003). This review will therefore focus more narrowly on the role of FGFs and their receptors in normal prostate and prostate cancer since there is an extensive and growing literature in this area.

The FGF signaling system

The human FGF gene family consists of at least 23 different genes encoding related polypeptides. FGFs are expressed in almost all tissues and play important roles in a variety of normal and pathological processes, including development, wound healing and neoplastic transformation. The FGFs are mitogenic for many cell types, both epithelial and mesenchymal. Some FGFs, like FGF2,

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have potent angiogenic activity and have been implicated as promoters of tumor angiogenesis. FGFs have also been shown to increase the motility and invasiveness of a variety of cell types. Finally, it has been shown that FGFs can inhibit cell death in the appropriate context. Thus FGFs have a broad range of biological activities that can play an important role in tumorigenesis.

FGFs interact with a family of four distinct, high-affinity tyrosine kinase receptors, designated FGFR-1 to -4 (Johnson & Williams 1993). The receptors consist of an extracellular portion containing three immunoglobulin-like domains and an intracellular tyrosine kinase domain and have variable affinities for the different FGFs. In addition, FGFRs-1 to -3 all undergo an alternative splicing event in which two alternative exons (IIIb and IIIc) can be used to encode the carboxy terminal portion of the third immunoglobulin-like loop, which results in receptor isoforms with dramatically altered binding specificity. The IIIa alternatively spliced isoform is secreted. A variety of other alternative splicing events have been described, including alternative splicing that results in loss of the first extracellular immunoglobulin-like domain.

The third component of the FGF system is extracellular matrix (ECM) and secreted proteins that help mobilize FGFs from the ECM. Heparin and heparin sulfate proteoglycans play a critical role in facilitating FGF signaling via FGF receptors, although there is controversy regarding the exact manner by which this occurs (Powers *et al*. 2000). FGFs are bound in the extracellular matrix and can be released by the activity of degradative enzymes such as proteases. In addition, FGF-binding protein (FGF-BP), a 17 kDa secreted polypeptide, can reversibly bind FGF1 and FGF2 and can facilitate release of FGFs from the extracellular matrix and interaction of these growth factors with cellular receptors (Aigner *et al*. 2001).

Binding of FGFs to the extracellular domains of FGF receptors results in receptor dimerization and transphosphorylation of tyrosine residues in the intracellular domain that is required for FGF receptor kinase activation. Ultimately, activation of FGF receptors leads to signal transduction through multiple pathways including phospholipase Cγ (PLCγ) (Burgess *et al*. 1990, Mohammadi *et al*. 1991), phosphatidylinositol 3-kinase (PI3K) (Hart *et al*. 2001), mitogen-activated protein kinases (MAPK) (Hadari *et al*. 2001) and signal transducers and activators of transcription (STATs) (Hart *et al*. 2000, Deo *et al*. 2002, Udayakumar *et al*. 2002). These effectors in turn disseminate the receptor tyrosine kinase signals by activating many target proteins, including transcription factors in the nucleus (Fig. 1). All of these pathways have been shown to be upregulated in prostate cancer and there is strong evidence linking each of these pathways to prostate cancer initiation and progression.

The docking proteins, FRS2α and FRS2β, play a critical role in mediating the intracellular signals that are generated at the cell surface by activation of the FGF receptors. Both FRS2α and FRS2β contain myristyl anchors and phosphotyrosine-binding sites in their C-terminal tails that serve as binding sites for the adaptor protein, Grb2, and for the Src homology (SH) 2 domain containing protein tyrosine phosphatase, Shp2 (Kouhara *et al*. 1997, Hadari *et al*. 1998). In response to FGF stimulation, Grb2 can also be recruited indirectly to FRS2α through its interaction with tyrosine-phosphorylated Shp2 molecules bound to the docking protein (Hadari *et al*. 1998). FGF-induced tyrosine phosphorylation of FRS2α results in complex formation with the adaptor protein Grb2 bound to Cbl by means of its SH3 domains. FGF-induced ternary complex formation among FRS2α, Grb2 and Cbl results in ubiquitination and degradation of FRS2α and FGF receptor (Wong *et al*. 2002). Thus, FRS2α functions as a central platform for recruitment of multiprotein complexes that are responsible for both signal activation and attenuation.

A new family of regulators of FGF activity has recently been identified. Sprouty inhibits signaling mediated by the FGF receptor and the epidermal growth factor (EGF) receptor during eye development and oogenesis in *Drosophila* (Casci *et al*. 1999, Kramer *et al*. 1999, Reich *et al*. 1999). Four mammalian genes (Sprouty1–4) have been identified with sequence similarity to *Drosophila* Sprouty (Tefft *et al*. 1999). *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/MAPK and Ras/Raf/ERK pathways and other proteins including c-Cbl, Grb2, Raf1, FRS2, Caveolin-1, dual specificity kinase TESK1, the protein tyrosine phosphatase PTP1B and the *Drosophila* Ras-GAP, Gap1 (Dikic & Giordano 2003) but the precise molecular mechanism by which the FGF receptor signal is blocked remains controversial.

FGFs and FGF receptors in normal prostate

The prostate is a mixed epithelial and stromal organ that requires androgenic stimulation for its development, maintenance and growth. There is a considerable body of evidence indicating that interaction between the stroma and epithelium plays a crucial role in the growth and development of the prostate. The mesenchymal elements in the prostate appear to mediate the development of this organ in response to androgens (Chung *et al*. 1991,

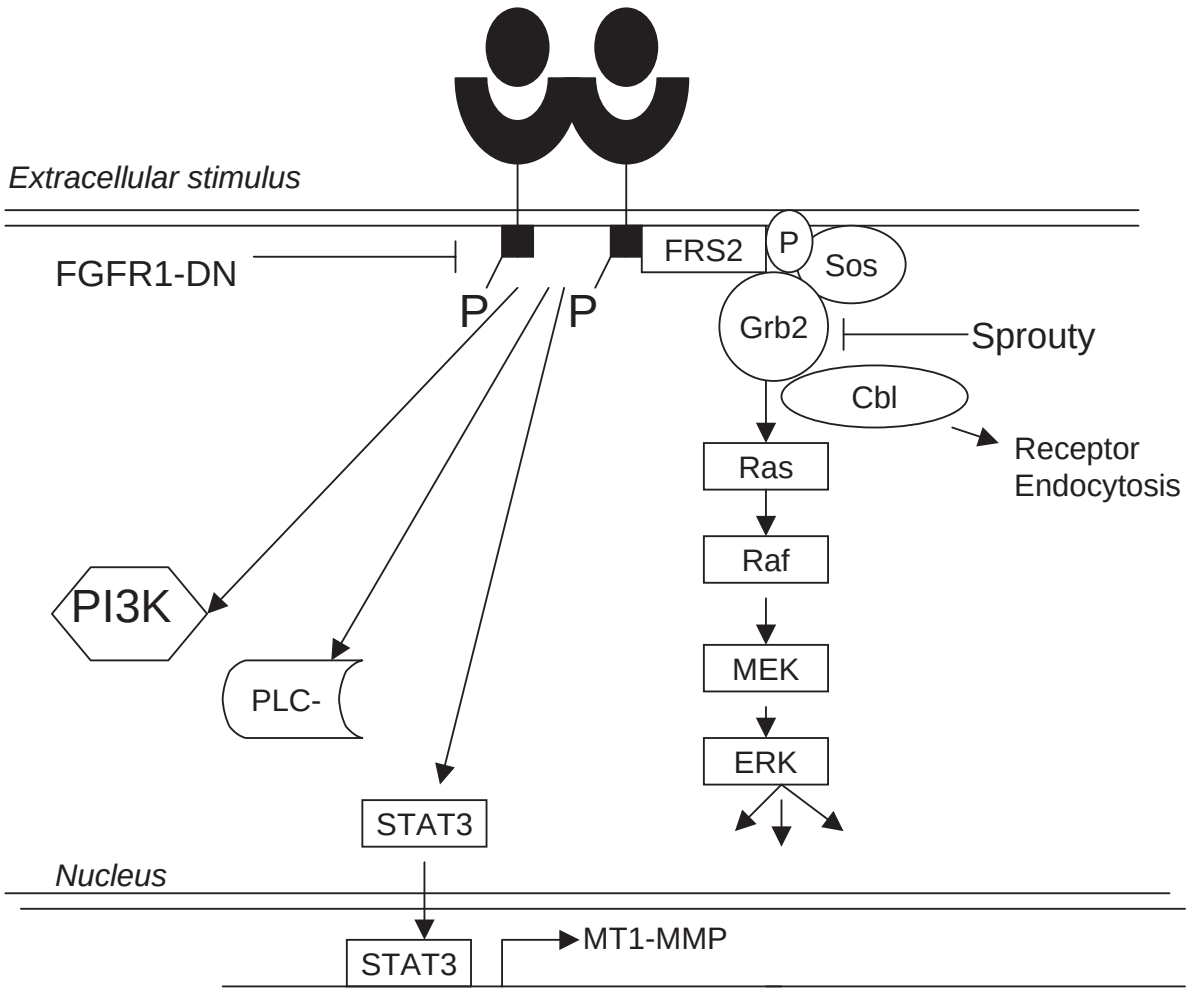

Figure 1 FGF signaling pathways in the prostate. Ligand binding initiates receptor dimerization and activation, promoting binding of the adaptor molecule FRS2 and the subsequent recruitment of adapter proteins Grb2 and Son of sevenless (Sos) to the FGF receptor complex. Sos facilitates guanine nucleotide exchange to activate Ras, which stimulates the Raf-to-MEK (MAPK)-to-extracellular signal-related kinase (ERK). These effectors, in turn, disseminate the receptor tyrosine kinase signals by phosphorylating many target proteins, including transcription factors in the nucleus. The PI3K, PLCγ and STAT pathways are also activated by FGF receptor signaling. Regulators of FGF signaling include Sprouty. Cbl mediates downregulation of receptor protein. FGF receptor dominant negative (DN) proteins can be used to block FGF receptor signaling experimentally. MT1-MMP, membrane-type-I matrix metalloproteinase; P indicates phosphorylation.

Hayward *et al.* 1997). In addition, it has been shown that prostatic stromal cells in culture secrete factors into their medium that stimulate or inhibit prostatic epithelial growth in a paracrine manner (Kabalin *et al.* 1989, Yan *et al.* 1992). Thus the accepted paradigm is that *in vivo* the stromal cells secrete paracrine factors, some of which are under the control of androgens, which are responsible for the maintenance and growth of the epithelium. The major source of FGFs in human prostate is the prostatic stromal cells and they can act as paracrine growth factors for the epithelial cells. FGF2 (basic FGF), FGF7 (KGF) and FGF9 are expressed by the stromal cells of the prostate in biologically significant quantities. The mean FGF2 content of normal peripheral zone tissue as measured by ELISA is 110 ng/g wet weight (Giri *et al.* 1999*a*). FGF2 does not contain a classical signal peptide and is not efficiently secreted (Basilico & Moscatelli 1992). Traditionally, it was thought that FGF2 is released by cellular damage. However, a number of investigations have indicated that FGF2 is actively (but inefficiently) secreted and recent evidence indicates that FGF2 can be actively transported across the plasma membrane (Schafer *et al.* 2004) and/or can be released by extracellular vesicle shedding (Taverna *et al.* 2003). FGF7 is present at approximately 28 ng/g wet weight in normal prostate (Giri *et al.* 1999*a*). FGF7 contains a classic signal peptide

and is actively secreted. It has been reported that FGF7 is induced by androgens in cultures of rat (Yan *et al*. 1992) and human (Planz *et al*. 1998) prostatic stromal cells, but observations on castrated rats indicated that FGF7 expression in the prostate may not be androgen regulated *in vivo* (Nemeth *et al*. 1998) so the effect of androgens on FGF7 production is controversial. Our laboratory has shown a small increase in FGF7 secretion in organ cultures of normal prostate tissue in response to androgens (authors' unpublished data), supporting the idea that FGF7 expression by stromal cells can be regulated at least partially by androgens, but other factors may be more important (Giri & Ittmann 2000). We have also found that FGF9 is expressed by prostatic stromal cells and is present in normal prostate tissue at approximately 51 ng/g wet weight by ELISA (authors' unpublished data). FGF9 is actively secreted and acts as a growth factor for both prostatic epithelial and stromal cells in culture (Giri *et al*. 1999b). All of these FGFs are potent growth factors for primary epithelial cells in culture at 1–10 ng/ml, so it is clear that the concentrations of FGFs present in normal prostate are biologically significant. FGF10 is expressed by stromal cells, but is present at low levels in normal adult prostate and probably does not act as a significant growth factor in this context, although it is important for prostatic development (Ropiquet *et al*. 2000*a*). In addition to these stromal FGFs, epithelial-derived FGFs are present in the normal prostate. Small amounts of FGF6 can be observed in basal cells of normal prostate gland (Ropiquet *et al*. 2000*b*) on immunohistochemistry (IHC). We have recently found that FGF17 is expressed in relatively small amounts by prostate epithelial cells (Polnaszek *et al*. 2004). Both of these FGFs are actively secreted and, given that they are expressed by the epithelial cells in an autocrine manner, they may have biological importance that is disproportionate to their relatively low expression level, since they would not have to diffuse across the ECM in order to interact with epithelial FGF receptors.

