

AD_____

Award Number: W81XWH-04-1-0878

TITLE: Mechanisms Down-Regulating Sprouty1, a Growth Inhibitor in Prostate Cancer

PRINCIPAL INVESTIGATOR: Bernard Kwabi-Addo Ph.D.

CONTRACTING ORGANIZATION: Howard University
Washington D.C. 20060

REPORT DATE: October 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-10-2007		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 20 SEP 2006 - 19 SEP 2007	
4. TITLE AND SUBTITLE Mechanisms Down-Regulating Sprouty1, a Growth Inhibitor in Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0878	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bernard Kwabi-Addo Ph.D. E-Mail: bernardk@bcm.tmc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Howard University Washington D.C. 20060				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT PURPOSE. The Sprouty gene family negatively regulates growth factor-induced receptor tyrosine kinase signaling with a potential tumor suppressor function in cancer. I have demonstrated that Sprouty1 is down-regulated in human prostate cancer. The purpose of the present study is to elucidate the relative contribution of transcription regulation and epigenetic DNA methylation changes in regulating Sprouty1 expression in human prostate cancer. RESULTS. Using transient transfection analysis in prostate cancer cell lines; I have shown that gene knockdown of two transcription factor families: EGR (1 and 2), and GATA (2 and 4) induced Sprouty1 protein expression as determined by western blot analysis suggesting that these transcription factors may negatively regulate Sprouty1 expression. In addition, methylation modification of the <i>Sprouty1</i> promoter by treatment with SssI methylase abolished promoter activity in transiently transfected prostate cancer cells, whereas global demethylation induced Sprouty1 expression. CONCLUSION. Observations reported here demonstrate transcriptional repression and/or epigenetic inactivation of Sprouty1 expression in prostate cancer cells. 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 result in a refractory transcriptional response even in the presence of necessary trans-acting activities.					
15. SUBJECT TERMS Sprouty1; transcriptional regulation; shRNA gene targeting; prostate cancer; Epigenetic DNA-methylation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	63	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	Page
Introduction.....	4-6
Body.....	6-12
Key Research Accomplishments.....	12-13
Reportable Outcomes.....	13
Conclusions.....	13-14
References.....	14-17
Appendices.....	18-63

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 [6-8]. Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [9-16]. 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 [17,18] or the inhibition of Raf [19,20]. 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 [21] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [22]. 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 [23,24]. McKie et al., [25] 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 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-2-deoxycytidine (5-aza-dC) a demethylation agent, reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 down-regulation [26]. Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated [27]. 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 [28] indicating cancer-specific mechanisms of Sprouty down-regulation [29].

In studying the molecular mechanisms regulating Sprouty1 expression, we 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. Thus, the present study seeks is to further evaluate the relative contribution of transcriptional regulation and epigenetic DNA methylation changes in down-regulating Sprouty1 protein expression in prostate cancer.

BODY

As outlined in my Statement of work, I seek to accomplish 3 main tasks during my 3 years of funding. I have made substantial progress in all of these tasks. For the remaining 12 months of this proposal, I have focused my attention on the role of transcription factors and epigenetic DNA methylation in regulating Sprouty1 expression in human prostate. A manuscript describing the use of siRNA knockdown experimentations of transcription factors is in preparation for re-submission to the Prostate Journal in response to the reviewers' critiques. A copy of this manuscript is

attached and will be referred to below. I have changed institutions from Baylor College of Medicine in Houston (Texas) to Howard University in Washington (D.C). I have therefore requested a 1 year no cost extension in order to complete my remaining studies, please see attached for the revised contract indicating extension of the project until September, 2008.

Task 3: Characterization of transcription factor(s) responsible for interaction with *Sprouty1* promoter (Months 18 – 36).

In vitro characterization of transcription factors identified using TranSignal Protein/DNA Arrays.

In the first part of task 3, I have reported the identified potential transcription factors binding sites for *Sprouty1* promoter using a computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). I have found potential binding sites for several TFs including GATA1, EGR2 [30], ZBP [31], ETS [32], HIC [33] and FKHD [34] in the proximal promoter region. Sequence homology analysis showed that over the entire 5'-flanking region of the human *Sprouty1* promoter, only a very short region in *Sprouty1b* promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Sprouty1* promoter, demonstrating that Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3, and WTE are conserved between the two species. The high sequence homology in the *Sprouty1* promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Sprouty1* gene regulation.

Furthermore, I reported that using a protein/DNA array technology I have identified the differential activation of a number of transcription factors with consensus binding sites on *Sprouty1* promoter in these cell lines. This includes AP-1/2, 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 (manuscript in preparation for re-submission; see attachment).

Impact of EGR and GATA activity on *Sprouty1* expression (Months 24-36)

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 were 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 1). 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 were 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.

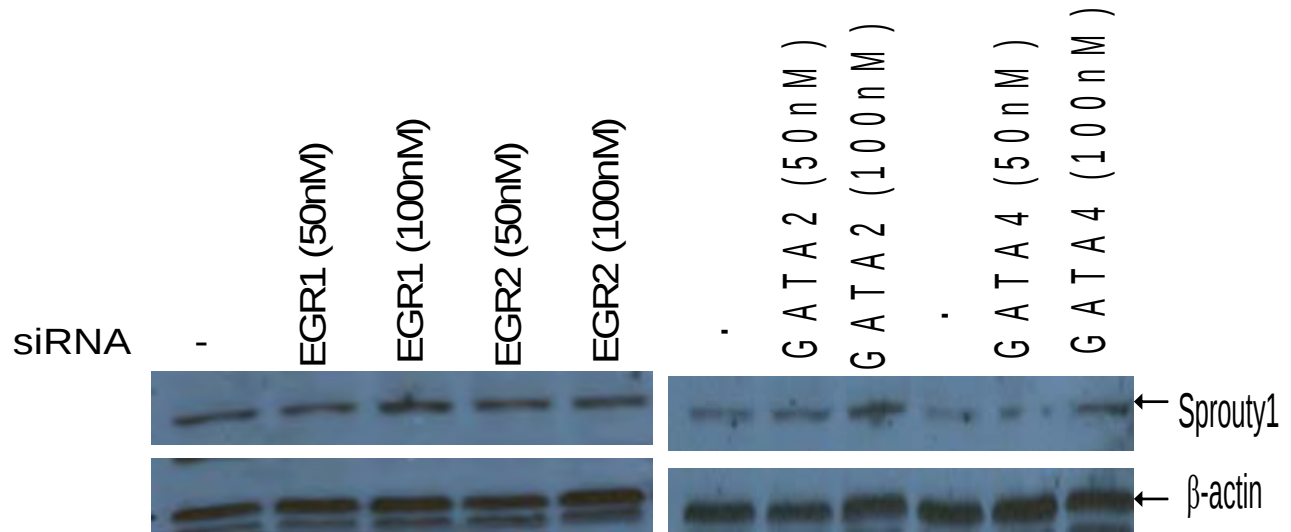


Figure 1. siRNA knock-down of EGR and GATA and Sprouty1 expression in LNCaP cells. The LNCaP cells were transiently transfected with either EGR1, EGR2, GATA2, or GATA4 siRNA duplexes for 72 hours. Total protein extracts from the transfected cells were used in western blotting with either anti-Sprouty1 or β -actin antibody.

The minimal increase in Sprouty1 expression due to EGR 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.

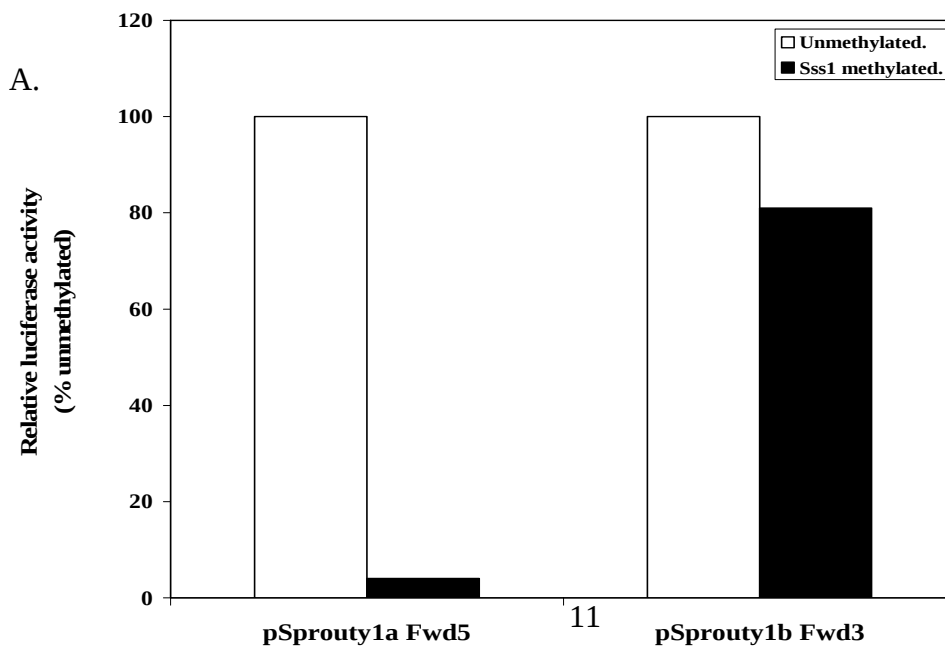
I have made recombinant GATA (2 and 4) and EGR (1 and 2) shRNA constructs. Stable transfection of these recombinant vectors into the prostate cancer cell lines LNCaP and PC3 showed detrimental effect on cell growth after a week of cell growth under media selection (data not shown). My hypothesis is that 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 [23] therefore 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.

Methylation analysis of *Sprouty1* promoter.