The expression of the other FGFs that we have evaluated to date in normal human prostate are substantially less than FGF2, FGF7 and FGF9. We have shown that expression of FGF1 (acidic FGF) is barely detectable by RT-PCR in normal prostate and in primary cultures of prostatic stromal cells, but cannot be detected by Northern blotting (Ittmann & Mansukhani 1997). We have also detected FGF5 and FGF8 mRNA in human prostate by RT-PCR but neither of these was detectable by Northern blotting (Ittmann & Mansukhani 1997). Our observations regarding the expression of FGF8 in normal human prostate are supported by similar observations by other laboratories (Ghosh *et al*. 1996). No expression of FGF3, FGF4 or FGF16 was detected by RT-PCR of normal peripheral zone tissue (Ittmann & Mansukhani 1997, authors' unpublished observations). Expression of FGF15, FGF19 and FGF20-23 in normal prostate has not been examined to date, to our knowledge. FGFs 11–14 do not appear to be secreted and at least two of these FGF family members act as intracellular signal transduction scaffolding molecules rather than as growth factors (Schoorlemmer & Goldfarb 2001). We have detected expression of FGF13 but not FGF14 in normal prostate by RT-PCR (authors' unpublished data).

Prostate epithelial cells express multiple FGF receptors. FGFR-1 and FGFR-2 are expressed in the basal epithelial cells of the prostate but not the luminal cells (Giri *et al*. 1999*a*). Based on studies of primary epithelial cells in culture, FGFR-1 is present exclusively as the IIIc isoform, while FGFR-2 is present exclusively as the IIIb (FGF7 specific) isoform in the epithelium (Ittmann & Mansukhani 1997). FGFR-3 is also present in prostatic epithelium, predominantly, but not exclusively, as the IIIb isoform (Kwabi-Addo *et al*. 2001). Finally, FGFR-4 is also expressed in prostatic epithelium and IHC has revealed that it is expressed in the luminal epithelial cells (Wang *et al*. 2004*b*). Based on the known properties of the various FGF receptor isoforms (Ornitz *et al*. 1996), prostatic epithelial cells express appropriate receptors to respond to the FGFs present in normal prostate, i.e. FGFR-1 IIIc binds FGF2 and FGF6, FGFR-2 IIIb binds FGF7, FGFR-3 IIIb binds FGF9, and FGFR-4 binds FGF2, FGF6 and FGF9.

Expression of FGFs in prostate cancer

FGF1 (acidic FGF) is a potentially important mitogen in prostate cancer due to the fact that it is mitogenic when it binds any type and isoform of FGF receptor (Ornitz *et al*. 1996). More than 80% of prostate cancers express FGF1 in the cancer cells by IHC and that strong expression was correlated with increased Gleason score (Dorkin *et al*. 1999*a*). FGF1 was also increased in prostatic intraepithelial neoplasia (PIN). Both the PC3 and LNCaP prostate cancer cell lines express FGF1 (Payson *et al*. 1998). Expression of FGF1 is detected by RT-PCR (Foster *et al*. 1999) or Western blotting (Polnaszek *et al*. 2003) in a significant fraction of prostate cancer tissues in the TRAMP mouse model of prostate cancer. FGF1 has a nuclear localization motif and has been detected in the nucleus in other systems (Klingenberg *et al*. 2000) but the role of nuclear FGF1 in prostate cancer is unclear.

FGF2 (basic FGF) is expressed in many human malignancies, including prostate cancer. Using ELISA we have shown that FGF2 is present at significantly higher concentrations in clinically localized cancer tissue (almost 2.5-fold) when compared with normal prostate (Giri *et al*.

1999a). Immunohistochemical analysis revealed that FGF2 was present in stromal cells within the prostate cancer, consistent with a paracrine effect of FGF2 in localized prostate cancer from radical prostatectomy specimens. We have also shown that prostate cancer expresses increased levels of interleukin-8 (IL-8) and that IL-8 can induce expression of FGF2 (Giri & Ittmann 2001), so one candidate mediator for the induction of FGF2 expression in stromal cells is IL-8 secreted by the cancer cells. In contrast, two groups, using primarily tissues from more advanced cancers than our laboratory has analyzed, have examined expression of FGF2 in prostate cancer by IHC and detected expression of FGF2 in prostate cancer epithelial cells in the majority of these cases (Cronauer *et al*. 1997, Dorkin *et al*. 1999a). Most of the prostate cancers in these studies were locally advanced or metastatic and/or poorly differentiated and thus are much more aggressive than the cancers from the radical prostatectomy specimens studied by our group. High levels of expression of FGF2 are present in PC3 and DU145 prostate cancer cells (Cronauer *et al*. 1997) and both of these cell lines were derived from metastatic prostate cancer. In addition, it has been demonstrated that prostate cancer patients have elevated levels of serum FGF2 (Cronauer *et al*. 1997). Thus it seems likely that initially FGF2 is expressed as a paracrine factor by stromal cells in localized prostate cancer and during tumor progression there is a switch to autocrine expression. In support of this hypothesis, there is increased expression of FGF2 during progression of TRAMP prostate cancers to a poorly differentiated phenotype (Huss *et al*. 2003). When TRAMP mice were crossed with FGF2 knockout mice, there was a significant increase in survival and decreased metastasis in mice bearing even one FGF2 knockout allele, which supports the hypothesis that the increased FGF2 expression seen during progression in the TRAMP model is biologically important in tumor progression (Polnaszek *et al*. 2003). One interesting aspect of FGF2 biology is the production of high molecular weight forms of FGF2 (22 and 25 kDa) that arise from alternative translation initiation from CUG codons that preferentially localize directly to the nucleus and can promote growth in low serum in some cell types (Arese *et al*. 1999). These higher molecular weight forms are present in TRAMP prostate cancers (Huss *et al*. 2003). If FGF2 is expressed as autocrine growth factors by the cancer cells, these higher molecular weight intranuclear forms may be biologically significant.

Our laboratory has demonstrated that FGF6 is increased in prostate cancer (Ropiquet *et al*. 2000*b*) and high grade PIN. ELISA of tissue extracts of normal prostate, PIN and prostate cancer for FGF6 showed that this growth factor was undetectable in normal prostate but was present at elevated levels in four of nine PIN lesions and in 15 of 24 prostate cancers. Immunohistochemical analysis with anti-FGF6 antibody revealed weak staining of prostatic basal cells in normal prostate that was markedly elevated in PIN. FGF6 may play a unique role in prostate cancer by acting as a paracrine factor secreted by residual basal cells in PIN that supports the growth of the dysplastic luminal epithelial cells. In the prostate cancers, IHC revealed autocrine expression of FGF6 by the prostate cancer cells in the majority of the cases.

The role of FGF7 in prostate cancer is unclear. We have measured expression of FGF7 in prostate cancer tissue from radical prostatectomy specimens by ELISA and have found that expression of FGF7 is similar in normal and cancer tissue and that, by IHC, as in normal tissue FGF7 is expressed by stromal cells (Giri *et al*. 1999*a*). We have recently confirmed that there is no increase in expression of FGF7 mRNA is prostate cancer tissue using quantitative RT-PCR (authors' unpublished data). In contrast, Planz *et al*. (1999) have reported expression of FGF7 in cancer cells on IHC of sections from radical prostatectomies using a different antibody. Serum FGF7 levels are lower in men with prostate cancer than in men with benign prostatic hyperplasia (BPH) (Mehta *et al*. 2000*a*), and we have shown that FGF7 is substantially elevated in BPH tissue (Ropiquet *et al*. 1999*b*), consistent with our observation that FGF7 is not elevated in prostate cancer. Further studies are needed to determine if there is increased expression of FGF7 in prostate cancer. In addition, as will be discussed below, it is unclear whether activation of FGFR-2, the only receptor that binds FGF7, promotes prostate cancer progression, so that even if FGF7 is increased it is not clear whether it would promote tumor progression.

Expression of FGF8 in human prostate cancer cells, both by *in situ* hybridization and IHC, has been well documented, while normal prostate expresses little detectable FGF8 (Leung *et al*. 1996, Tanaka *et al*. 1998, Dorkin *et al*. 1999*a,b*, Valve *et al*. 2001, West *et al*. 2001, Gnanapragasm *et al*. 2003). Overall, about 50% of clinically localized cancers express increased FGF8 while 80% or more of advanced cancers express increased FGF8. Dorkin *et al*. (1999*b*) observed strong correlations of FGF8 expression with tumor grade, stage and patient survival, although FGF8 did not appear to be an independent predictor of survival on multivariate analysis. An interesting aspect of FGF8 is that its mRNA undergoes alternative splicing, yielding multiple isoforms designated FGF8a, b, e and f (Ghosh *et al*. 1996). FGF8b is apparently the major isoform expressed in prostate cancer, although expression of the other alternatively spliced isoforms has also been reported in prostate cancers (Valve *et al*. 2001). FGF8b is

expressed by LNCaP, DU145 and PC3 cells and it has transforming activity in NIH3T3 cells (Tanaka *et al*. 1995, Ghosh *et al*. 1996). Increased expression of FGF8b in LNCaP cells using a lentivirus vector resulted in increased growth, colony formation in soft agar, invasion and tumorigenesis *in vivo* and facilitated growth-promoting stromal–epithelial interactions (Song *et al*. 2000*a*). Taking the opposite approach, Rudra-Ganguly *et al*. (1998) showed that antisense inhibition of FGF8 expression in DU145 cells decreased soft agar colony formation and tumorigenicity *in vivo*. Prostate-specific expression of FGF8b in transgenic mice under the control of an enhanced probasin promoter results in progressive epithelial hyperplasia and ultimately PIN, although invasive carcinoma was not reported (Song *et al*. 2002). Taken together, the finding of expression in human tissues that correlates with clinical and pathological parameters of aggressive disease and the biological observations *in vitro* and *in vivo* are convincing for the importance of FGF8 in human prostate cancer progression.

We have detected expression of FGF17 in normal prostatic epithelium and this expression is maintained in cancer cells (Polnaszek *et al*. 2004). Although, in samples from clinically localized cancers, the amount of FGF17 per cancer cell is similar to that in normal epithelial cells, the cancer cells constitute most of the tissue volume within the cancer tissue, leading to increased local FGF17 concentration. The FGF17 present in such cancers can act as an autocrine growth factor for the prostate cancer cells. We also observed increased expression of FGF17 in the DU145 cell line, suggesting that FGF17 may be expressed at increased levels in advanced prostate cancers. Further studies of FGF17 expression in advanced cancer tissues are needed to clarify this question.

In summary, multiple FGFs are expressed either as autocrine or paracrine growth factors in PIN and prostate cancer tissues. Many questions remain to be answered. Due to the fragmentary and relatively small scale of most the studies reported to date, the extent to which the expression of the different FGFs overlap is unclear, although Dorkin *et al*. (1999*a*) did examine FGF1, FGF2 and FGF8 by IHC in relatively advanced cancers and found that the expression of these growth factors was only partially overlapping. Expression of any one FGF has not been shown to be an independent prognostic factor on multivariate analysis, but the relatively small number of specimens involved in the published studies limits the power of this analysis and the possibility that the FGFs may act synergistically should be examined. The advent of tissue microarrays should greatly facilitate simultaneous analysis of multiple FGFs in large numbers of prostate cancers. In addition, not all FGFs have been studied quantitatively, so it is difficult to determine the relative expression levels of the different FGFs in cancer tissues. Finally, the most important question is the mechanism by which expression of FGFs is regulated in prostate cancer cells. FGF expression can be modulated by transcriptional, post-transcriptional and translational mechanisms, but little is known about what underlies the expression of FGFs as autocrine growth factors in prostate cancer cells. Although androgen may modulate expression of some FGFs, it seems unlikely that androgen receptor alone can selectively increase expression in cancer cells, since androgen receptor is also active in the benign cells. Further studies are needed to determine how FGF expression in upregulated in prostate cancer.

Expression of FGF receptors in prostate cancer

Our laboratory has shown that FGFR-1 is expressed in approximately 20% of moderately differentiated cancers and 40% of poorly differentiated clinically localized cancers based on IHC and Western blotting of prostate cancer extracts, but was not detected in well-differentiated cancers (Giri *et al*. 1999a). Prostate cancer cells most closely resemble prostatic luminal epithelial cells in their differentiation, in that they express cytokeratins that are similar to these cells and prostate-specific antegen (PSA). Given that luminal epithelial cells do not express FGFR-1, it appears that with transformation and progressive loss of differentiation, there is increasing expression of FGFR-1. Takahashi (1998) found increased expression of FGFR-1 mRNA in poorly differentiated prostate cancer, with which our results are consistent. Naimi *et al*. (2002) found relatively equal expression of FGFR-1 IIIc in normal prostate and cancer tissues by quantitative RT-PCR. However, FGFR-1 IIIc is expressed in stromal cells and as the cancer epithelial cells replace stroma in the cancer tissues (which were 90% cancer in this study), there would be a marked decrease in FGFR-1 IIIc unless the cancer epithelial cells express FGFR-1 IIIc. Thus, these quantitative RT-PCR results are also consistent with our IHC observations. FGFR-1 is also expressed preferentially in poorly differentiated TRAMP prostate cancers (Huss *et al*. 2003). In the Dunning rat prostate carcinoma model, FGFR-1 expression promotes tumor progression (Feng *et al*. 1997). Transgenic models that express constitutively active FGFR-1 in the prostate epithelium develop hyperplasia and PIN (Wang *et al*. 2002, 2004*a*) and increased expression accelerates the appearance of this phenotype (Jin *et al*. 2003). Transgenic mice in which FGFR-1 kinase is activated by a chemical dimerizer also develop PIN when treated with dimerizer drug (Freeman *et al*. 2003*a*). Finally, activation of FGFR-1 in TRAMP cell lines using chemical dimerizer enhanced tumor

proliferation *in vitro* and *in vivo* (Freeman *et al*. 2003*b*). It should be noted that FGFR-1 IIIc binds FGF2 and FGF6, both of which are increased in prostate cancer tissues. Thus, all evidence to date strongly supports the hypothesis that FGFR-1 can promote prostate cancer progression.

In contrast to the consistent evidence linking FGFR-1 to prostate cancer progression, the role of FGFR-2 in prostate cancer is far less clear. Activation of FGFR2-2 IIIb by FGF7 can enhance proliferation of primary or immortalized prostate epithelial cells *in vitro* (Ropiquet *et al*. 1999*a,b*) and prostate-specific expression of FGF7 expression promotes hyperplasia of prostatic epithelium in a transgenic model *in vivo* (Foster *et al*. 2002). As described above, FGF7 is expressed in prostate cancer tissues at levels similar to those in normal prostate. However, McKeehan and his colleagues have shown that in the Dunning rat prostate cancer model FGFR-2 IIIb expression inhibits neoplastic progression (Feng *et al*. 1997, Matsubara *et al*. 1998). Furthermore, when FGFR-2 kinase is activated in a transgenic mouse prostate epithelium using a chemical dimerizer, mice do not develop PIN (Freeman *et al*. 2003*a*), while activation of FGFR-2 by chemical dimerizer in TRAMP cell lines does not enhance tumor proliferation as observed for FGFR-1 (Freeman *et al*. 2003*b*). Finally, decreased FGFR-2 activity enhances the progression to PIN in mice expressing activated FGFR-1 (Jin *et al*. 2003). Thus most *in vivo* studies have found that FGFR-2 either inhibits or does not promote prostate cancer initiation and progression. Another complication is that FGFR-2 is expressed in normal prostate epithelium as the IIIb isoform, which binds almost exclusively to FGF7 and FGF10. During progression in the Dunning model there is a change in alternative splicing of FGFR-2, with increased expression of the IIIc isoform (Yan *et al*. 1993). In the DU145 cell line and in one of three prostate cancer xenografts studied, there was predominant expression of the FGFR-2 IIIc isoform, suggesting that exon switching to the FGFR-2 IIIc isoform by changes in alternative splicing may occur in human cancers (Carstens *et al*. 1997). Using a PCR-based approach we found that such isoform switching occurs in a subset of clinically localized cancers *in vivo* (Kwabi-Addo *et al*. 2001). TRAMP prostate cancers express increasing amounts of FGFR-2 during progression to the poorly differentiated phenotype and express the IIIc isoform even at the PIN stage (Huss *et al*. 2003). Such isoform switching would allow FGFR-2 to be activated by FGF2, FGF6 and FGF9 in human cancer tissue. However, if FGFR-2 activation does not promote or even inhibits tumor progression, it is difficult to see any selective advantage to the tumor cells in this isoform switching, unless the FGFR-2 IIIc is forming heterodimers with other FGF receptors such as FGFR-1 and activating them to promote cancer progression. It is also possible that such isoform switching is a manifestation of an epithelial to mesenchymal transition and does not in itself yield a selective advantage for tumor cells. Our laboratory has shown that FGFR-2 is expressed in approximately 30% of clinically localized cancers by IHC (Giri *et al*. 1999*a*). By quantitative RT-PCR, Naimi *et al*. (2002) found a decreased mean expression of both FGFR-2 IIIb and IIIc in prostate cancer tissues. However, if 70% of cancers do not express FGFR-2 and the cancer tissue is replacing normal epithelium and stroma (which expresses FGFR-2 IIIb and IIIc), one might expect a marked decrease in average FGFR-2 mRNA expression and this might not be compensated for by the 30% of cancers that do express FGFR-2, particularly if they express relatively low levels of the FGFR-2 mRNA. Further investigations are necessary to establish the role of FGFR-2 in prostate cancer, bearing in mind that it is possible that human prostate cancer may be heterogeneous in its response to FGFR-2 activation depending on the presence of other genetic alterations. A major goal is to understand the underlying differences in signal transduction between FGFR-1 and FGFR-2 that could lead to enhancement or inhibition of tumor progression by these two related receptors. In this regard, Freeman *et al*. (2003*b*) have shown that FGFR-1 but not FGFR-2 activation can induce osteopontin, which is known to facilitate tumor growth, although the basis for this difference in expression is still unclear.

FGFR-3 is expressed in normal prostate epithelium predominantly as the IIIb isoform and, based on PCR studies, it continues to be expressed in prostate cancer tissues predominantly as the IIIB isoform (Kwabi-Addo *et al*. 2001), which will bind FGF1 and FGF9. To date no immunohistochemical studies of FGFR-3 expression in prostate cancer have been reported. Using ELISA we have found that cancer tissues contain FGF9 at levels similar to normal prostate. Thus, FGFR-3 may have a role in FGF signaling in prostate cancer.

FGFR-4 is expressed in normal human prostate, in prostate cancer cell lines and in the immortalized human prostate epithelial cell line PNT1A by RT-PCR (Kwabi-Addo *et al*. 2001). Of note is the observation that FGF2, FGF6, FGF8 and FGF17, which are all present in human prostate cancer tissues, are potent activators of FGFR-4 (Ornitz *et al*. 1996). We have recently reported that FGFR-4 is expressed in luminal epithelial cells, PIN and in all of the prostate cancers examined (Wang *et al*. 2004*b*). A germline polymorphism in the FGFR-4 gene, resulting in expression of FGFR-4 containing either glycine (Gly^{388}) or arginine (Arg^{388}) at codon 388 has been identified and the presence of the FGFR-4 Arg388 allele is associated with decreased disease-free survival in breast cancer patients with lymph node metastasis as well

as with metastasis and poor prognosis in colon cancer (Bange *et al*. 2002). We have found that the presence of homozygosity for the FGFR-4 Arg^{388} allele is significantly associated with prostate cancer incidence. In addition, the presence of the FGFR-4 Arg^{388} polymorphism is correlated with the occurrence of pelvic lymph node metastasis and PSA recurrence in men undergoing radical prostatectomy. Expression of the FGFR-4 Arg^{388} in immortalized prostate epithelial cells results in increased cell motility and invasion and upregulation of the urokinase-type plasminogen activator receptor, which is known to promote invasion and metastasis (Sidenius & Blasi 2003). This may explain, in part, the increased aggressiveness of prostate cancers in men bearing this polymorphism. These findings indicate that FGFR-4 plays a significant role in prostate cancer initiation and progression.

There are several known mutations that activate FGF receptor signaling and play a role in genetic disorders of bone formation (Naski & Ornitz 1998). Mutations of FGFR-3 that are associated with thantophoric dysplasia have been found to occur in a significant fraction of bladder cancers (Cappellen *et al*. 1999). However, these mutations do not occur in prostate cancers (Naimi *et al*. 2000, Sibley *et al*. 2001). A similar activating mutation in FGFR-2 also does not occur in prostate cancer (Mehta *et al*. 2000*b*). In addition to point mutations, fusion transcripts can lead to aberrant activity and fusions involving FGF receptor genes have been detected in hematopoietic malignancies (Xiao *et al*. 1998, Demiroglu *et al*. 2001, Li *et al*. 2001, Grand *et al*. 2004, Roumiantsev *et al*. 2004). Although such fusion transcripts have not been reported in prostate cancer, they may be quite hard to detect in a solid malignancy in which the cancer cells are difficult to grow in culture. Thus the major alteration that has been observed to date in FGF receptors during prostate cancer progression is increased expression.

Many questions remain regarding the role of FGF receptors in prostate cancer. As described above, the role of FGFR-2 and changes in alternative splicing of this receptor in prostate cancer progression are still not clear. The different FGF receptors display differences in signaling and biological activities in various systems, but whether these differences are fundamentally quantitative or qualitative in nature is unclear. FGFR-1 appears to activate the MAPK pathway more robustly than other FGF receptors (Shaoul *et al*. 1995, Raffioni *et al*. 1999). FGFR-4, in particular, appears to activate MAPK weakly and yet it can promote proliferation. In addition, FGFR-4 is the only FGFR that can promote membrane ruffling when tranfected into COS-7 cells (Johnston *et al*. 1995). Such membrane ruffling is associated with changes in the actin cytoskeleton related to increased motility. Thus, FGFR-4 activation may be more important in altering motility or other properties when compared with similar stimulation by other FGFRs. Further work is needed to understand these differences in FGF receptor activities in the context of prostate cancer. In this regard, the chemical dimerizer system for FGF receptor activation used by Spencer and his colleagues is attractive (Freeman *et al*. 2003*a*,*b*), since multiple FGF receptors and FGFs are expressed in the prostate cancer cell lines examined to date, making clean analysis of the effects of any single receptor difficult using FGF ligands. Finally, the basis for increased expression of FGF receptors is not clear. If one considers that the differentiation displayed by prostate cancers is more luminal than basal, it is not surprising that FGFR-4 which is expressed in normal luminal cells is widely expressed in prostate cancer. In contrast, expression of FGFR-1 and FGFR-2 is normally in basal cells and tends to be increased in more advanced and poorly differentiated cancer, implying that additional genetic alterations are needed to increase expression of these receptors. The nature of these alterations is unknown. Amplification of FGFR-1 and FGFR-2 has been reported in advanced prostate cancers (Edwards *et al*. 2003) but the observed amplifications are relatively low level (less than two-fold). It is likely that other genetic alterations enhance expression of FGF receptors in cancer cells. For example, overexpression of cyclin D1 in fibroblastic cells leads to upregulation of FGFR-1 (Tashiro *et al*. 2003). Interestingly, translation of both FGF2 and cyclin D1 are enhanced by expression of eIF4e, a cap-binding protein that can regulate protein synthesis, which is overexpressed in prostate cancer. Thus eIF4e could potentially have synergistic effects on proliferation by increasing both FGF ligand and receptor (de Benedetti & Harris 1999). Thus genetic alterations that are known to affect prostate cancer cells can affect FGF receptor expression but further work is needed to determine if there is a linkage of these alterations to changes in FGF receptor expression in prostate cancer.

The extracellular matrix, proteoglycans and FGF activity in prostate cancer

Heparin and heparin sulfate proteoglycans play a critical role in FGF signaling, but little is known of how these molecules are modulated during prostate cancer initiation and progression. Syndecan-1 is a heparin sulfate proteoglycan that can potentially modulate FGFR-1 activity. Immunohistochemical analysis has revealed overexpression of syndecan-1 in prostate cancer and such overexpression is associated with increased Gleason score, early recurrence and decreased survival (Zellweger *et al*. 2003). However, syndecan-1 can interact with both matrix proteins and a number of different growth factors so that

it is unclear whether the effect of syndecan-1 on clinical outcome is mediated via its potential interactions with FGFs. FGFs are extensively bound in the extracellular matrix and a variety of degradative enzymes, particularly proteases, can release them from the ECM. Thus, one potential way in which proteases can enhance tumor progression is by local release of FGFs. FGF-BP can reversibly bind FGF1 and FGF2 and release them from the extracellular matrix (Aigner *et al*. 2001). PC3, DU145 and LNCaP prostate cancer cells all express FGF-BP. Using ribozymes to FGF-BP, Aigner *et al*. (2002) were able to demonstrate that decreased FGF-BP is associated with decreased proliferation and tumorigenicity in PC3 cells. Thus FGF-BP can significantly enhance the biological activities of FGFs. The extent to which FGF-BP is expressed in clinical prostate cancer specimens has not been reported to date. Overall, how the ECM and factors releasing FGFs from the ECM contribute to FGF receptor activation modulation in prostate cancer is not well understood and warrants further investigation.

Alterations of modulators of FGF signal transduction in prostate cancer

As described above, members of the Sprouty gene family negatively regulate FGF signaling in a variety of systems and could potentially limit the biological activity of FGFs in prostate cancer. Recently Kwabi-Addo *et al*. (2004) have established that decreased Sprouty1 expression may play a role in prostate cancer. Immunohistochemical analysis of normal and neoplastic prostate tissues using tissue microarrays revealed that Sprouty1 protein is downregulated in approximately 40% of prostate cancers. By quantitative real-time PCR, Sprouty1 mRNA levels were significantly decreased in prostate cancers *in vivo* in comparison with normal prostate. In prostate cancer cell lines there was loss of the normal upregulation of Sprouty1 mRNA 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. Alterations of expression of other Sprouty family members and other proteins that may regulate FGF signal transduction in prostate cancer are currently being investigated.

Biological effects of FGFs and FGF receptors in prostate cancer

As described above, there is extensive evidence that FGF receptor signaling is enhanced in prostate cancer by multiple mechanisms including increased expression of FGF ligands and receptors, increased mobilization of FGFs from the ECM and loss of negative regulation of FGF signaling. This increased FGF receptor signaling results in activation of multiple signal transduction pathways. The effects of such increased FGF receptor signaling are wide ranging and involve both the cancer cells and surrounding stroma, particularly the vasculature. For prostate cancer, the biological consequences of increased FGF signaling include enhanced proliferation, resistance to cell death, increased motility and invasiveness, increased angiogenesis, enhanced metastasis, resistance to chemotherapy and radiation and androgen independence, all of which can enhance tumor progression and clinical aggressiveness.