Previously, I reported that DNA methylation analysis does not appear to play a role in the down-regulation of Sprouty1 expression in prostate cancer. This observation was based on pyrosequencing analysis that quantitatively measured DNA methylation of bisulfite modified genomic DNA. The pyrosequencing reaction was carried out in the 200 bp proximal region containing the CpG islands of Sprouty1a and Sprouty1b promoters respectively (data not shown). However, Sprouty1a promoter CpG island spans about 2 Kbp sequence whereas Sprouty1b promoter CpG region is only 110 bp (see attached manuscript) suggesting that perhaps other sites of the Sprouty1b promoter might be methylated. This is supported by the observation that methylation of Sprouty1a and not Sprouty1b with SssI methylase treatment abolished Sprouty1a activity. To investigate whether constitutively active *Sprouty1a* and/or *Sprouty1b* promoter activity was inhibited

by the methylation of the promoter CpG island, I modified the promoter constructs with SssI methylase treatment and examined the activity of the methylated promoter. When the SssI methylated or non-methylated *Sprouty1a* and *Sprouty1b* 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. On the other hand, CpG methylation of *Sprouty1b* construct did not show significant effect on its activity when compared to the control unmethylated construct (figure 2A). This observation indicates that methylation of the *Sprouty1a* promoter may be involved in the control of *Sprouty1* gene expression.

Furthermore, treatment of pNT1, DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2'deoxyctidine (5-aza-dC) led to a significant increase in *Sprouty1* mRNA expression in the prostate cancer cell lines (figure 2B). Taken together, these data indicates that other CpG sites of *Sprouty1a* that were not analyzed in the previous report might be methylated in the tumor samples that were analyzed. Currently, I am investigating the methylation status of other CpG sites in the *Sprouty1a* region in the tumor samples as well as prostate cancer cell lines.





Methylation analysis of *Sprouty1* gene. **A.** Effect of *in vitro* methylation on the activity of the *Sprouty1* promoter. *Sprouty1a* and *1b* promoters were methylated *in vitro* by SssI methylase. Methylated and unmethylated *Sprouty1a* and *1b* promoters were transfected into LNCaP cells and assayed for activity. Results are shown as percentages, with luciferase activity due to unmethylated promoter designated as 100%. Results are the mean for 2 independent experiments each of which was performed with triplicate samples. **B.** 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 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.

KEY RESEARCH ACCOMPLISHMENTS

- I have constructed recombinant GATA (2 and 4) and EGR (1 and 2) shRNA lentivirus that would be useful for the efficient knockdown of these transcription factors in in-vitro prostate cancer cell lines in order to ascertain their biological effect on *Sprouty1* expression.
- In vitro methylation of *Sprouty1b* promoter by Sss1 methylase modification completely abolished *Sprouty1b* promoter activity. Whereas, the treatment of prostate cancer cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine restored *Sprouty1* mRNA expression demonstrating that epigenetic DNA methylation changes plays a role in down-regulating *Sprouty1* expression. To verify that *Sprouty1a* promoter is methylated in human prostate tumors, I performed methylation analysis of 12 pairs of matched normal and tumor prostate tissue samples using bisulfite pyrosequencing. I did not observe methylation in any of the samples analyzed (data not shown). However, because

Sprouty1a promoter region has a 2 kbp region of CpG island it is likely that other CpG sites are methylated in the these samples and this remains to be investigated.

- I have demonstrated that transcriptional repression and epigenetic DNA methylation changes constitutes key mechanisms for the down-regulation of sprouty1 expression in prostate cancer.

REPORTABLE OUTCOMES

- **AACR Annual Meeting- Age-Related DNA Methylation Changes in Normal Human Prostate. (2007) Los Angeles CA.**
- **Manuscript in preparation for re-submission to the Prostate Journal**

CONCLUSION

The Sprouty gene family functions as negative regulators of receptor tyrosine kinase signaling. I have identified functional regions of the human *Sprouty1* 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 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.

FUTURE WORK WILL FOCUS ON:

- 1) Complete the evaluation of GATA and EGR transactivation of the Sprouty1 promoter in human prostate cancer cells by optimizing the knockdown efficiency of GATA and EGR shRNA targeting to achieve significant effect on Sprouty1 protein expression. In addition, the use of Protein/DNA Arrays technology has identified additional transcription factors (TFs) whose expression are increased in response to FGF2 stimulation of prostate cancer cell lines. I want to investigate the functional activity of these TFs in Sprouty1 expression that may contribute to further understanding the regulation of Sprouty1 expression.
- 2) I will continue to investigate the relative contribution of epigenetic DNA methylation changes in matched normal and prostate cancer tissues to ascertain the frequency of methylation in the tumor samples and determine whether there is an inverse co-relation between DNA methylation and Sprouty1 expression at the mRNA level. Because Sprouty1 promoter region encompass about 2 kbp promoter extension methylation of multiple sites are being analyzed in other to identify what CpG sites are methylated in the tumor samples.

REFERENCES

1. Kwabi-Addo B, Ozen M, Ittmann M: **The role of fibroblast growth factors and their receptors in prostate cancer.** *Endocr Relat Cancer* 2004, **11**: 709-724.
2. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA: **sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways.** *Cell* 1998, **92**: 253-263.
3. Casci T, Vinos J, Freeman M: **Sprouty, an intracellular inhibitor of Ras signaling.** *Cell* 1999, **96**: 655-665.

4. Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y: **Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila.** *Development* 1999, **126**: 2515-2525.
5. Reich A, Sapir A, Shilo BZ: **Sprouty is a general inhibitor of receptor tyrosine kinase signaling.** *Development* 1999, **126**: 4139-4147.
6. de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S: **Cloning and expression pattern of a mouse homologue of Drosophila sprouty in the mouse embryo.** *Mechanisms of Development* 1999, **81**: 213-216.
7. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA: **sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways.** *Cell* 1998, **92**: 253-263.
8. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N *et al.*: **Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed.** *Development* 1999, **126**: 4465-4475.
9. Hanafusa H, Torii S, Yasunaga T, Matsumoto K, Nishida E: **Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty.** *J Biol Chem* 2004, **279**: 22992-22995.
10. Gross I, Bassit B, Benezra M, Licht JD: **Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation.** *J Biol Chem* 2001, **276**: 46460-46468.
11. Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB: **The C terminus of sprouty is important for modulation of cellular migration and proliferation.** *J Biol Chem* 2001, **276**: 22742-22747.
12. Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G: **Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells.** *J Cell Biol* 2001, **152**: 1087-1098.
13. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M *et al.*: **Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1.** *Nat Cell Biol* 2003, **5**: 427-432.
14. Yusoff P, Lao DH, Ong SH, Wong ESM, Lim J, Lo TL *et al.*: **Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of raf.** *Journal of Biological Chemistry* 2002, **277**: 3195-3201.
15. Hanafusa H, Torii S, Yasunaga T, Nishida E: **Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway.** *Nature Cell Biology* 2002, **4**: 850-858.

16. Lee SH, Schloss DJ, Jarvis L, Krasnow MA, Swain JL: **Inhibition of angiogenesis by a mouse sprouty protein.** *Journal of Biological Chemistry* 2001, **276**: 4128-4133.
17. Casci T, Vinos J, Freeman M: **Sprouty, an intracellular inhibitor of Ras signaling.** *Cell* 1999, **96**: 655-665.
18. Hanafusa H, Torii S, Yasunaga T, Nishida E: **Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway.** *Nature Cell Biology* 2002, **4**: 850-858.
19. Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G: **Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells.** *J Cell Biol* 2001, **152**: 1087-1098.
20. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M *et al.*: **Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1.** *Nat Cell Biol* 2003, **5**: 427-432.
21. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N *et al.*: **Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed.** *Development* 1999, **126**: 4465-4475.
22. Lim J, Wong ES, Ong SH, Yusoff P, Low BC, Guy GR: **Sprouty proteins are targeted to membrane ruffles upon growth factor receptor tyrosine kinase activation. Identification of a novel translocation domain.** *J Biol Chem* 2000, **275**: 32837-32845.
23. Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G *et al.*: **The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer.** *Cancer Res* 2004, **64**: 4728-4735.
24. Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B: **Sprouty4, a suppressor of tumor cell motility, is downregulated by DNA methylation in human prostate cancer.** *Prostate* 2005.
25. McKie AB, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R *et al.*: **Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer.** *Oncogene* 2005, **24**: 2166-2174.
26. Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B: **Sprouty4, a suppressor of tumor cell motility, is downregulated by DNA methylation in human prostate cancer.** *Prostate* 2005.

27. McKie AB, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R *et al.*: **Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer.** *Oncogene* 2005, **24**: 2166-2174.
28. Lo TL, Yusoff P, Fong CW, Guo K, McCaw BJ, Phillips WA *et al.*: **The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer.** *Cancer Res* 2004, **64**: 6127-6136.
29. Lo TL, Yusoff P, Fong CW, Guo K, McCaw BJ, Phillips WA *et al.*: **The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer.** *Cancer Res* 2004, **64**: 6127-6136.
30. Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ: **Expression of early growth response genes in human prostate cancer.** *Cancer Res* 1998, **58**: 2461-2468.
31. Sander TL, Stringer KF, Maki JL, Szauter P, Stone JR, Collins T: **The SCAN domain defines a large family of zinc finger transcription factors.** *Gene* 2003, **310**: 29-38.
32. Liu AY, Corey E, Vessella RL, Lange PH, True LD, Huang GM *et al.*: **Identification of differentially expressed prostate genes: increased expression of transcription factor ETS-2 in prostate cancer.** *Prostate* 1997, **30**: 145-153.
33. Yamanaka M, Watanabe M, Yamada Y, Takagi A, Murata T, Takahashi H *et al.*: **Altered methylation of multiple genes in carcinogenesis of the prostate.** *Int J Cancer* 2003, **106**: 382-387.
34. Tasheva ES, Klocke B, Conrad GW: **Analysis of transcriptional regulation of the small leucine rich proteoglycans.** *Mol Vis* 2004, **10**: 758-772.