Exogenous FGFs can promote proliferation of normal, immortalized and fully transformed prostatic epithelial cells. Our laboratory has shown that FGF2, FGF6, FGF7, FGF9 and FGF17 can all enhance normal and/or neoplastic prostatic epithelial cell proliferation. Song *et al*. (2000a) investigated the biological effects of overexpression of FGF8b in prostate cancer and demonstrated increased growth rate in comparison with controls. In addition, FGF-BP depletion reduces proliferation (Aigner *et al*. 2002). Activation of FGF signaling *in vivo* in multiple transgenic mouse models also leads to enhanced proliferation. Thus one major effect of FGF signaling in prostate cancer cells is to enhance cell proliferation.

It has been shown in different systems that FGFs can inhibit cell death in the appropriate context (Fox & Shanley 1996, Fujiwara *et al*. 2003, Erez *et al*. 2004). Our laboratory has demonstrated that FGF receptor signaling may act to provide an important survival signal in prostate cancer cells. Adenovirus-mediated expression of dominant negative FGF receptors, which blocks FGF signaling, leads to an arrest in G2 in prostate cancer cells followed by cell death (Ozen *et al*. 2001). Primary cultures of epithelial cells show reduced growth after infection with adenovirus-expressing dominant negative FGF receptors but not increased cell death, suggesting that cancer cells are particularly dependent on FGF signaling. We have made similar observations in melanoma cells and primary melanocytes (Ozen *et al*. 2004). As prostate cancers progress, the amount of stroma decreases and the amount of epithelium increases, particularly as cancers develop Gleason patterns 4 and 5, in which large areas of fused, cribiform tumor or sheets of cells are seen, so that there is far less stroma per unit volume to provide stromal FGFs to the epithelial cells. If the cancer cells require FGF signaling for survival, there would be a strong selective pressure for the emergence of cells that have genetic or epigenetic alterations that enhance FGF signaling, and such changes are common in human prostate cancers, as

described above. Based on these observations, disruption of FGF signaling is an attractive therapeutic target in prostate cancer.

FGFs have significant biological function as positive regulators of angiogenesis. FGF1 and FGF2 were among the first angiogenic factors to be identified (Folkman & Shing 1992, Powers *et al*. 2000) and other FGFs can also have angiogenic activity. When FGF2 is expressed in prostate cancer cells and cancer stroma it can induce the formation of tumor vasculature. PC3M cells that express high levels of secreted FGF2 were more angiogenic when growing as solid tumors in nude mice in contrast to DU145 cells (Connolly & Rose 1998). In high grade prostate cancers, production of thrombospondin-1, a major inhibitor of angiogenesis, is downregulated while that of stimulatory FGF2 and/or vascular endothelial growth factor (VEGF) rise, and this is associated with increased microvessel density (Doll *et al*. 2001). Sugamoto *et al*. (2001) reported similar correlations. It should be noted that the angiogenic factors VEGF and IL-8 are increased along with FGF2 in prostate cancer and can act synergistically to promote angiogenesis. Hypoxia can increase expression of all of these factors by prostatic stromal cells (Berger *et al*. 2003) and presumably in cancer cells as well, and hypoxia may be responsible, at least in part, for the increased expression of these three angiogenic factors in prostate cancer. Huss *et al*. (2003) studied the process of angiogenesis and the temporal and spatial expression of the FGF axis during prostate tumor progression in the TRAMP model. They have demonstrated that FGFR-1 IIIb is specifically expressed in new vasculature associated with prostate cancer but not in the vessels of normal mouse prostate. Such increased expression of FGFR-1 could potentiate the effect of increased FGF2 on the tumor vasculature.

Growth factors are key regulators of proliferative and migratory events and FGFs play a role in wound repair (Ortega *et al*. 1998), which is characterized by both cellular proliferation and migration. In response to FGF2, endothelial cells increase the activation and ligation of integrin $\alpha_v\beta_3$ to facilitate cellular migration process through the ERK pathway (Eliceiri *et al*. 1998). MMPs are elevated in many types of cancer including prostate cancer (Basset *et al*. 1990, Matrisian *et al*. 1991, Pajouh *et al*. 1991) and are involved in the invasion and metastasis of prostate cancer. MMPs are a family of endopeptidases that require zinc for catalytic activity and are capable of digesting ECM and basement membrane components (Newell *et al*. 1994). Increased expression of MMPs in prostate cancer leads to proteolytic breakdown of the basement membrane and ECM structures leading to release of FGFs (Mack *et al*. 1993). Matrilysin, a matrix metalloproteinase, can degrade the extracellular proteins, including proteoglycans, fibronectin, entactin, laminin, gelatin and elastin (Wilson & Matrisian 1996). Its expression has been shown in prostate cancer (Pajouh *et al*. 1991) and can enhance the invasiveness of the DU145 prostate cancer cell line (Knox *et al*. 1996, Powell *et al*. 1993). It has been demonstrated that overexpression of matrilysin in prostate cancer is partially due to the paracrine factors secreted from the surrounding stroma (Klein *et al*. 1999) and inhibition of FGF receptor signaling can inhibit promatrilysin expression along with PSA expression and tumor growth in LNCaP prostate cancer cells (Udayakumar *et al*. 2003). Thus it is possible that a positive feedback loop could be established in which FGFs promote activity of MMPs which, in turn, leads to increased availability of FGFs by release from the ECM. We have recently demonstrated that immortalized prostatic epithelial cells expressing the Arg^{388} form of FGFR-4 have enhanced migration, invasiveness and expression of urokinase-type plasminogen activator receptor (Wang *et al*. 2004*b*), which plays a key role in cell motility and invasiveness. Thus FGFs can play an important role in invasion and migration by enhancing the activity of multiple proteolytic systems.

Given the biological effects described above it is not surprising that FGFs can enhance metastasis *in vivo*. Highly metastatic variants of PC3 express higher levels of FGF2 (Greene *et al*. 1997). Our laboratory has recently demonstrated, by crossing TRAMP mice with FGF2 knockout mice, that inactivation of even one FGF2 allele is associated with decreased metastasis (Polnaszek *et al*. 2003). Other FGFs probably have a similar effect on metastasis although this has not been established experimentally to date.

FGF2 could affect the cytotoxicity of chemical and other non-physiological stresses inflicted upon the cell. This is particularly important for the interaction of cancer cells with chemotherapy drugs and other DNA-damaging agents, including radiation. Depending on the type of cells studied, the chemotherapeutic or DNA-damaging agent used and the method of exposure to FGF2, such treatment can be either protective or sensitizing. Song *et al*. (2000*b*) have shown that the chemoresistance in lung metastases is caused by acidic and basic FGFs (aFGF and bFGF) expressed in solid tumors. Subsequently the same group investigated the effect of FGF inhibitors on doxorubicin activity in human prostate PC3 tumors (Zhang *et al*. 2001). Addition of suramin (which can inhibit FGF receptor activation) to doxorubicin therapy significantly enhanced the anti-tumor effect, resulting in complete inhibition of tumor growth. The protective effects of FGFs can also involve endothelial cells. For example, FGF2 can increase survival of radiated endothelial cells, which could limit the effectiveness of radiation

therapy in inhibiting tumor angiogenesis (Abdollahi *et al.* 2003). While further studies are needed, the evidence to date indicates that increased FGF expression could contribute to the resistance of many prostate cancers to radiation and/or chemotherapy.

Androgen receptor activity is critical for prostate cancer cell survival and androgen ablation plays an important role in the treatment of prostate cancer, particularly in men with advanced disease. Unfortunately androgen-independent prostate cancer almost always emerges following androgen ablation, limiting the effectiveness of this therapy. The vast majority of androgen-independent prostate cancers continue to express androgen receptor and androgen receptor target genes such as PSA, indicating that the emergence of androgen independence is usually due to genetic or epigenetic changes that allow for activation of androgen receptor at extremely low androgen levels. Such changes include amplification and overexpression of androgen receptor, mutations of androgen receptor, increased expression of androgen receptor coactivators and activation of androgen receptor transcription by growth factor signaling. Culig *et al.* (1994) originally demonstrated that FGF7, insulin-like growth factor-I and EGF activate androgen receptor-dependent transcription in prostate cancer cells. It is now clear that multiple growth factors and cytokines can activate androgen receptor-dependent transcription, but the mechanism by which this occurs is still unclear. One potential mechanism is through activation of androgen receptor coactivators by the MAPK pathway (Debes *et al.* 2003, Rowan *et al.* 2000) but further detailed mechanistic studies are needed to fully understand the crosstalk between FGF receptor signaling and androgen receptor activity. However, given the evidence that FGF receptor signaling is increased in advanced prostate cancer, it is possible that FGFs contribute significantly to androgen receptor activity in androgen-independent disease.

FGF receptors and FGF receptor signaling as therapeutic targets in prostate cancer

Tyrosine kinases have emerged as a major potential therapeutic target in cancer therapy. Imatinib, which inhibits the BCR-abl kinase and c-kit, is highly effective in the treatment of chronic myelogenous leukemia and malignant gastrointestinal stromal tumors. Similarly, Her2/neu is an important target in breast cancer therapy. As described above, expression of FGF receptors and increased FGF receptor signaling are ubiquitous in human prostate cancer. Furthermore, since FGF signaling enhances multiple biological processes that promote tumor progression, most critically cell survival, it is an attractive therapeutic target, particularly since therapies targeting FGF receptors and/or FGF signaling can affect both the tumor cells directly and tumor angiogenesis. There are numerous approaches that could target FGF receptors and/or FGF receptor signaling in prostate cancer. One approach is to target cancer cells by conjugating FGF ligands to toxins (Davol & Frackelton 1999) or adenoviruses carrying toxic genes (Lanciotti *et al.* 2003). Another approach would be to develop antibodies targeting FGF receptors that could either directly inhibit their activity or be used to target therapeutic molecules to the cancer cells and tumor vasculature. He *et al.* (2003) have used an interesting approach in which FGF receptor from a non-mammalian species (*Xenopus*) was used as a vaccine in mice and which led to inhibition of tumor growth. Finally, small molecule inhibitors of FGF signaling are under active development. SU5402 is a specific inhibitor of FGFR activity (Mohammadi *et al.* 1997). It has been shown to specifically inhibit the growth of chronic myeloid leukemia cell lines bearing an unusual translocation that results in the production of a BCR–FGFR1 fusion protein (Demiroglu *et al.* 2001) as well as multiple myeloma cells bearing a rearrangement of FGFR-3 (Grand *et al.* 2004). In LNCaP cells, low doses of SU5402 have been shown to inhibit secretion of promatrilysin (Klein *et al.* 1999). Another FGF receptor inhibitor, PD173074, shows activity against breast cancer cell lines (Koziczak *et al.* 2004) and multiple myeloma cells expressing FGFR-3 fusion protein (Grand *et al.* 2004) and has anti-angiogenic activities *in vivo* (Dimitroff *et al.* 1999). SU5416 and SU668 are broad-spectrum anti-angiogenic tyrosine kinase inhibitors that inhibit FGF receptors as well as VEGF and platelet-derived growth factor receptors and have anti-tumor effects *in vivo* (Griffin *et al.* 2002). These two agents are currently undergoing clinical trials for treatment of a variety of malignancies, although SU5416 has recently been reported not to be effective in advanced prostate cancer (Stadler *et al.* 2004). Finally, it should be noted that a number of chemotherapeutic agents such pegylated interferon-α-2b (Huang *et al.* 2002) and taxanes (Cassinelli *et al.* 2002) downregulate expression of FGF2 and this may be part of their therapeutic effectiveness. While this effort is only beginning, agents targeting the FGF signaling system in prostate cancer may have therapeutic effectiveness and may well be integrated into patient treatment in the future.

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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 NH_2-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 database[3] and the Cancer Genome Anatomy Project database[4] 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

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

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

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′-AAGCTTA*G*AC**ATG-**GATC-CCCAAAATC-3′; and reverse primer, 5′-*GAATTC***TGA**TGGTTTAC-CCTGACCCC-3′ (The underlined sequence indicates *Hin*dIII 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 *Hin*dIII 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′-ACGAGCA-CAGACACACAAG-3′, and reverse, 5′-CAACCCACCTCCAAAAATCA-3′; set 2 primers (SF2): forward, 5′-CCTTCTTTGGATAGCCGTCA-3′, and reverse, 5′-CCCTTCAAGTCATCCACAATC-3′; set 3 primers (SF3): forward, 5′-AGGACCCCAGCATCATTGTA-3′, and reverse, 5′-GTGGCTTGTGT-GTCTGTGCT-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′-TGTCCGAAAAGGATTTCAGATGC-3′, and reverse, 5′-ACTGCCACTGCCA TGTTGAT-3′; for β-actin were forward, 5′-AGCACGGCATCGTCA CCAACT-3′, and reverse 5′-TGGCT-GGGGTGTTGAAGGTCT-3′; for keratin 18 were forward, 5′-AGGGCTCA-GATCT-TCGCAAAT-3′, and reverse, 5′-GTCATCAATGACCTTGCGGAG-3′; for FGF2 were forward, 5′-CCACTTCAAGGACCCCAAG-3′, and reverse, 5′-ATAGCCAGGTAACGGTTAGC-3′; and for FGF7 were forward, 5′-CCT-TCTGCCTGTTGATTTATGG-3′, and reverse, 5′-GTTGCTGTGACGCT-GTTTG-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 μg 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 μg/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 μg 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 μg of pEGFP (BD Biosciences Clontech) alone or 6 μg of pEGFP with 18 μg 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-Alpra 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 analyzed 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. 1*F*). 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. 2*A*). 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. 2*B*). 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).