Category: Cellular and Molecular Biology 35

Session Title: DNA Methylation 2

#2870 Age-related DNA methylation changes in normal human prostate tissues. Bernard Kwabi-Addo¹, Woonbok Chung², Lanlan Chen², Michael Ittmann¹, Jaroslav Jelinek², Jean-Pierre Issa². ¹Baylor College of Medicine, Houston, TX and ²MD Anderson Cancer Center, Houston, TX.

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. To quantitatively assess the extent of gene methylation in the normal prostate, we used pyrosequencing and investigated the methylation status of 9 CpG islands in DNAs extracted from 43 normal human prostate tissues samples. We found a significant increase in promoter methylation correlating with age for the CpG islands at GSTPI, RASSF1a and Clstn1 genes. CpG islands at p16, ESR1 and MyoD1 also exhibited low but significant increase in methylation as a function of age in normal prostate samples. We also demonstrate that for GSTPI, higher methylation levels accounted for decreased GSTPI mRNA expression in prostate cancer patient samples. Our findings demonstrate global aberrant methylation in normal aging cells. By affecting genes that regulate the growth and/or differentiation of these cells, such age-related methylation may precede and predispose to full-blown malignancy. Therefore the identification of genes which undergoes age-related methylation in the normal prostate would be potentially useful in understanding the etiology of prostate cancer.



The Molecular Mechanisms Controlling the Expression of Sprouty1, a Negative Regulator of Fibroblast Growth Factor Signaling in Prostate Cancer

Journal:	<i>The Prostate</i>
Manuscript ID:	PROS-07-129.R1
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	01-Jun-2007
Complete List of Authors:	Kwabi-Addo, Bernard; Howard University Cancer Center, Medicine Ren, Chengxi; Baylor College of Medicine, Pathology Dixit, Shantu; Baylor College of Medicine, Pathology Ittmann, Michael; Baylor college of Medicine, Pathology
Key Words:	Sprouty1, transcription, DNA methylation, prostate cancer



**The Molecular Mechanisms Controlling the Expression of Sprouty1, a Negative
Regulator of Fibroblast Growth Factor Signaling in Prostate Cancer**

*Bernard Kwabi-Addo¹, Chengxi Ren², Dixit Shantu² and Michael Ittmann^{2,3}

¹Howard University Cancer Center, 2041 Georgia Ave, N.W. Washington D.C. 20060.

²Department of Pathology, Baylor College of Medicine, 1 Baylor Plaza, Houston TX
77030. ³Michael E. Debakey Department of Veterans Affairs Medical Center, 2002
Holcombe Blvd. Houston TX 77030 USA.

RUNNING TITLE: Transcriptional and epigenetic regulation of Sprouty1

KEY WORDS: Sprouty1; transcription; DNA methylation; prostate cancer

*Correspondence:

Bernard Kwabi-Addo, PhD,
Howard University Cancer Center
2041 Georgia Ave. N.W. Washington D. C. 20060
Tel: 202 865 4653
Fax: 202 667 1686
E-mail: bkwabi-addo@howard.edu

PURPOSE. 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. In this report, we investigated the molecular mechanisms regulating Sprouty1 expression in human PCa cells.

EXPERIMENTAL DESIGN. Deletion analysis coupled with reporter gene assays were used to characterize *Sprouty1* promoter activity. Electrophoretic mobility shift assays, and chromatin immunoprecipitation were used to demonstrate binding interaction of Transcription factors (TFs) with *Sprouty1* promoter. Methylation of the *Sprouty1* promoter was assessed using Sss1 methylase or 5'-Aza-2'-Deoxycytidine treatment.

RESULTS. Deletion analysis showed a strong promoter activity in the proximal 0.3-kb region of *Sprouty1* promoter. Several potential binding sites for transcription factors (TFs) such as: AP-1/2, CREB, EGR1, GATA1, and SP1 were found within this region. Gene knockdown of two such transcription factor families: EGR (1 and 2), and GATA (2 and 4) induced *Sprouty1* expression. Methylation modification of the *Sprouty1* promoter abolished promoter activity whereas global demethylation induced *Sprouty1* expression.

CONCLUSION. Our observation demonstrates transcriptional repression and/or epigenetic inactivation of *Sprouty1* expression in prostate cancer cells.

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 [3,14] or the inhibition of Raf [11,16]. 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 [17]. 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 [18,19]. McKie et al., [20] 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 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 [19]. Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated [20]. 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 [21]. Therefore a full understanding of the molecular mechanisms regulating Sprouty1 must include knowledge of Sprouty1 transcriptional regulation. Thus, in the present study, we sought to investigate the relative contribution of transcription and epigenetic mechanisms regulating *Sprouty1* expression in prostate cancer.

MATERIALS AND METHODS

Cell Culture and antibodies

The human prostate cancer cell lines, PC3, DU145 and LNCaP, and the immortalized normal prostate epithelial cell line pNT1A were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) unless otherwise stated. Antibodies used for western blotting, electrophoretic mobility shift assays and chromatin immunoprecipitation were the following: anti-Sprouty1 (C-12) goat polyclonal IgG; anti-PBX1 (P-20) rabbit polyclonal IgG; anti-GATA1 (C-20) goat polyclonal IgG; anti-GATA2 (C-20) goat polyclonal IgG; anti-GATA4 (C-20) goat polyclonal IgG; anti-EGR1 (588) rabbit polyclonal IgG; anti-EGR2 (N-20) goat polyclonal IgG; anti-SP1 mouse monoclonal IgG; anti-HNF4a (C-19) goat polyclonal IgG were all purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-acetyl-Histone H4 rabbit antiserum was purchased from Upstate Biotech (Lake Placid, NY).

Rapid amplification of cDNA ends

Transcription start sites were identified by 5'-RACE following the protocol from the GeneRacer Kit (Invitrogen) and using the human fetal lung Poly(A)⁺ RNA (250 ng; Clontech, Palo Alto, CA) to create RACEready cDNA. A PCR reaction was carried out using RACEready cDNA as template and GeneRacer RNA oligo (5'-CGACTGGAGCACGAGGACACTGA-3') as the forward primer and either the Sprouty1-specific primer Spry1ARACE (5'-CTTGTCT-TGGTGCTGTCCGAGGAGC-

AGGT-3') or Spry1BRACE (5'-CTGCAAAGCACGCAGTGGTTTGCAGAGCGGA-3') as the reverse primer and Advantage-GC Genomic Polymerase Mix (Clontech). The PCR conditions consisted of one cycle of 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 68°C, and 1 min at 72°C. PCR products were cloned into the pCR-Blunt II-TOPO vector (Invitrogen) followed by sequencing.

Construction of plasmids for promoter analysis

Progressive deletion constructs of *Sprouty1a* and *Sprouty1b* 5'-flanking regions were accomplished by unidirectional cloning of PCR fragments from the *Sprouty1* 5'-flanking region into the *Kpn1/Nhe1* site of the promoterless and enhancerless firefly luciferase reporter vector pGL3-Basic (Promega). For each region the PCR fragments were generated using a common reverse primer and different forward primers. The *Kpn1* and *Nhe1* sites (showed in lowercase) were engineered into the 5' ends of forward and reverse primers, respectively. The numbers indicated after the primer sequences correspond to the distance in nucleotides from the 5-end of the sequence in uppercase to the 5'-most transcription start site determined by 5'-RACE. For *Sprouty1a* 5'flanking region: Forward (Fwd) 13 (-93) 5'-ggtaccTCCTACCACAGAGAGAGGGAGAAA-3'; Fwd12 (-133) 5'-ggtaccCCCTCCTGAGCTCATGGTAACCT-3'; Fwd 6 (-509) 5'-ggtaccCTTCTGGTTTGGAGCACAGTGCAAAG-3'; Fwd 5 (-1318) 5'ggtaccAGAAG-ACCTCCCGAGGTGGATGTTA-3'; Fwd3 (-2025) 5'-ggtaccCTGTCAATCACCGGG-AGC-3'; Reverse (+8) 5'-gctagcAATCCGCACTGAATAAATAGTTGAC-3'. For *Sprouty1b* 5-flanking region: Fwd2 (-70) 5'-ggtaccCATGATATCACCGGAGGCGTGT-

CCTG-3'; Fwd3 (-175) 5'-ggtaccGAGTCTGTAGGGCAACATTTCCAAGTTGG-3'; Fwd 4 (-233) 5'-ggtaccCTGCATTTGCAGAATTTTATAGAGGCAC-3'; Fwd 5 (-305) 5'-ggtacc-CACCAATCCTTTTAATTGAGATCGAC-3'; Fwd6 (-530) 5'-ggtaccCATATGCTTATATTACATTTGCAGTAAGG-3'; Fwd 7 (-960) 5'-ggtaccGTTTTGCCAGACTTTAAGCTACTCC-3'; Reverse (+50) 5'-gctagcGGGAATGTGCTGATAATCACTCG-3'. We used the Advantage Genomic PCR kit (Clontech) to amplify these promoter fragments and according to the manufacturer's protocol. The human genomic DNA was used as template. The PCR products were first cloned into TOPO TA vector (Invitrogen) and then excised by *Kpn*I and *Nhe*I digestion and subcloned into the pGL3-Basic vector. Every construct was sequenced to ensure correct orientation and sequence integrity.

Transient transfections

The PC3, DU145, LNCaP and PNT1A 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. To investigate FGF2 stimulatory effects cells were grown in serum free medium supplemented with 1% ITS for 24 hours. Cells were then transfected with the individual reporter plasmids as above. After 24 hours post transfection, cells were stimulated with or without FGF2 (20 ng/ml) for an additional 24 hours. Each transfection was done in triplicate.

Reporter gene luciferase assay

Cells were lysed 48 hours posttransfection 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's 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.

siRNA cell transfection

The LNCaP (1×10^6) cells were each transfected with either 50 or 100 nM of siGENOME SMART pool GATA2 siRNA, GATA4 siRNA, EGR1 siRNA and EGR2 siRNA (Dharmacon Inc.) using 60 μ l of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Seventy-two hours post-transfection cells were harvested and total protein extracted used in western blot analysis as previously described [21].

In vitro Methylation

The *Sprouty1a* Fwd5 (-1318/+8) and *Sprouty1b* Fwd 3 (-175/+50) promoter fragments (1 µg) were in vitro methylated with *SssI* (CpG) methylase (New England Biolabs, Beverly, MA) as recommended by the manufacturer. The *SssI* methylation, which methylates all cystocine residues within the double-stranded dinucleotide recognition sequence (5'-CG-3'), was performed at 10mM Tris, pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 160 µM *S*-adenosylmethionine at 37°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 *NheI* and *KpnI* 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 *SssI* or *HpaII* or *HhaI* restriction enzymes.

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

LNCaP cells were seeded at 5 X 10⁵ 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. Cells were then harvested for RNA extraction and the extracted RNA used in real-time quantitative PCR as previously described [18].

Preparation of nuclear extracts

Nuclear extracts were prepared from PC3, DU145, LNCaP and PNT1A essentially as previously described by [22].

Oligonucleotide probe preparation and Electrophoretic mobility shift assays
(EMSA)

Oligonucleotide sequences were synthesized by Sigma Genosys. Oligonucleotide sequences encompassing binding sites for wild-type or mutant (mut) transcription factors were synthesized as sense (F) or antisense (R) strands: EGRF 5'-GGATCCAGCGGGGCGAGCGGGGGCCA-3'; EGRR 5'-TGGCCCCCGCTCGCC-CCCGCTGGATCC-3'; mut- EGRF 5'-GGATCCATTTTTTTTCGATTTTTTTTCCA-3'; mutEGRR 5'-TGGAAAAAATCGAAAAAATGGATCC-3'; PBX1F 5'-CGAATTG-ATTGATGCACTAATTGGAG-3'; PBX1R 5'-CTCCAATTAGTGCATCAATCAATT-CG-3'; HNF4F 5'-ACAGGGTCAAAGGTCACGA-3'; HNF4R 5'-TCGTGACCTTGA-CCCTGT-3'; SP1F 5'-ATTCGATCGATCGGGGCGGGGCGAG-3'; SP1R 5'-CTCG-CCCCGCCCCGATCGAAT-3'. Each oligonucleotide sense strand was end-labeled with [γ - 32 P] dATP (Amersham Biochemicals; 3000 Ci/mmol) and 1X polynucleotide kinase reaction buffer (Invitrogen). The labeled oligonucleotide was annealed to 50 fold molar excess of the complementary anti-sense strand by heating at 85°C for 5 min and slowly cooling to room temperature. Unincorporated [γ - 32 P] dATP were removed by purifying the probes using a G-25 (Fine) Sephadex Quick spin columns (Roche). The EMSAs were carried out as described previously [23]. Briefly, the 32 P-labeled oligonucleotide probes were incubated with or without nuclear extract in a total reaction volume of 25 μ l containing the binding assay buffer (50 mM Tris-HCl, pH 7.4; 50 mM NaCl; 1 mg/ml BSA; 5 μ g/ml poly dI-dC; 20% glycerol). The reactions were started by the addition of nuclear extract (5 μ l per reaction) and incubated at room temperature for 30 minutes. Competition reactions were pre-incubated in the binding buffer for 30 min at room

temperature with 100 fold molar excess of the corresponding unlabelled oligonucleotides followed by a 30 min incubation at room temperature with the labeled oligonucleotide. For supershift analysis 1 μ l of antibody was preincubated with the binding buffer for 45 minutes at room temperature prior to the addition of the 32 P-labeled probe. The bound and free DNA were resolved by electrophoresis through a 5% polyacrylamide gel at 175 V in 0.5 X TBE at room temperature for 2.5 h. Dried gels were exposed to Kodak Bio-Max film at -80°C with intensifying screens.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously [24]. For PCR reaction, DNA solution (50 ng) was used as a template with *Sprouty1b* Fwd 5 (forward) and Rev (reverse) primers as described above.

Western blot analysis

Western blotting for Sprouty1 protein and quantitative analysis of Western blot signals has been previously described [18].

RESULTS

Genomic organization of the Sprouty1 gene

The human *Sprouty1* gene consists of two splice variants, 1a [2] and 1b [25] that maps 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 *Sprouty1a* and *Sprouty1b* splice variants, we performed 5'-RACE using poly (A)⁺ RNA from fetal human lung and a *Sprouty1* 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).

Functional characterization of Sprouty1 promoter region

To localize the DNA elements that are important for promoter activity, we carried out a series of unidirectional deletion analyses of up to 2 kb and approximately 1 kb of the 5'-flanking region of *Sprouty1a* of *Sprouty1b* 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 2, the promoter activities demonstrated significant difference between Splice 1a (Fig 2A) and 1b (Fig 2B) variants. *Sprouty1a* promoter strength was between 2 to 5 fold above the basal level. Whereas *Sprouty1b* 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 [18] 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 *Sprouty1b* Fwd3 (-175 to +50) construct. In the androgen independent prostate cancer cell lines PC3 and DU145, maximum promoter activity was observed from the *Sprouty1b* Fwd4 (-233 to + 50) and *Sprouty1b* Fwd6 (-530 to + 50) constructs respectively. In the

immortalized normal prostate cell line pNT1A, maximum promoter activity was seen with the *Sprouty1b* Fwd5 (-305 to + 50) construct. Because strong promoter activity was observed at the *Sprouty1b* promoter region, we believe this region has the transcriptional elements and enhancer sequence(s) necessary for *Sprouty1* gene regulation. Therefore all subsequent promoter analysis was done at the *Sprouty1b* locus and is hereafter referred to as *Sprouty1* promoter.

Comparative sequence analysis of the *Sprouty1* promoter locus

To further characterize the *Sprouty1* promoter region, we searched for transcription factor binding sites using the MatInspector program [26]. We analyzed 2 kb of the genomic AC026402 sequence upstream of the *Sprouty1b* transcription start sites, using computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). We found potential binding sites for several TFs including GATA1 [27], EGR [28], ZBP [29], ETS [30], HIC [31] and FKHD [32] in the proximal promoter region. The human and murine [33] *Sprouty1* 5'-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 5'-flanking region of the human *Sprouty1* promoter, only a very short region in *Sprouty1b* promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Sprouty1* promoter. As illustrated in Fig 3, Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3 [34], and WTE [35] are conserved between the two species. Interestingly, the nucleotide sequences immediately upstream from the EGR motif diverge in these species. Furthermore, we did not see any sequence homology between the human *Sprouty1* promoter region and that of the published

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sprouty2 [36] or *Sprouty4* [37] promoters. The high sequence homology in the *Sprouty1* promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Sprouty1* gene regulation.

Electrophoretic mobility shift assay

To verify the binding interaction of the *Sprouty1* consensus sequence in vivo, we 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 4A. 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. We 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), we observed a new complex migrating very close with complex C2. Cold competition assay with wild-type EGR1 oligonucleotide

1
2
3 competed out complex C2 totally but only partially competed the new complex.
4
5 Furthermore, supershift assay successfully competed C2. This indicates that EGR1
6
7 protein preferentially recognize and interact with the wild-type EGR1 consensus binding
8
9 sequence.
10
11

12
13
14
15 **Chromatin immunoprecipitation (ChIP)**
16

17
18 We next studied whether these TFs bound to the *Sprouty1* promoter *in vivo* using
19
20 ChIP assay. Figure 4B showed that indeed these TFs bound to *Sprouty1* promoter *in vivo*
21
22 as demonstrated by the same PCR product in the assay precipitation with different
23
24 antibodies compared to the Anti-acetyl-Histone H4 antibody control (positive control).
25
26 Conversely precipitation with normal goat IgG (negative control) did not show any
27
28 binding. These studies clearly demonstrate that *Sprouty1* proximal promoter region
29
30 contain several sequence motifs (i.e., EGR, GATA, SP1, PBX1 and HNF4) which are
31
32 specifically recognized by known as well as uncharacterized transcription factors and are
33
34 functionally important and likely to be responsible for driving the basal transcription of
35
36 the *Sprouty1* gene.
37
38
39
40
41
42

43
44 **Impact of EGR and GATA activity on Sprouty1 expression**
45

46
47 To verify the involvement of transcription factor binding activity in regulating
48
49 *Sprouty1* expression, we investigated 2 transcription factors; EGR and GATA. We
50
51 studied EGR because the EGR binding site appears to be highly conserved between
52
53 human and mouse, and GATA because of its consensus binding site in *Sprouty1* proximal
54
55 promoter region. We transiently transfected LNCaP cells with siRNA duplexes
56
57
58
59
60

corresponding to EGR1, EGR2, GATA2 and GATA4. Western blot analysis were 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 Fig 5A). The Western blot signals were quantified and expressed relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; data not shown). Figure 5B shows that when compared with the LNCaP cells transfected with the scrambled siRNA oligos, LNCaP cells transfected with EGR1 siRNA (100 mM) and EGR2 siRNA (100 mM) showed a 1.3 fold And 1.6 fold increase in Sprouty1 protein expression respectively. On the other hand, when LNCaP cells were transfected with GATA2 siRNA (100 mM) and GATA4 siRNA (100 mM), there were approximately 4 and 3 fold increases in Sprouty1 protein expression respectively. The observed Sprouty1 expression levels were 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). Our data indicates that the blockade of EGR1 and EGR2, GATA2 and 4 by small inhibitory RNA can increase Sprouty1 protein expression.