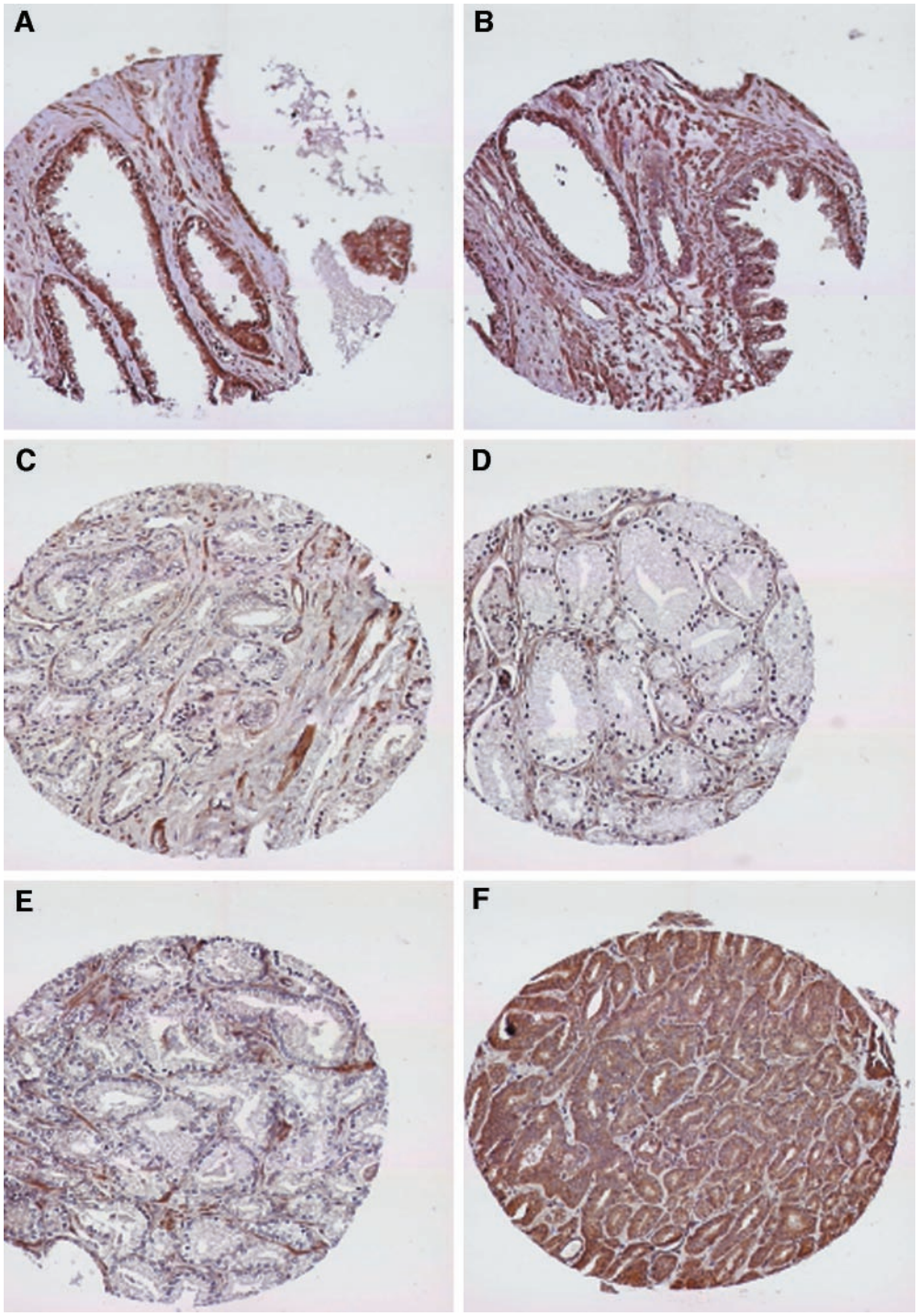

Fig. 1. Immunohistochemical analysis of Sprouty1 expression in tissue microarrays. Expression of Sprouty1 in normal prostate (*A* and *B*) and prostate cancer (*C*--*F*) was determined using tissue microarrays as described in "Materials and Methods." *A* and *B*, normal prostate peripheral zone tissue 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

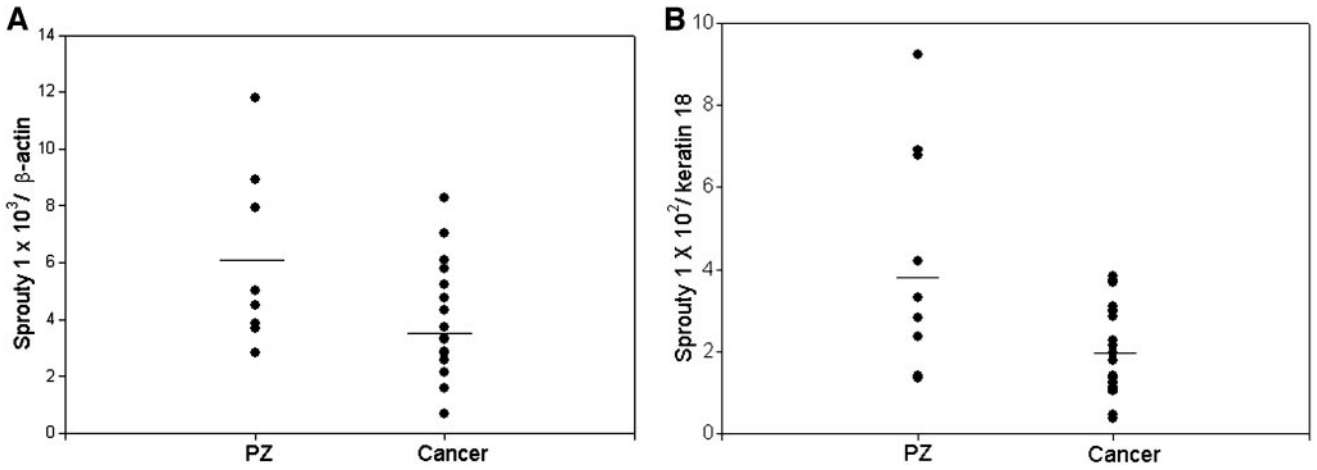

Fig. 2. Sprouty1 expression in normal prostate and prostate cancer as determined by quantitative reverse transcription-PCR. Sprouty1 expression in normal prostatic peripheral zone (*PZ*) and cancer tissues was assessed by quantitative reverse transcription-PCR using a real-time thermal cycler (iCycler; 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. 4*A* 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. 4*B* 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,

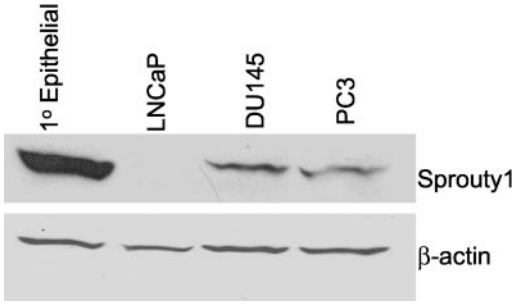

Fig. 3. Expression of Sprouty1 protein in primary prostatic epithelial cells and prostate cancer cells lines. Protein extracts of primary cultures of prostatic epithelial cells and the prostate cancer cell lines LNCaP, PC3, and DU145 were analyzed by Western blotting with anti-Sprouty1 antibodies as 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*.

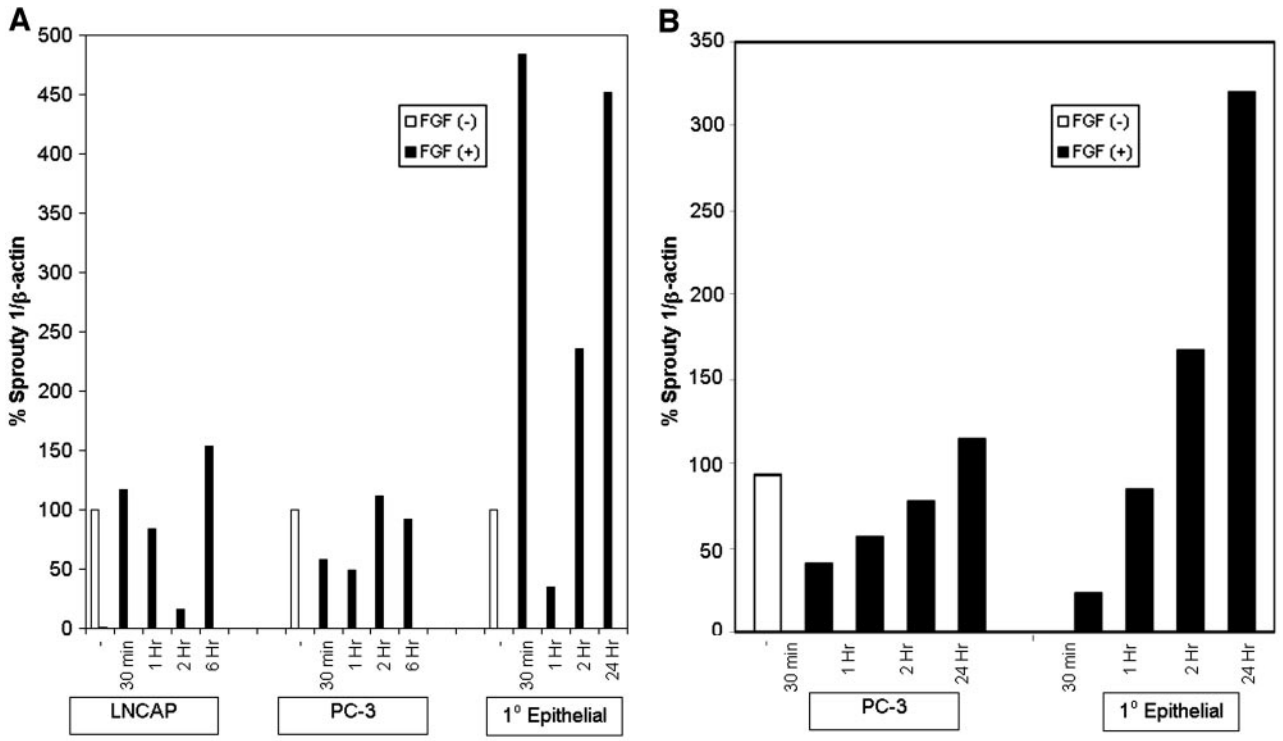

Fig. 4. Sprouty1 expression in response to FGF2 stimulation of normal or neoplastic prostate epithelial cells. *A*, prostatic primary epithelial cells, LNCaP, and PC3 cells were grown in serum-free medium for 24 h. Cells were stimulated with serum-free medium with or without 25 ng/ml recombinant FGF2. 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 ± 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. 6*A*), there was a profound decrease in proliferation in the cells transfected with the Sprouty1 expression construct (Fig. 6*B*). 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. 6*C* 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).