In vitro methylation analysis of the Sprouty1 promoter region

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 *Sprouty1a* promoter region and the other spanning about 110 bp of *Sprouty1b* promoter region (not shown). To investigate whether constitutively active *Sprouty1a* and/or *Sprouty1b* promoter activity was inhibited by the methylation of

the promoter CpG island, we modified the promoter constructs with *SssI* methylase treatment and examined the activity of the methylated promoter. When the *SssI* methylated or non-methylated *Sproutyl1a* and *Sproutyl1b* promoter constructs were each transiently transfected into LNCaP cells the activity of the methylated *Sproutyl1a* promoter was only 5 % of that of the unmethylated construct (see Fig 6A) On the other hand, CpG methylation of *Sproutyl1b* construct did not show significant effect on its activity when compared to the control unmethylated construct (Fig 6B). This observation indicates that methylation of the *Sproutyl1a* promoter may be involved in the control of *Sproutyl1* gene expression.

To verify that *Sproutyl1a* promoter is methylated in human prostate tumors, we performed methylation analysis of 12 pairs of matched normal and tumor prostate tissue samples using bisulfite pyrosequencing. We observed methylation in 1 tumor sample when compared to normal tissue (data not shown) indicating that decreased *Sproutyl1* expression could be due to methylation in some of the tumor samples, however this needs to be confirmed using more tumor samples. Furthermore, because *Sproutyl1a* promoter region has 2kbp region of CpG island it is likely that other CpG sites are methylated in the tumor samples which remains to be investigated.

To further confirm that DNA methylation plays a role *Sproutyl1* expression, we tested the hypothesis that pharmacological modulation of methylation can reactivate gene expression [38]. To achieve this we treated pNT1, DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2’deoxycytidine (5-aza-dC). As shown in Fig 7C, treatment of the prostate cancer cell lines, DU145, PC3 and LNCaP with 5-aza-dC (2 μ M) led to a significant increase in *Sproutyl1* mRNA expression in the

prostate cancer cell lines. Taken together, these data suggests that promoter methylation maybe a key mechanism in the regulation of Sprouty1 expression in these cell lines.

For Peer Review

DISCUSSION

In the present study, we have cloned and functionally characterized the 5'-flanking region of the human *Sprouty1* gene which is responsible for its transcriptional regulation in prostate cancer cell culture. We 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 *Sprouty1* promoter region (including GATA1, EGR, ZBP, ETS, HIC and FKHD) that confers responsiveness to growth factor signaling.

We have identified a highly conserved nucleotide binding site for the early growth response (EGR1) in the human and mouse *Sprouty1* promoter region which underscore the importance of this motif in the regulation of the *Sprouty1* promoter expression. In the human prostate, there is strong evidence to suggest that EGR1 overexpression is involved in prostate cancer progression [39]. 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* [40]. We 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, we 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. Our 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.

Data from luciferase reporter gene assays from transiently transfected cells demonstrated strong promoter activity of the Sprouty1 gene in the prostate cancer cell lines, however this cell line expressed the least Sprouty1 protein compared when compared to the primary prostatic epithelial cells [18]. Because of the presence of a large CpG island of the Sprouty1 promoter region, we speculated that other mechanism such as DNA methylation may contribute to the silencing of Sprouty1 expression. Transcriptional silencing via methylation of promoter associated CpG islands has been shown to be caused by the direct interference with the binding of transcription factor(s) [41]. The Sprouty1 proximal promoter region contains GC-rich region with a potential binding motif for the SP1 transcription factor and in vitro electrophoretic mobility shift assays studies indicated the direct binding of SP1 oligonucleotide consensus sequence with total nuclear extracts from prostate cancer cells suggesting that binding interaction of SP1 to the sprouty1 promoter may lead to transcriptional silencing. An alternative mechanism for transcriptional silencing is the recruitment of methyl-CpG binding proteins (MeCPs) which inhibits the binding of transcription factors to the promoter regions [42,43]. Our studies indicate that Sprouty1 expression was restored after treatment with the demethylating agent, 5'aza-2'-deoxycytidine. This also implies that transcriptional

silencing via promoter-associated CpG methylation could be mediated by binding of MeCPs to methylated CpG dinucleotide.

CONCLUSION

We have identified functional regions of the human *Sprouty1* gene promoter, which are responsible for constitutive gene expression. We have shown that transcription repression and DNA methylation constitutes key mechanisms for the down-regulation of *sprouty1* expression in prostate cancer. We have observed strong transcriptional activity in the prostate cancer cell lines eventhough *Sprouty1* expression is down-regulated, 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.

ACKNOWLEDGEMENTS

The helpful comments of Dr. S. Belaguli are gratefully acknowledged. This work was supported by grant from the Department of Defense Prostate Cancer Research Program New Investigator Award; PC040326 to Bernard Kwabi-Addo. The Department of Veterans Affairs Merit Review program to Michael Ittmann and the National Cancer Institute to the Baylor prostate cancer SPORE program (P50CA058204).

REFERENCES

1. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr Relat Cancer* 2004;11:709-724.

2. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* 1998;92:253-263.

3. Casci T, Vinos J, Freeman M. Sprouty, an intracellular inhibitor of Ras signaling. *Cell* 1999;96:655-665.

4. Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y. Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. *Development* 1999;126: 2515-2525.

5. Reich A, Sapir A, Shilo B. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* 1999;126:4139-4147.

6. de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S. Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. *Mech Dev* 1999;81:213-216.

7. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N *et al*. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* 1999;126:4465-4475.

8. Hanafusa H, Torii S, Yasunaga T, Matsumoto K, Nishida E. Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. *J Biol Chem* 2004;279: 22992-22995.

9. Gross I, Bassit B, Benezra M, Licht JD. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *J Biol Chem* 2001;276:46460-46468.

10. Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB. The C terminus of sprouty is important for modulation of cellular migration and proliferation. *J Biol Chem* 2001;276: 22742-22747.

11. Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *J Cell Biol* 2001;152:1087-1098.

12. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M *et al*. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat Cell Biol* 2003;5:427-432.

13. Yusoff P, Lao DH, Ong SH, Wong ES, Lim J, Lo TL *et al.* Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. *J Biol Chem* 2002;277:3195-3201.
14. Hanafusa H, Torii S, Yasunaga T, Nishida E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat Cell Biol* 2002;4:850-858.
15. Lee SH, Schloss DJ, Jarvis L, Krasnow MA, Swain JL. Inhibition of angiogenesis by a mouse sprouty protein. *J Biol Chem* 2001;276:4128-4133.
16. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M *et al.* Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat Cell Biol* 2003;5:427-432.
17. Lim J, Wong ES, Ong SH, Yusoff P, Low BC, Guy GR. Sprouty proteins are targeted to membrane ruffles upon growth factor receptor tyrosine kinase activation. Identification of a novel translocation domain. *J Biol Chem* 2000;275:32837-32845.
18. Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G *et al.* The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. *Cancer Res* 2004;64:4728-4735.
19. Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B. Sprouty4, a suppressor of tumor cell motility, is down regulated by DNA methylation in human prostate cancer. *Prostate* 2006;66:613-624.
20. McKie AB, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R *et al.* Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. *Oncogene* 2005;24:2166-2174.
21. Lo TL, Yusoff P, Fong CW, Guo K, McCaw BJ, Phillips WA *et al.* The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer. *Cancer Res* 2004;64:6127-6136.
22. Schiff R, Reddy P, Ahotupa M, Coronado-Heinsohn E, Grim M, Hilsenbeck SG *et al.* Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. *J Natl Cancer Inst* 2000;92:1926-1934.
23. Zimarino V, Wu C. Induction of sequence-specific binding of Drosophila heat shock activator protein without protein synthesis. *Nature* 1987;327:727-730.
24. Rosa AL, Wu YQ, Kwabi-Addo B, Coveler KJ, Reid Sutton V, Shaffer LG. Allele-specific methylation of a functional CTCF binding site upstream of MEG3 in the human imprinted domain of 14q32. *Chromosome Res* 2005;13:809-818.
25. Wang L, Ji C, Wu H, Xu J, Wu Q, Dai J *et al.* Identification and expression analysis of a novel splice variant of human Sprouty1 gene. *Int J Mol Med* 2003;12:783-787.
26. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995;23:4878-4884.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

27. Gong QH, Stern J, Dean A. Transcriptional role of a conserved GATA-1 site in the human epsilon-globin gene promoter. *Mol Cell Biol* 1991;11:2558-2566.

28. Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ. Expression of early growth response genes in human prostate cancer. *Cancer Res* 1998;58:2461-2468.

29. Sander TL, Stringer KF, Maki JL, Szauter P, Stone JR, Collins T. The SCAN domain defines a large family of zinc finger transcription factors. *Gene* 2003;310:29-38.

30. Liu AY, Corey E, Vessella RL, Lange PH, True LD, Huang GM *et al.* Identification of differentially expressed prostate genes: increased expression of transcription factor ETS-2 in prostate cancer. *Prostate* 1997;30:145-153.

31. Yamanaka M, Watanabe M, Yamada Y, Takagi A, Murata T, Takahashi H *et al.* Altered methylation of multiple genes in carcinogenesis of the prostate. *Int J Cancer* 2003;106:382-387.

32. Tasheva ES, Klocke B, Conrad GW. Analysis of transcriptional regulation of the small leucine rich proteoglycans. *Mol Vis* 2004;10:758-772.

33. Gross I, Morrison DJ, Hyink DP, Georgas K, English MA, Mericskay M *et al.* The receptor tyrosine kinase regulator Sprouty1 is a target of the tumor suppressor WT1 and important for kidney development. *J Biol Chem* 2003;278:41420-41430.

34. Rauscher FJ 3rd, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* 1990;250:1259-1262.

35. Nakagama H, Heinrich G, Pelletier J, Housman DE. Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. *Mol Cell Biol* 1995;15:1489-1498.

36. Ding W, Bellusci S, Shi W, Warburton D. Functional analysis of the human Sprouty2 gene promoter. *Gene* 2003;322:175-185.

37. Ding W, Bellusci S, Shi W, Warburton D. Genomic structure and promoter characterization of the human Sprouty4 gene, a novel regulator of lung morphogenesis. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L52-L59.

38. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 1980;20:85-93.

39. Baron V, Duss S, Rhim J, Mercola D. Antisense to the early growth response-1 gene (Egr-1) inhibits prostate tumor development in TRAMP mice. *Ann N Y Acad Sci* 2003;1002:197-216.

40. Albuissou F, Baron V, Belleannee G, Bergeron C, Bretz-Grenier MF, Cartier I *et al.* [False negatives in France: multicenter study from the French Society of Clinical Cytology]. *Ann Pathol* 1999, 19: S84-S87.

41. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 2000;16:168-174.
42. Yu F, Thiesen J, Stratling WH. Histone deacetylase-independent transcriptional repression by methyl-CpG-binding protein 2. *Nucleic Acids Res* 2000;28:2201-2206.
43. Nguyen CT, Gonzales FA, Jones PA. Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: correlation of accessibility, methylation, MeCP2 binding and acetylation. *Nucleic Acids Res* 2001;29:4598-4606.

For Peer Review

FIGURE LEGENDS

Fig. 1. Schematic representation of the *Sprouty1* 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. 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 *Sprouty1a* (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.

Fig. 3. 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 were 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.

Fig. 4. Identification of transcription factors binding to the Sprouty1 promoter. **A.** The analysis includes EGR1, SP1, PBX1 and HNF4. Radiolabelled double-stranded DNA oligonucleotides (probes) were 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. **B.** Chromatin immunoprecipitation assays shows *in vivo* binding of different antibodies to the proximal *Sprouty1b* 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.

Fig. 5. siRNA knock-down of EGR and GATA and Sprouty1 expression in LNCaP cells. **A.** The LNCaP cells were transiently transfected with either EGR1, EGR2, GATA2, or GATA4 siRNA duplexes for 72 hours and total protein extracts used in Western blot analysis. **B.** The Western blot signals were quantified using NucleoVision imaging workstation and calculated as the ratio of Sprouty1 protein to β -actin protein. For each transfection, the Sprouty1 expression level was expressed as a ratio relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; where the Sprouty1 expression in the negative control was set at 1).

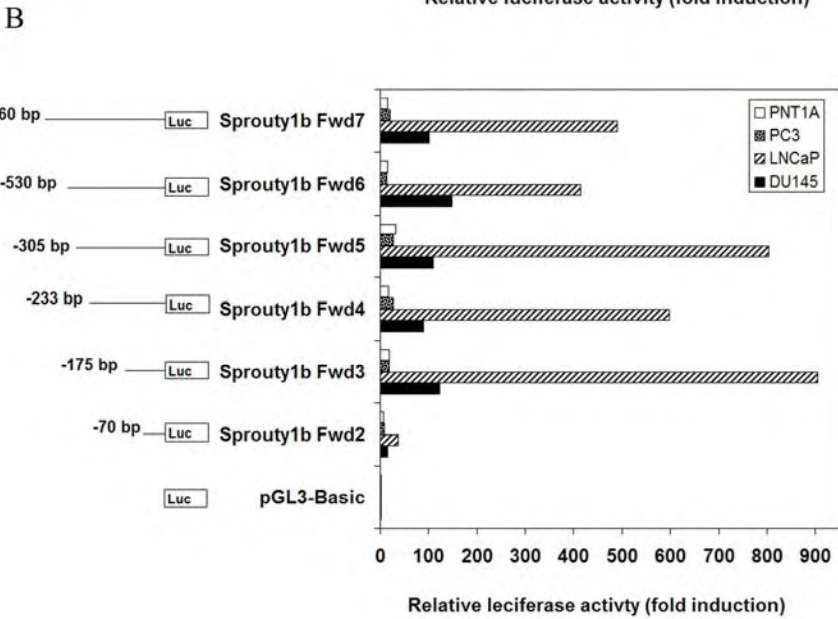
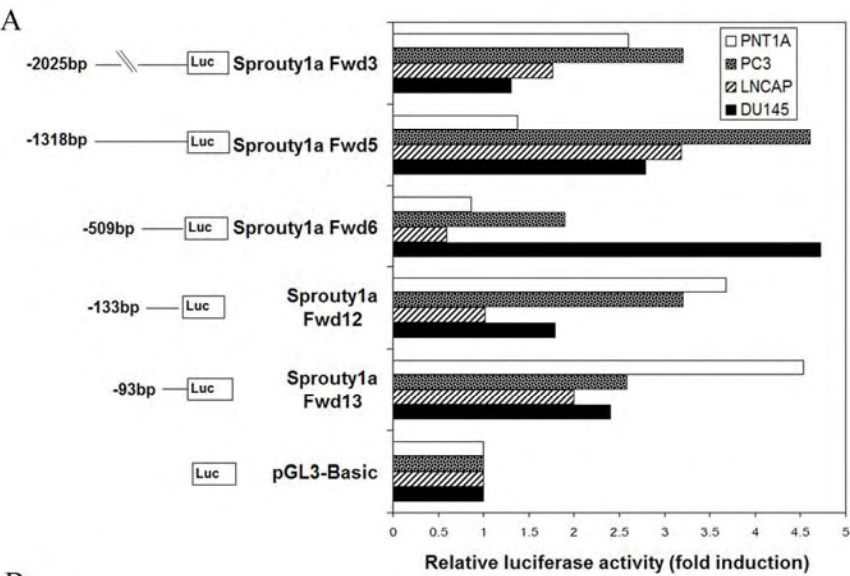
Fig. 6. Methylation analysis of *Sprouty1* gene. **A.** Effect of *in vitro* methylation on the activity of the *Sprouty1* promoter. *Sprouty1a* and *1b* promoters were methylated *in vitro* by SssI methylase. Methylated and unmethylated *Sprouty1a* and *1b* promoters were

transfected into LNCaP cells and assayed for activity. Results are shown as percentages, with luciferase activity due to unmethylated promoter designated as 100%. Results are the mean for 2 independent experiments each of which was performed with triplicate samples. **B.** 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 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.



schematic map of sprouty1 promoter
215x279mm (300 x 300 DPI)

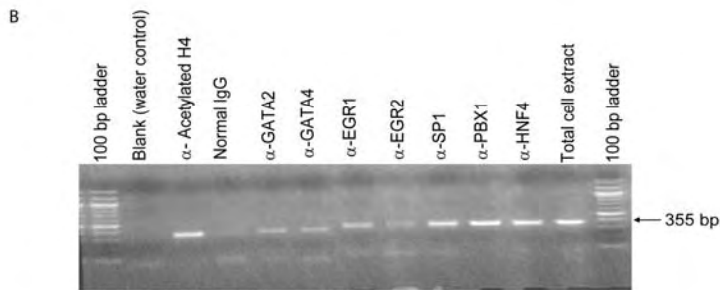
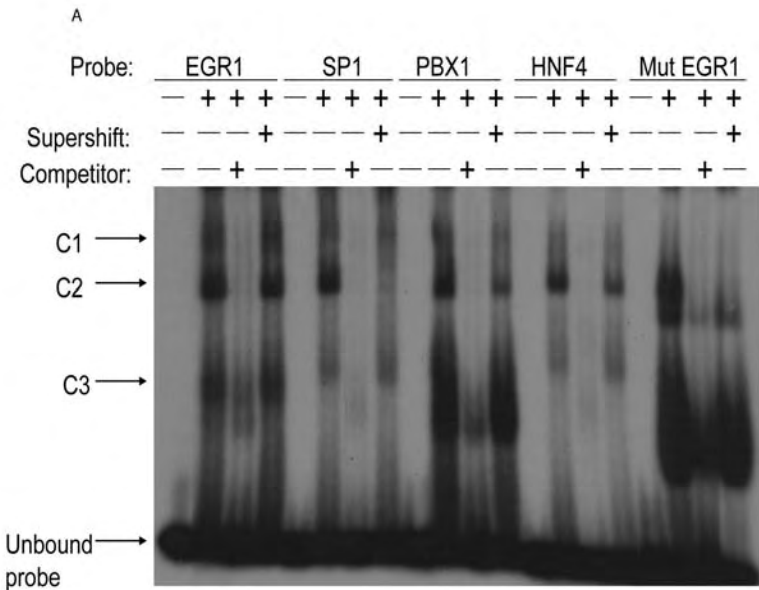


promoter assay
215x279mm (300 x 300 DPI)

Figure 3

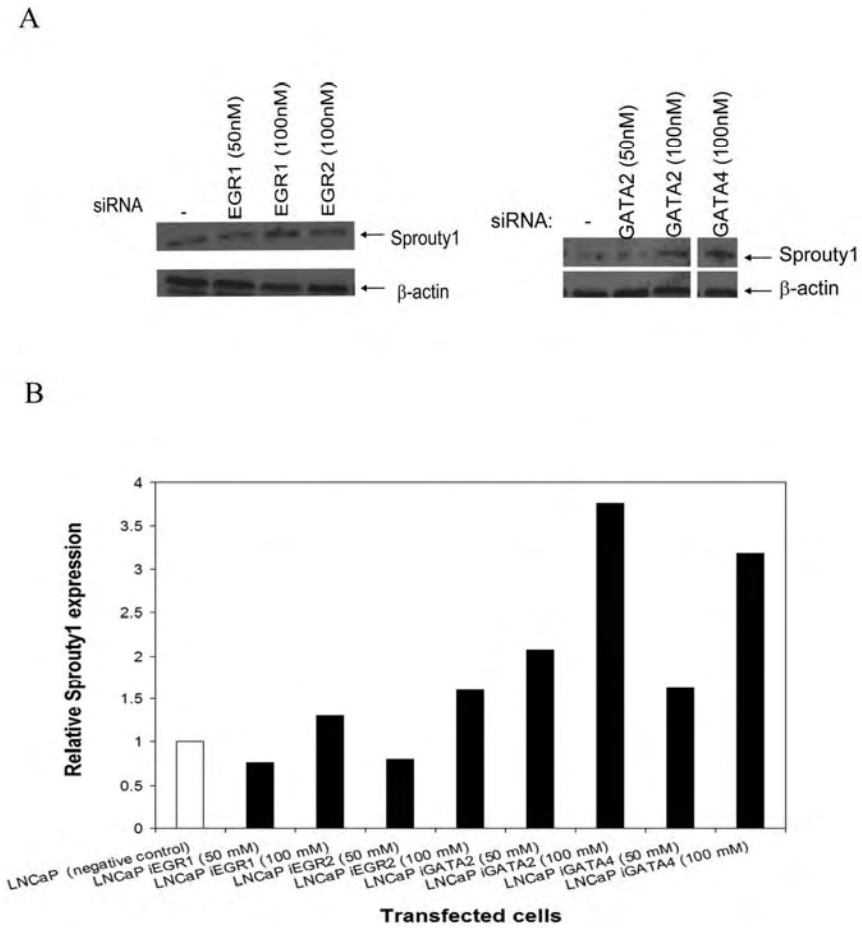


homology sequence search
215x279mm (300 x 300 DPI)



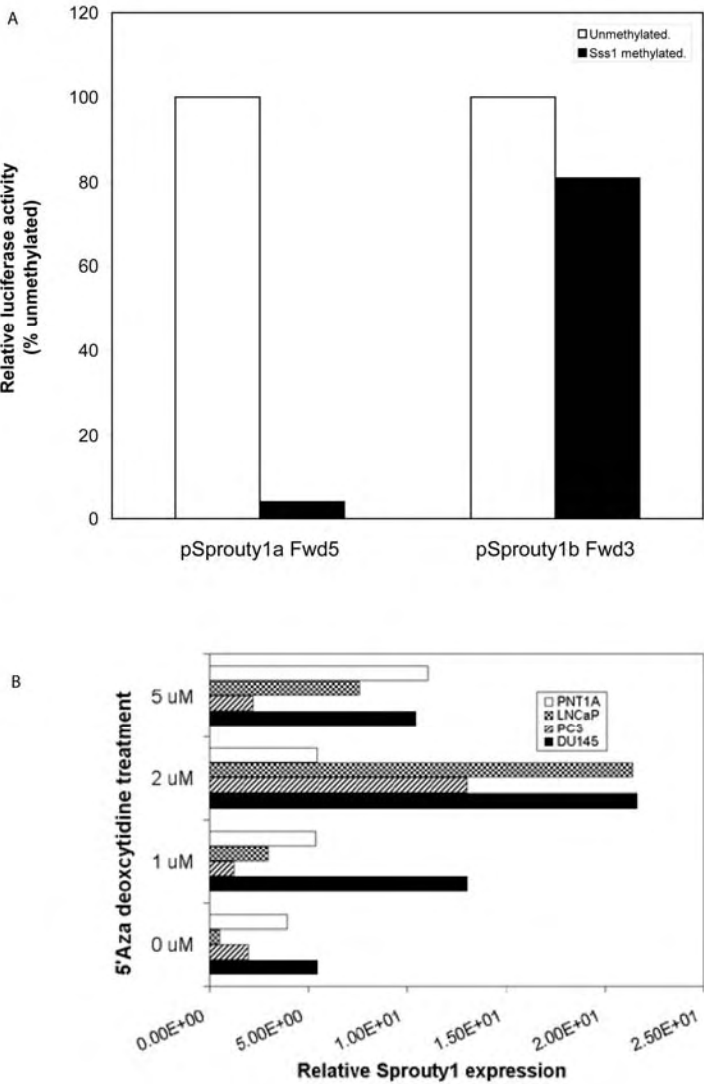
emsa and chip assay
215x279mm (300 x 300 DPI)

Figure 5



gene ko assay
215x279mm (300 x 300 DPI)

Figure 6



methylation analysis
215x279mm (300 x 300 DPI)

AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT				1. CONTRACT ID CODE S		PAGE OF PAGES 1 7	
2. AMENDMENT/MODIFICATION NO. P00001		3. EFFECTIVE DATE 01-Oct-2007		4. REQUISITION/PURCHASE REQ. NO. W23RYX-4206-N651		5. PROJECT NO. (If applicable)	
6. ISSUED BY USA MED RESEARCH ACQ ACTIVITY 820 CHANDLER ST FORT DETRICK MD 21702-5014		CODE W81XWH		7. ADMINISTERED BY (If other than item 6) USA MED RESEARCH ACQ ACTIVITY ATTN: CHRIS SHERMAN 301-619-2375 FORT DETRICK MD 21702		CODE W81XWH	
8. NAME AND ADDRESS OF CONTRACTOR (No., Street, County, State and Zip Code) HOWARD UNIVERSITY STACY-ANN K. WHITE 2400 6TH STREET, NW WASHINGTON DC 20059				9A. AMENDMENT OF SOLICITATION NO.			
				9B. DATED (SEE ITEM 11)			
				X 10A. MOD. OF CONTRACT/ORDER NO. W81XWH-04-1-0878			
				X 10B. DATED (SEE ITEM 13) 15-Sep-2004			
CODE 4B806		FACILITY CODE					
11. THIS ITEM ONLY APPLIES TO AMENDMENTS OF SOLICITATIONS							
☐ The above numbered solicitation is amended as set forth in Item 14. The hour and date specified for receipt of Offer ☐ is extended, ☐ is not extended. Offer must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation or as amended by one of the following methods: (a) By completing Items 8 and 15, and returning _____ copies of the amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKNOWLEDGMENT TO BE RECEIVED AT THE PLACE DESIGNATED FOR THE RECEIPT OF OFFERS PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided each telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.							
12. ACCOUNTING AND APPROPRIATION DATA (If required)							
13. THIS ITEM APPLIES ONLY TO MODIFICATIONS OF CONTRACTS/ORDERS. IT MODIFIES THE CONTRACT/ORDER NO. AS DESCRIBED IN ITEM 14.							
A. THIS CHANGE ORDER IS ISSUED PURSUANT TO: (Specify authority) THE CHANGES SET FORTH IN ITEM 14 ARE MADE IN THE CONTRACT ORDER NO. IN ITEM 10A.							
B. THE ABOVE NUMBERED CONTRACT/ORDER IS MODIFIED TO REFLECT THE ADMINISTRATIVE CHANGES (such as changes in paying office, appropriation date, etc.) SET FORTH IN ITEM 14, PURSUANT TO THE AUTHORITY OF FAR 43.103(B).							
C. THIS SUPPLEMENTAL AGREEMENT IS ENTERED INTO PURSUANT TO AUTHORITY OF:							
X D. OTHER (Specify type of modification and authority) Grant Transfer							
E. IMPORTANT: Contractor ☒ is not, ☐ is required to sign this document and return _____ copies to the issuing office.							
14. DESCRIPTION OF AMENDMENT/MODIFICATION (Organized by UCF section headings, including solicitation/contract subject matter where feasible.) Modification Control Number: aayai076533 Transfer from: Baylor College of Medicine Office of Research One Baylor Plaza Houston, TX 77030 Transfer to: Howard University 2400 6th St NW Washington, DC 20059 See attached pages							
Except as provided herein, all terms and conditions of the document referenced in Item 9A or 10A, as heretofore changed, remains unchanged and in full force and effect.							
15A. NAME AND TITLE OF SIGNER (Type or print)				16A. NAME AND TITLE OF CONTRACTING OFFICER (Type or print) JOSEPH S. LITTLE / CONTRACTING OFFICER TEL: 301-619-2546 EMAIL: joseph.s.little@us.army.mil			
15B. CONTRACTOR/OFFEROR		15C. DATE SIGNED		16B. UNITED STATES OF AMERICA BY <u>Joseph S. Little</u> (Signature of Contracting Officer)		16C. DATE SIGNED 27-Sep-2007	
(Signature of person authorized to sign)							

SECTION SF 30 BLOCK 14 CONTINUATION PAGE

SUMMARY OF CHANGES

SECTION 00010 - SOLICITATION CONTRACT FORM

The contractor organization has changed from
BAYLOR COLLEGE OF MEDICINE
BARBARA COCHRAN
OFFICE OF RESEARCH
ONE BAYLOR PLAZA
HOUSTON TX 77030
to
HOWARD UNIVERSITY
STACY-ANN K. WHITE
2400 6TH STREET, NW
WASHINGTON DC 20059

CLIN 0001

The CLIN extended description has changed from PERIOD OF PERFORMANCE: 20 SEP 2004 - 19 OCT 2007(RESEARCH ENDS 19 SEP 2007) to PERIOD OF PERFORMANCE: 20 SEP 2004 - 19 OCT 2008 (RESEARCH ENDS 19 SEP 2008).

DELIVERIES AND PERFORMANCE

The following Delivery Schedule item for CLIN 0001 has been changed from:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
POP 20-SEP-2004 TO 19-OCT-2007	N/A	USA MED RESEARCH AND MATERIEL COM JUDY PAWLUS COMMANDER USAMRMC ATTN: MCMR-ZC-I BLDG 504XX FORT DETRICK MD 21702 FOB: Destination	FORM9

To:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
POP 20-SEP-2004 TO 19-OCT-2008	N/A	USA MED RESEARCH AND MATERIEL COM JUDY PAWLUS COMMANDER USAMRMC ATTN: MCMR-ZC-I BLDG 504XX FORT DETRICK MD 21702 FOB: Destination	FORM9

SECTION 00800 - SPECIAL CONTRACT REQUIREMENTS

The following have been added by full text:

PI: Bernard Kwabi-Addo, PhD

Title: Mechanisms Down-regulating Sprouty1, a Growth Inhibitor in Prostate Cancer

The following items are applicable to this modification:

GRANT TRANSFER

1. This modification incorporates the May 15, 2007 letter from Baylor College of Medicine (hereafter referred to as the "relinquishing institute"), requesting permission to transfer the assistance agreement by mutual consent from the relinquishing institute to Howard University (hereafter referred to as the "gaining institute") under the original award number cited above. The relinquishing institute hereby relinquishes all future claims under this award. All closeout documents have been submitted on behalf of the relinquishing institute.
2. The total cost to the Government for the full performance of this award is \$338,625.00. The relinquishing institute is entitled to payment of \$297,517.48 for performance under this award.
3. The amount of \$41,107.52 is hereby awarded by the U.S. Army Medical Research Acquisition Activity (USAMRAA) on behalf of the United States of America to the gaining institute.
4. The revised period of performance of this award is:

FROM: September 20, 2004 – September 28, 2007 (Baylor College of Medicine)

TO: October 1, 2007 – October 19, 2008 (Research ends September 19, 2008) (Howard University)

The transfer of this award is effective as of the signature date of this modification.