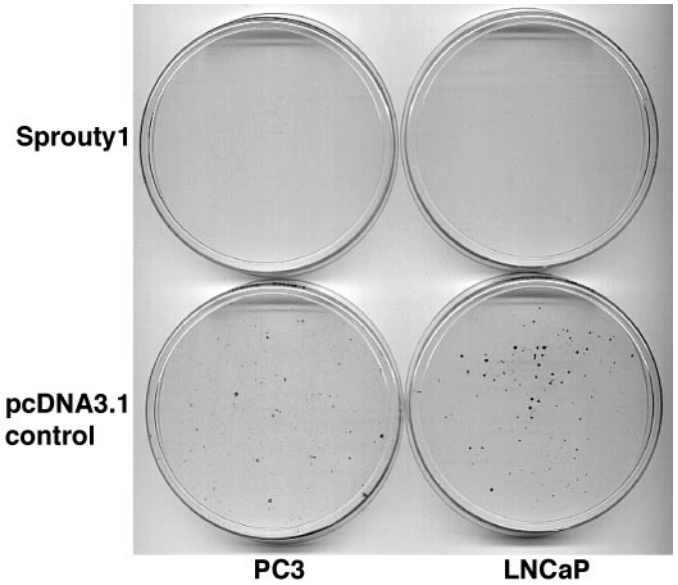

Fig. 5. Stable transfections of Sprouty1 plasmid into prostate cancer cells. Prostate cancer cell lines LNCaP and PC3 were each transfected with a Sprouty1 cDNA cloned into pcDNA3.1 or the pcDNA3.1 vector alone. After 2 weeks of selection in Geneticin, cells were fixed and stained with crystal violet.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

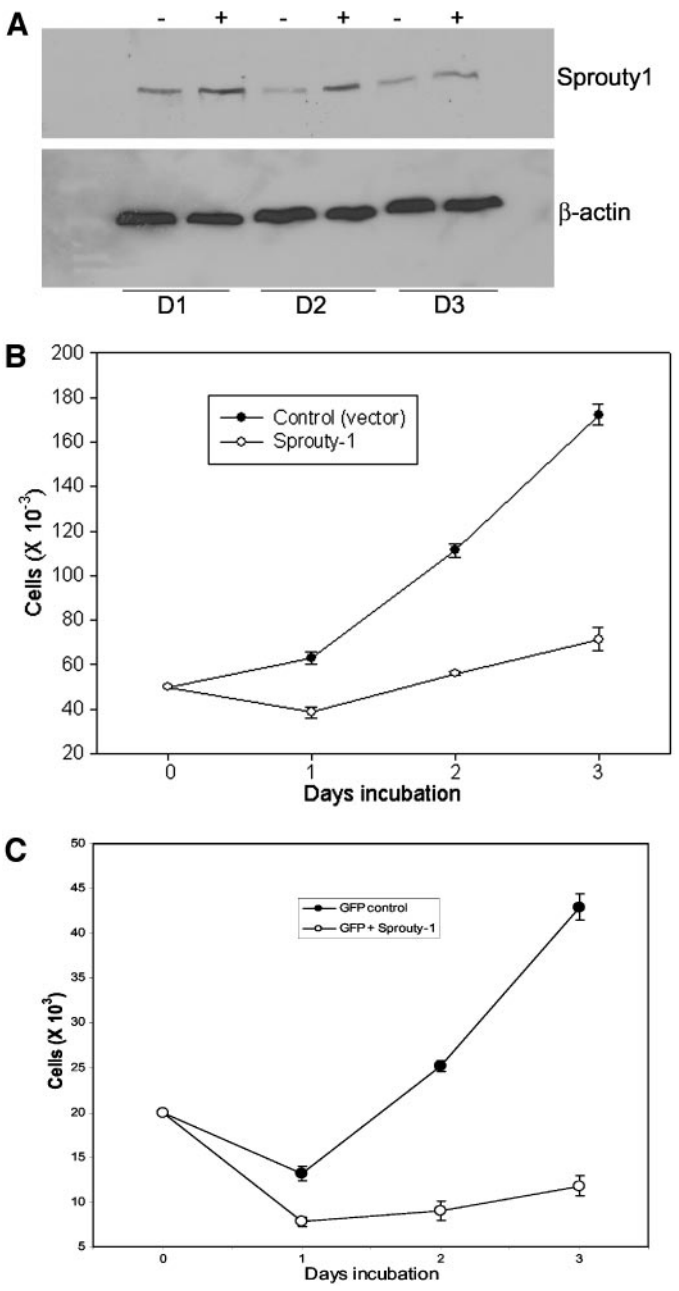

Fig. 6. Transient transfection of Sprouty1 in LNCaP prostate cancer cells. *A*, protein extracts were collected from LNCaP cells 1, 2, or 3 days after transfection with pcDNA3.1 (−) or Sprouty1 cDNA in pcDNA3.1 (+) and analyzed by Western blotting with either anti-Spouty1 antibody or control anti-β-actin 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

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DNA Methylation and Aberrant Expression of Sprouty1 in Human Prostate Cancer

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Abstract

Sprouty1 is a negative regulator of fibroblast growth factor signaling with a potential tumor suppressor function in prostate cancer (PCa). Sprouty1 is downregulated in human PCa and Sprouty1 expression can markedly inhibit PCa proliferation in vitro. The aim of this study was to investigate the role of DNA methylation in Sprouty1 expression in human prostate tumors. We used pyrosequencing to quantitatively measure the methylation status of the Sprouty1 promoter region in matched normal and adjacent prostate cancer tissues from same prostate cancer patients, the immortalized normal prostate epithelial cell line pNT1A and prostate cancer cell line LNCaP. Sprouty1 mRNA expression in tissue samples were assessed by quantitative RT-PCR. Further methylation analysis of the Sprouty1 promoter was studied using *Sss*1 methylase or 5'-Aza-2'-Deoxycytidine treatment. The % methylation of Sprouty1 promoter was significantly higher in the prostate cancer tissues when compared to the matched normal tissues. Quantitative RT-PCR analysis confirms that Sprouty1 expression was decreased in the prostate cancer tissues compared to the benign prostate tissues. Hypermethylation of Sprouty1 promoter was detected in LNCaP cells which showed barely detectable Sprouty1 expression, whereas pNT1A cells which showed low methylation demonstrated higher Sprouty1 protein expression. Methylation modification of the *Sprouty*1 promoter abolished promoter activity whereas global demethylation with 5'-Aza-2'-Deoxycytidine treatment induced Sprouty1 expression. Our data demonstrates that DNA methylation in the Sprouty1 promoter region is responsible for down-regulating Sprouty1 expression in prostate cancer.

INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths among men in the United States. There is abundant evidence to indicate that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (for review see). [1] Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development. [2] Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis. [3-5] While Drosophila has only one Sprouty gene, at least four Sprouty homologues (Sprouty1-4) have been found in humans and mice. [6, 7] Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway. [8-14] Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein, FRS2 [3, 15] or the inhibition of Raf. [11, 12] Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript [7] and in some systems, the recruitment of Sprouty proteins to the plasma membrane. [16] Given that Sprouty proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 and Sprouty4 are down-regulated in a subset of prostate cancers tissues when compared with normal prostate tissues. [17, 18] McKie *et al*., [19] have observed that Sprouty2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). The decrease in Sprouty 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 FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

We and others have also shown epigenetic inactivation to be a key mechanism for silencing Sprouty proteins in the prostate. For instance, we have observed promoter methylation at Sprouty4 CpG islands in prostate cancer. More than half of all prostate cancer tissue DNAs were methylated in this region and methylation significantly correlated with decreased Sprouty4 expression. Furthermore the treatment of prostate cancer cells with 5-aza-dC reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 down-regulation. [18] Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated. [19] The suppressed Sprouty2 expression correlated with methylation of the CpG region in clinical samples indicating that methylation of the Sprouty2 promoter was the likely cause of its transcriptional inactivation in the prostate. However, promoter methylation does not seem to explain Sprouty2 inactivation in breast cancer. Cultured breast cancer cell lines in the

presence of 5'Aza-2-deoxycytidine (5-aza-dC) a demethylation agent, did not reactivate the expression of Sprouty2 and only minimal and patient specific methylation of the Sprouty2 CpG islands was found indicating cancer-specific mechanisms of Sprouty down-regulation. [20]

The Sprouty1 promoter contains a CpG island, and DNA methylation events that affect promoter activity offers a likely mechanism for epigenetic alteration in prostate cancer. Thus, in the present study, we sought to investigate the epigenetic mechanisms regulating *Sprouty*1 expression in prostate cancer.

RESULTS

Genomic organization of the Sprouty1 gene

The human *Sprouty*1 transcript consists of two splice variants, 1a [21] and 1b [2] arising from two alternative promoters that map to human chromosome 4q27-28 and 4q25-28 respectively. Each splice variant has 2 exons and one intron. Exon 1 encodes the 5'-untranslated region of the cDNA, whereas exon 2 encodes the remainder of the 5'-untranslated region, the entire open-reading frame and the entire 3'-untranslated region. While the splice variants share the same second exon, they have different first exons, located very close to each other on the same chromosome (Fig. 1). The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site. In order to identify the transcription start sites of *Sprouty*1a and *Sprouty*1b splice variants, we performed 5'-RACE using poly $(A)^+$ RNA from fetal human lung and a *Sprouty*1 specific primer. We observed multiple bands after amplification, the largest of about 275 bp (data

not shown). Sequence analysis identified multiple transcription initiation sites within the region -315 to -305 nucleotides from the first ATG codon in a Kozak consensus sequence. The 5'-most start site found is located at nucleotide position 160026 of the published sequence (GenBank accession no. AC026402). Because this region corresponds to the 5'-UTR of Splice variant 1b, this may represent the corresponding promoter region. Using similar approach we identified the transcription start site for Splice variant 1a to be at nucleotide position 162754 in the same published sequence (GenBank accession no. AC026402).

Global DNA methylation analysis using the *Sss* I methylase assay.

Using the MethPrimer software package for CpG islands identification (http://www.urogene.org/methprimer/), we have identified 2 separate CpG islands: 1 spanning about 2 kbp of the *Sprouty*1a promoter region and the other spanning about 110 bp of *Sprouty*1b promoter region (not shown). Using a series of unidirectional PCR based deletion analysis followed by luciferase reporter assay, we have identified optimal promoter activity for Sproutyla Fwd5 and Sproutylb Fwd3 (data not shown) and these are here after referred to as Sprouty1a and Sprouty1b promoter respectively. To investigate whether constitutively active *Sprouty*1a and/or *Sprouty*1b promoter activity was inhibited by the methylation of the promoter CpG island, we modified the promoter constructs with *Sss*I methylase treatment and examined the activity of the methylated promoter. When the *Sss*I methylated or non-methylated *Sprouty*1a and *Sprouty*1b promoter constructs were each transiently transfected into LNCaP cells the activity of the methylated Sprouty1a promoter was only 5 % of that of the unmethylated construct (Fig. 2). On the

other hand, CpG methylation of *Sprouty*1b construct did not show significant effect on its activity when compared to the control unmethylated construct (Fig. 2). This observation indicates that methylation of the *Sprouty*1a promoter may be involved in the control of *Sprouty*1 gene expression.

In vitro methylation analysis of the Sprouty1 promoter region.

To investigate whether Sprouty1a promoter is methylated in human prostate tumors, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA. Typical examples of bisulfite methylation profiles at 3 CpG sites of the Sprouty1 promoter is presented as pyrogram for pNT1A and LNCaP cells (Fig. 3A). As shown in the pyrogram, the pNT1A cells demonstrated on average 5% methylation at each CpG site. On the other hand, LNCaP cells demonstrated an average of 30% methylation at each CpG site suggesting that Sprouty1 is hypermethylated in LNCaP cells but shows low methylation in pNT1A cells. Interestingly, the immortalized normal prostate epithelial cells, pNT1A expressed high levels of Sprouty1 protein whereas LNCaP cells show barely detectable levels of the Sprouty1 proteins (Fig. 3B). We also observed higher methylation in the prostate cancer cell lines DU145 and PC3 and these cell lines showed lower Sprouty1 expression when compared with pNT1A (data not shown). The inverse association between Sprouty1 mRNA expression and % DNA methylation level suggests that DNA methylation maybe a mechanism for down-regulating Sprouty1 expression in the prostate cancer cell line.

Next, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA in matched normal and prostate cancer tissue samples from 15

individuals. The average percentage of methylation at the 3 CpG sites of the Sprouy1 promoter was compared between the matched normal and prostate cancer tissue for each patient (Fig. 3C). Our data showed approximately 1.6 fold higher % methylation level in the prostate cancer tissues (6.967 $\pm$ 0.682, SEM) when compared with the matched normal prostate tissues (4.40 $\pm$ 0.387, SEM). We next used quantitative RT-PCR to measure Sprouty1 mRNA in these same prostate tissue samples. We found that Sprouty1 expression was approximately 1.5 fold higher in the normal prostate tissues (8.44 $\pm$ 1.716, SEM) compared to prostate cancer tissues (5.572 $\pm$ 1.261, SEM; Fig. 3D). The reduced Sprouty1 mRNA expression in the prostate cancer tissues correlated with a significantly (T-test) correlated with increased in % DNA methylation in the prostate cancer tissues compared to the normal prostate tissues. This data indicates that the reduced Sprouty1 expression in prostate cancer tissues may reflect an increased in promoter methylation in this same tissue samples.

Treatment of prostate cancer cell lines with 5'-Aza-2'Deoxycytidine can restore Spry1 Expression.

To further investigate whether DNA methylation plays a role in Sprouty1 expression, we tested the hypothesis that pharmacological modulation of methylation can reactivate gene expression. [22] To achieve this we treated pNT1A, DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (5-aza-dC). As shown in Fig 4, treatment of the prostate cancer cell lines, DU145, PC3 and LNCaP with 5-aza-dC (2 μM) led to a significant increase in Spry1 mRNA expression in the prostate cancer cell lines. Taken together, these data

suggests that promoter methylation may play a role in down-regulating Spry1 expression in these cell lines and human prostate tumors.

DISCUSSION

In the present study, we have investigated DNA methylation and Sprouty1 expression in human prostate cancer. DNA methylation is a common event in cancer and several genes promoter methylation has been reported. For example, aberrant methylation of GSTP1 gene is perhaps the most common genomic alteration in human prostate cancer and occurs in the earliest stages of prostate carcinogenesis. [23] Because of the presence of a large CpG island (2 kbp) of the Sprouty1 promoter region, we speculated that DNA methylation may contribute to the silencing of Sprouty1 expression. We used pyrosequencing to quantify the methylation status of 3 CpG sites in the Sprouty1 promoter. Pyrosequencing offers a semi-quantitative high-throughput and reliable method with an in built internal control for adequacy of bisulfite treatment. [24] Using this approach, we have observed methylation in normal as well as prostate cancer tissues analyzed. It is not unusual to detect DNA methylation in normal prostate tissues as we have recently demonstrated that DNA methylation changes starts in the normal prostate tissue as a function of age and this dramatically increases in prostate cancer. [25] The samples we used in our analysis are derived from patients 50 years of age and older so we would expect some degree of methylation in the normal prostate tissues. On average, we observed a significantly higher methylation in the 15 prostate cancer cases when compared to the matched normal prostate tissues. However, because of the limited sample size that was analyzed, we were unable to observe direct statistical correlation between methylation and mRNA expression in our studies. However, our observation of higher methylation in the prostate cancer cell lines prostate cancer tissues which showed

reduced Sprouty1 expression compared to normal prostate cells and prostate tissues clearly indicate that methylation is responsible for reduced expression of Sprouty1 in prostate cancer. This observation is supported by our published data showing that methylation of Sprouty4 significantly correlated with decrease in Sprouty4 expression in prostate cancer. [18] My data clearly demonstrate methylation as a key mechanism for the inactivation of Sprouty genes in human prostate cancer.

However, in 3 cases where we detected low methylation in the cancer tissues compared to the normal tissues, we also observed low Sprouty1 expression. Other mechanisms of gene inactivation, such as alterarions in trans-acting factors, and heterozygous or homozygous deletion could also affect Sprouty1 expression and this remains to be explored. Another mechanism for the decrease in Sprouty1 expression is the recruitment of methyl-CpG binding proteins (MeCPs) which inhibits the binding of transcription factors to the promoter regions. [26, 27] Our studies indicate that Sprouty1 expression was restored after treatment with the demethylating agent, 5'aza-2'-deoxycytidine. This also implies that silencing via promoter-associated CpG methylation could be mediated by binding of MeCPs to methylated CpG dinucleotide to other Sprouty1 promoter CpG sites not investigated in this study. The expression of Sprouty1 is driven by two promoters which are responsible for the synthesis of one transcript. The upstream promoter has a very large CpG island and our studies demonstrate that methylation of this promoter site is responsible for decreased expression of Sprouty1 protein. The internal promoter does not seem to be affected by methylation, however, this region contains key regulators sites for basal transcription. Whether additional mechanism(s) of gene inactivation such as alteration in trans-acting factors is being actively investigated by our laboratory.

CONCLUSION

We have identified a 2 kbp CpG island of the human *Sprouty*1 gene promoter and DNA methylation of this promoter region appears to play a role in down-regulating Sprouty1 expression in human prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostate cancer cell lines, PC3, DU145 and LNCaP were obtained from the American Type Culture Collection (Manassas, VA). The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Cultures (Salisbury, U.K). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) unless otherwise stated.

Human Prostate Tissue Samples

All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank. [28] Fresh frozen tissue punches of normal and tumor tissues were obtained at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70%-100% with Gleason scores of 6-8.

Bisulfite Modification, PCR and Pyrosequencing Analysis of the Sprouty1 promoter.

DNA samples prepared from prostate tissues and cell lines were modified by sodium bisulfite treatment using MethylEasy kit (Human Genetic Signatures, Sydney, AUS) according to the manufacturer's protocol.

Bisulfite PCR primers were designed based on bisulfite/converted sequence from the Sprouty1 CpG island ensuring that the bisulfite-PCR primers avoid CpG sites and that they are designed as close to the transcription start site as possible. A two step PCR reaction was carried out using 2 μl of bisulfite converted genomic DNA and 2 sets of different bisulfite PCR primers in a standard PCR reaction. One of the primers (reverse primer) in the 2nd step PCR reaction was biotinylated in order to create a single-stranded DNA template for the pyrosequencing reaction. Primers used in the 1st step PCR reaction were forward 5'-AGGGTTTTTAGAGAGGATAATTTGGGTTAT-3' and reverse 5'-CCCCCACTTCTAAAAACTCAAAATTAAATA and a reverse primer tailed with a universal sequence (shown as underlined sequence) 5'-GGGACACCGCTGATCGTTTACCCCCACTTCTAAAAACTCAAAATTAAATA-3'. The reverse primer and the reverse primer tailed with the universal primer were mixed at a1:9 in the PCR reaction respectively. The primers used in the 2nd step PCR reaction were forward 5'- TTTAGGGTAATAGGGGATGGAGGA-3' and biotinylated universal primer 5'-GGGACACCGCTGATCGTTTA-3'. Integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The product from the 2nd step PCR reaction was immobilized on streptavidin-sepharose beads (Amersham), washed, denatured and the biotinylated strands released into an annealing buffer containing the sequencing primer. Pyrosequencing was performed using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Biotage). Each pyrosequencing reaction was done at least twice.

The PCR amplification step was as follows: 95^{o}C for 3 min, then denature at 95^{o}C for 30 sec, anneal at 54^{o}C (1st step) or 58^{o}C (2nd step) for 30 sec, extension at 72^{o}C for 30 sec for 50 cycles, and a final 10 min extension at 72^{o}C.

In vitro Methylation

The *Sprouty*1a Fwd5 and *Sprouty*1b Fwd 3 promoter fragments (1 μg) were *in vitro* methylated with *Sss*I (CpG) methylase (New England Biolabs, Beverly, MA) as recommended by the manufacturer. The *Sss*I methylation, which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence (5'-CG-3'), was performed at 10mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 160 μM *S*-adenosylmethionine at 37^{o}C for 1h. After the methylation reaction the promoter fragments were purified by phenol chloroform extraction and ethanol precipitation. The methylated DNA fragments were then digested with *Nhe*1 and *Kpn*1 and then sub-cloned into the pGL3-Basic vector. The methylated promoter constructs were used for transient transfection assays. Individual reactions were monitored by digestion with *Sss*I or *Hpa*II or *Hha*I restriction enzymes.

Transient transfections

The LNCaP cells were seeded on a six-well tissue plates in RPMI-1640 medium and supplemented with 10% FBS and grown for 16-24 hours to 80% confluence. Next, cells were transiently transfected with the individual luciferase reporter plasmid by using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's procedure. The pSV-β-galactosidase control vector (Promega) was cotransfected with various luciferase

reporter plasmids into cells to normalize the variations in transfection efficiency. Each transfection was done in triplicate.

Reporter gene luciferase assay

Cells were lysed 48 hours post-transfection by freeze thaw (3 cycles) in luciferase reporter lysis buffer (Promega). The lysates were centrifuged at 12,000 g for 2 min to remove cell debris. The supernatant was used for both luciferase and β-galactosidase activity assays. Luciferase activity was determined by using a luciferase assay kit (Promega) according to the manufacturer's protocol and measured in a luminometer. The β-galactosidase activity was assayed using the β-galactosidase enzyme assay kit (Roche) according to the manufacturer' protocol. Variation in transfection efficiency was normalized by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The promoterless pGL3-Basic vector was used as negative control, and the pGL3-CMV plasmid (which has CMV promoter and enhancer to drive the luciferase gene) was used as positive control for each transfection assay. Each reporter gene assay was done in triplicate.

Induction of Sprouty1 expression by 5'-aza-2'-deoxycytidine (5'-aza-dC)

The pNT1A, DU145, PC3 and LNCaP cells were seeded at 5 X 10^5 cells/100-mm tissue culture dish. After 24 hours of incubation, the culture media was changed to media containing 5'-aza-dC for 96 hours. Total RNA extracted from cells and tissues using TRIzol Reagent (Invitrogen) was first used in first strand DNA (cDNA) synthesis using

Invitrogen Super-ScriptTM first strand synthesis and then used in real-time quantitative PCR as previously described. [17]

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FIGURE LEGENDS

Fig. 1. Schematic representation of the *Sprouty*1 gene. Exons are shown as open-boxes and translational start site, ATG is shown as a thick black bar. Promoter region is shown as blackened arrows. The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site.

Fig. 2. Effect of *in vitro* methylation and *Sprouty*1 promoter activity. *Sprouty*1a (pSprouty1a Fwd5) and 1b (pSprouty1b Fwd3) promoters were methylated *in vitro* by *Sss*I methylase. Methylated and unmethylated *Sprouty*1a and 1b promoters were transfected into LNCaP cells and assayed for luciferase activity. The luciferase activity

was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Results are shown as percentages, with luciferase activity due to unmethylated promoter designated as 100%. Data represents the mean of triplicate experiments.

Fig. 3. Methylation and expression analysis of the Sprouty1. **A.** Representative program traces for Sprouty1. Gray colums represents regions of C to T polymorphic sites. Genomic DNA sample extracted from immortalized normal prostate epithelial cell line, pNT1A cells (top panel), and genomic DNA sample extracted from prostate cancer cell line LNCaP cells (bottom panel). Top, percentage of methylation at each CpG site. Y-axis; signal peaks proportional to the number of nucleotides incorporated. X-axis; the nucleotide sequence incorporated. **B.** Protein extracts were collected from prostate cancer LNCaP cells and the immortalized normal prostate epithelial cell line pNT1A and analyzed by western blotting with either anti-Spouty1 antibody or control anti-ß-actin antibody. **C.** The % methylation level in match pair of normal prostate tissues (Nl) and cancer tissues (Ca) was analyzed using pyrosequencing. **D.** The Sprouty1 mRNA expression in normal prostate and prostate cancer as determined by quantitative reverse transcription-PCR. The Sprouty1 expression in normal prostate tissues (Nl) and cancer tissues (Ca) 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 x 10^3 to β-actin transcripts The data is a representative of duplicate experiments. The mean expression level is indicated as a horizontal bar.

Fig. 4. Demethylation and Sprouty1 expression. Prostate cancer cell lines; LNCaP, PC3 and DU145, and immortalized primary prostatic epithelial cells; pNT1A were each treated with 5'-aza-2'-deoxycytidine (5'-aza-dC) at the indicated concentrations for 96 hours.Sprouty1 mRNA expression, expressed as relative Sprouty1 expression was determined by quantitative RT-PCR using iCycler and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

Fig. 1

Fig. 2

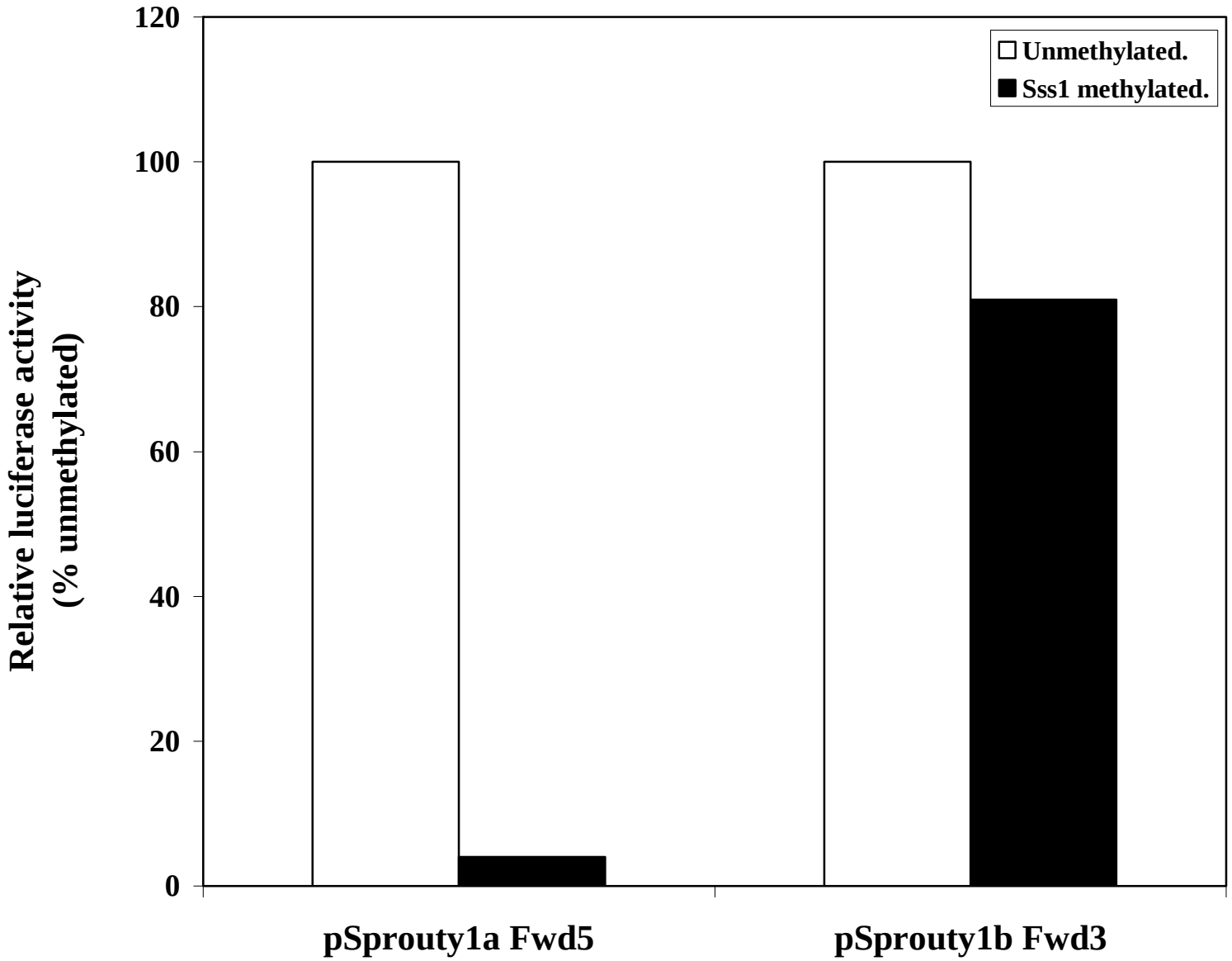

120
100
80
60
40
20
0
Relative luciferase activity
(% unmethylated)
Unmethylated.
Sss1 methylated.
pSprouty1a Fwd5
pSprouty1b Fwd3