5. The final report shall encompass the entire period of the project, which covers the performance under both institutions.
6. Additionally, this award is subject to the following USAMRAA Terms and Conditions for Assistance Agreements.

A. This award is made under the authority of 31 U.S.C. 6305 and 10 U.S.C. 2358. The recipient's statement of work dated 09/18/2007 and the budget dated 09/05/2007 are incorporated herein by reference. The Catalog of Federal Domestic Assistance Number relative to this award is CFDA 12.420.

B. ACCEPTANCE OF AWARD: The recipient is not required to countersign this assistance award. In case of disagreement, the recipient shall notify the Grants Officer and not assess the grant any costs until such disagreement(s) is resolved.

C. USAMRAA GENERAL TERMS AND CONDITIONS: This assistance agreement is subject to the USAMRAA General Terms and Conditions and to any special considerations as contained in the below mentioned Section titled "Special Terms and Conditions". These USAMRAA General Terms and Conditions are incorporated

by reference with the same force and effect as if they were given in full text. The full text of the USAMRAA General Terms and Conditions may be accessed electronically at <http://www.usamraa.army.mil/pages/index.cfm>.

D. SPECIAL TERMS AND CONDITIONS

1. RESEARCH TECHNICAL REPORTING REQUIREMENTS (JAN 2007) (USAMRAA)

Format Requirements for Annual/Final Reports

a. Annual reports must provide a complete summary of the research accomplishments to date with respect to the approved Statement of Work. Journal articles can be substituted for detailed descriptions of specific aspects of the research, but the original articles must be attached to the report as an appendix and appropriately referenced in the text. The importance of the report to decisions relating to continued support of the research can not be over-emphasized. An annual report shall be submitted within 30 calendar days of the anniversary date of the award for the preceding 12 month period. If the award period of performance is extended by the Grants Officer, then an annual report must still be submitted within 30 days of the anniversary date of the award. A final report will be due upon completion of the extended performance date that describes the entire research effort.

b. A final report summarizing the entire research effort, citing data in the annual reports and appended publications shall be submitted at the end of the award performance period. The final report will provide a complete reporting of the research findings. Journal publications can be substituted for detailed descriptions of specific aspects of the research, but an original copy of each publication must be attached as an appendix and appropriately referenced in the text. All final reports must include a bibliography of all publications and meeting abstracts and a list of personnel (not salaries) receiving pay from the research effort.

Although there is no page limitation for the reports, each report shall be of sufficient length to provide a thorough description of the accomplishments with respect to the approved Statement of Work. Submission of the report in electronic format (PDF or Word file only), shall be submitted to <https://ers.detrack.army.mil>.

All reports shall have the following elements in this order

FRONT COVER: Sample front cover provided at <https://mrmc.detrack.army.mil/rpindex.asp>. The Accession Document (AD) Number should remain blank.

STANDARD FORM 298: Sample SF 298 provided at <https://mrmc.detrack.army.mil/rpindex.asp>. The abstract in Block 13 must state the purpose, scope, major findings and be an up-to-date report of the progress in terms of results and significance. Subject terms are keywords that may have previously assigned to the proposal abstract or are keywords that may be significant to the research. The number of pages shall include all pages that have printed data (including the front cover, SF 298, table of contents, and all appendices). Please count pages carefully to ensure legibility and that there are no missing pages as this delays processing of reports. Page numbers should be typed: please do not hand number pages.

TABLE OF CONTENTS: Sample table of contents provided at <https://mrmc.detrack.army.mil/rpindex.asp>.

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible.

Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; informatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Pages shall be consecutively numbered throughout the report. DO NOT RENUMBER PAGES IN THE APPENDICES.

Mark all pages of the report which contain proprietary or unpublished data that should be protected by the U.S. Government. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the U.S. Army Medical Research and Materiel Command when restricted limitation assigned to a document can be downgraded to Approved for Public Release. DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS.

Manuscripts/Reprints, Abstracts

A copy of manuscripts or subsequent reprints resulting from the research shall be submitted to the USAMRMC. An extended abstract suitable for publication in the proceedings of the applicable research program is required in relation to a DOD meeting planned during the term of this award. The extended abstract shall (1) identify the accomplishments since award and (2) follow instructions to be prepared by the USAMRMC and promulgated at a later date. The extended abstract style will be dependent on the discipline.

2. ADVANCE PAYMENTS AND FULL FUNDING (NOV 2000) (USAMRAA)

a. Payments. Advance payments will be made to the recipient. Questions relative to payment issues involving Defense Finance and Accounting Service shall be directed to Chris Sherman, (301) 619-2375.

b. Electronic Funds Transfer. All advance payments to the recipient will be made by electronic funds transfer (EFT). The recipient shall contact the Defense Finance and Accounting System (DFAS) named on the face page of this award to make arrangements for EFT. Failure to do so may result in nonpayment.

- c. If the recipient fails to perform, the Grants Officer shall notify DFAS in writing to withhold payments.
- d. Revised Advance Payment Schedule to Howard University

<u>Amount</u>	<u>On or About</u>
\$41,107.52	Upon execution of this award check # 807733 from Baylor College of Medicine

e. Financial Reporting Requirements: The recipient shall submit on a quarterly basis a Standard Form 272, Federal Cash Transactions Report (form available on web site <http://www.usamraa.army.mil>). Each report shall be submitted to the U.S. Army Medical Research Acquisition Activity, ATTN: MCMR-AAA-R, 820 Chandler Street, Fort Detrick MD 21702-5014 in accordance with the following schedule:

<u>Period Covered</u>	<u>Due Date</u>
Jan - Mar	15 Apr
Apr - Jun	15 Jul
Jul - Sep	15 Oct
Oct - Dec	15 Jan

f. Interest Bearing Account. Unless exempted by applicable Treasury-State agreements in accordance with the Cash Management Improvement Act (CMIA) (31 U.S.C. 3335), the recipient shall deposit all advance payments in an interest bearing account. Interest over the amount of \$250 per year shall be remitted annually to the Department of Health and Human Services, Payment Management System, P.O. Box 6021, Rockville, MD 20852. A copy of the transmittal letter stating the amount of interest remitted shall be sent to the U.S. Army Medical Research Acquisition Activity, ATTN:MCMR-AAA-R, 820 Chandler Street, Fort Detrick, MD 21702-5014.

3. PROHIBITION OF USE OF LABORATORY ANIMALS (JAN 2007) (USAMRAA)

**** PROHIBITION – READ FURTHER FOR DETAILS ****

Notwithstanding any other provisions contained in this award or incorporated by reference herein, the contractor is expressly forbidden to use or subcontract for the use of laboratory animals in any manner whatsoever without the express written approval of the US Army Medical Research and Materiel Command, Animal Care and Use Office (ACURO). The contractor will receive written approval to begin research under the applicable protocol proposed for this award from the US Army Medical Research and Materiel Command, ACURO, under separate letter. A copy of this approval will be provided to the US Army Medical Research and Acquisition Activity for the official file. Non-compliance with any provision of this clause may result in the termination of the award.

4. PROHIBITION OF HUMAN RESEARCH (JAN 2007) (USAMRAA)

**** PROHIBITION – READ FURTHER FOR DETAILS ****

Research under this award involving the use of human subjects, to include the use of human anatomical substances and/or human data, may not begin until the U.S. Army Medical Research and Materiel Command's Office of Research Protections, Human Research Protections Office (HRPO) approves the protocol. Written approval to begin research or subcontract for the use of human subjects under the applicable protocol proposed for this award will be issued from the US Army Medical Research and Materiel Command, HRPO, under separate letter to the contractor. A copy of this approval will be provided to the US Army Medical Research Acquisition Activity for the official file. Non-compliance with any provision of this clause may result in withholding of funds and or the termination of the award.

5. PROHIBITION OF USE OF HUMAN CADAVERS (JAN 2007) (USAMRAA)

**** PROHIBITION – READ FURTHER FOR DETAILS****

Research under this award using human cadavers may not begin until the U.S. Army Medical Research and Materiel Command's Office of Research Protections, Human Research Protections Office (HRPO) approves the protocol. Written approval to begin research or subcontract for the use of human cadavers under the applicable protocol proposed for this award will be issued from the US Army Medical Research and Materiel Command, HRPO, under separate letter to the contractor. A copy of this approval will be provided to the US Army Medical Research Acquisition Activity for the official file. Non-compliance with any provision of this clause may result in withholding of funds and or the termination of the award.

6. MAXIMUM OBLIGATION (SEP 2006) (USAMRAA)

The maximum obligation for support of the project will not exceed the amount specified in the award, as amended. USAMRAA does not amend assistance agreements to provide additional funds for such purposes as reimbursement for unrecovered indirect costs resulting from the establishment of final negotiated rates or for increases in salaries, fringe benefits and other costs.

The following have been deleted:

USAMRAA- USAMRAA SPECIFIC TERMS
XXXX-0001

JUN 2004

(End of Summary of Changes)