Fig. 3A

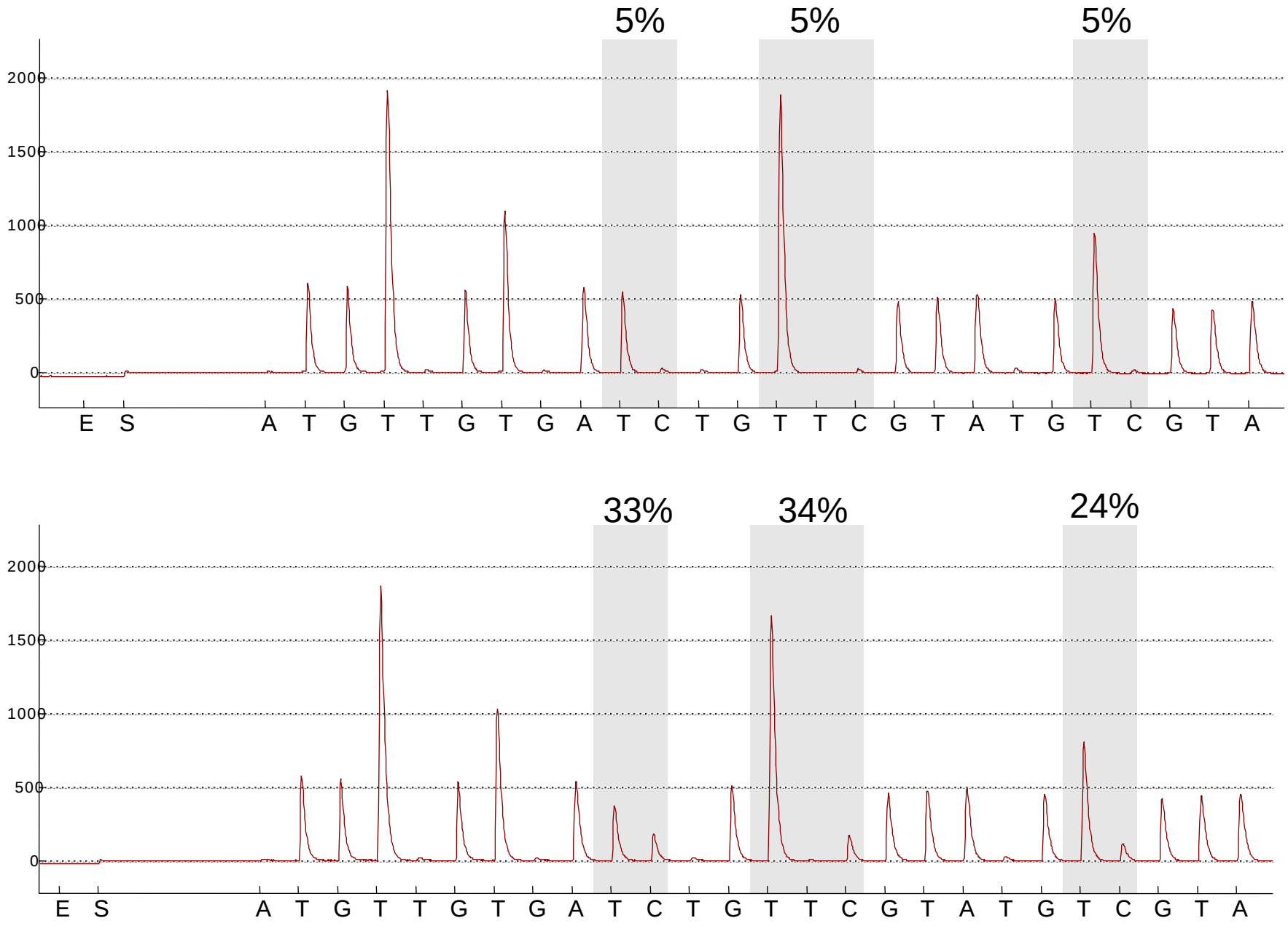

5%
5%
5%
2000
1500
1000
500
0
E S A T G T T G T G A T C T G T T C G T A T G T C G T A
33%
34%
24%
2000
1500
1000
500
0
E S A T G T T G T G A T C T G T T C G T A T G T C G T A

Fig. 3B

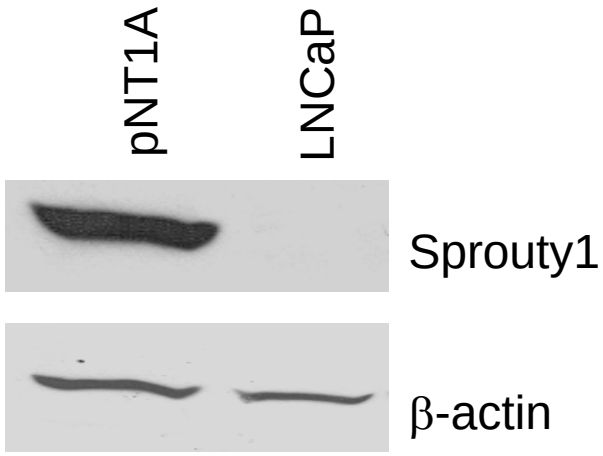


Fig 3C

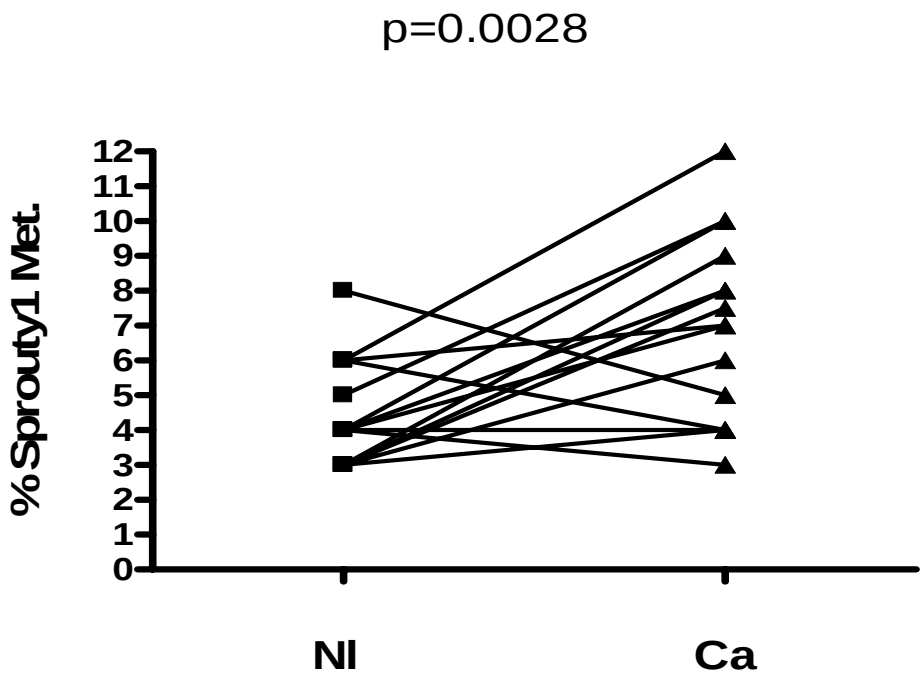


Fig. 3D

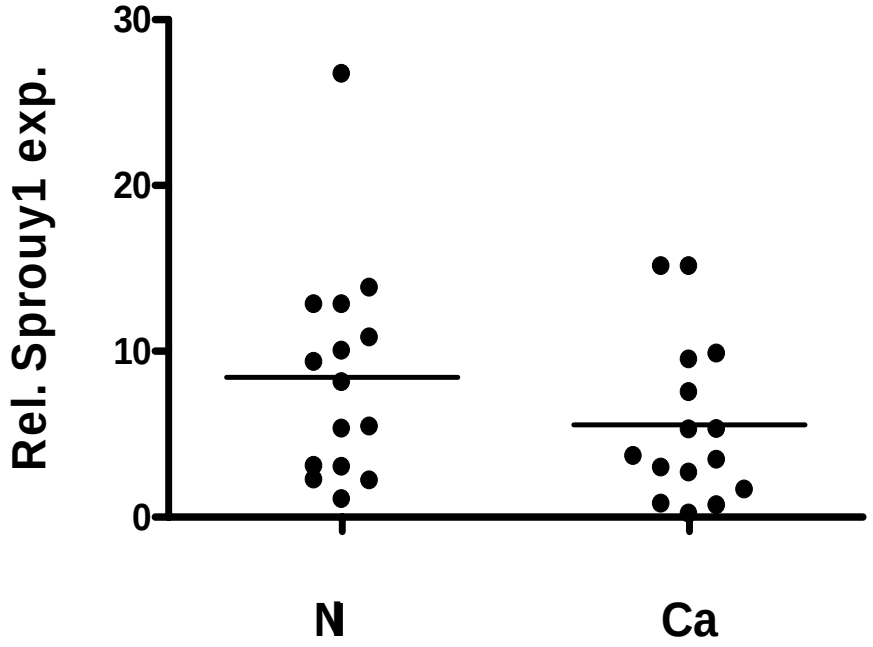
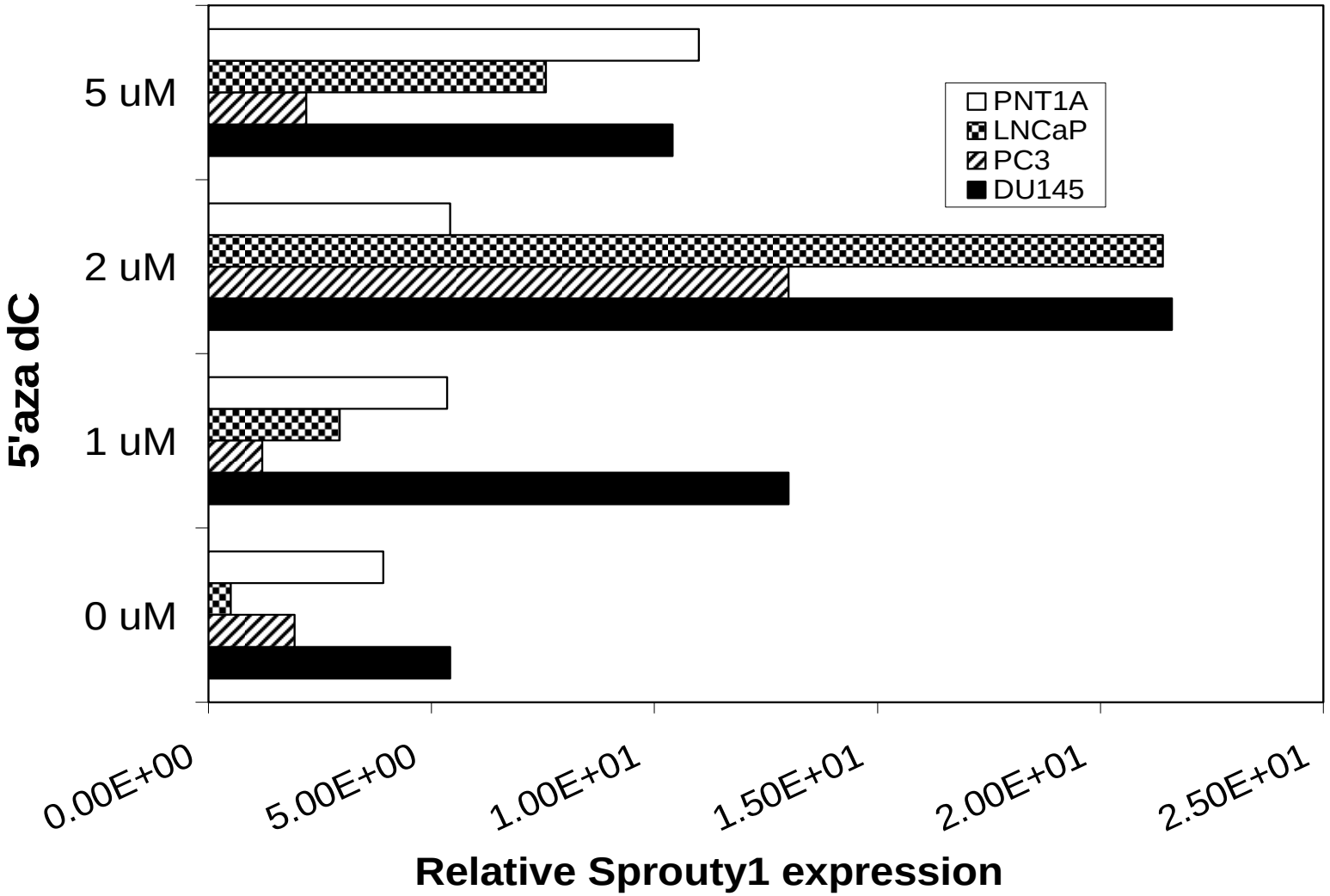


Fig. 4


5 uM
2 uM
1 uM
0 uM
5'aza dC
PNT1A
LNCaP
PC3
DU145
0.00E+00
5.00E+00
1.00E+01
1.50E+01
2.00E+01
2.50E+01
Relative Sprouty1 expression