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TITLE: Identification of Sonic Hedgehog-Induced Stromal Factors That Stimulate Prostate Tumor Growth

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14. ABSTRACT We will determine the mechanism by which Shh signaling accelerates prostate tumor growth, identify Shh targets in prostate tumor stroma, and test the effect of individual target genes on tumor growth. The purpose of the report is to evaluate the first year of research. Sonic hedgehog induces tumor growth by a paracrine mechanism using tumor stroma. Shh signaling independent of Shh ligand in tumor stroma accelerates tumor growth. We have identified potential stromal Shh target genes in xenograft tumors and have begun to evaluate the genes in human prostate cancer. Noggin was identified as a Shh-induced paracrine factors that may drive tumor growth by interfering with BMP signaling. Noggin was overexpressed in tumor stroma. Stromal-derived Noggin does not accelerate tumor growth in the presence or absence of Shh. Future research will continue to identify and evaluate Shh-induced paracrine factors that accelerate prostate tumor growth.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	14
Reportable Outcomes.....	15
Conclusions.....	17
References.....	17
Appendices.....	17

INTRODUCTION

Background: Prostate cancer is the most prevalent cancer in men after skin cancer. The advent of PSA testing has led to a surge in the number of prostate cancer cases detected, but most men diagnosed with prostate cancer do not die of prostate cancer. One of the primary goals of prostate research today is to understand what makes some prostate tumors grow, while others remain indolent for decades. Prostate cancer growth is directed by stromal-epithelial interactions within the tumor. A stromal response is required for progression of prostate tumors and a normal stroma can revert the phenotype of a tumor to a less aggressive state. For these reasons, we suspect that alterations in prostate stroma are responsible for growth of aggressive tumors.

Many cancers arise from aberrant activation of developmental signaling pathways. Sonic hedgehog (Shh) directs stromal-epithelial interactions during embryonic prostate development. After budding initiates, Shh localizes to epithelial regions where prostatic buds appear. Shh signaling occurs in mesenchymal cells adjacent to the Shh expressing epithelium. Shh signaling in mesenchyme is presumed to induce epithelial proliferation, but the mechanism is unknown.

The Hedgehog signaling pathway has been implicated in prostate cancer. Shh ligand is expressed in adult prostate and signaling becomes activated in advanced prostate cancer. Shh overexpression increases the growth rate of tumors via an effect on the stroma.

Hypothesis: This proposal will address the hypothesis that Hedgehog regulated stromal target genes stimulate tumor growth.

Specific Aims: We will (1) determine the mechanism by which Shh signaling accelerates tumor growth, (2) identify Shh targets in prostate tumor stroma, and (3) test the effect of individual target genes on tumor growth.

Study Design: We will use our bi-clonal xenograft model to characterize the nature of Shh-induced signaling in LNCaP xenograft tumors in vitro and in vivo. We will determine if Shh-induced accelerated tumor growth is due to the action of a soluble, secreted factor, if the growth acceleration can be achieved by ligand-independent activation of Shh signaling in tumor stroma, and if the signaling components Gli1 and Gli2 are necessary for accelerated tumor growth. Then we will use the list of Shh target genes obtained previously by microarray analysis to identify Shh target genes that are induced in the stroma of LNCaP xenograft tumors, and also

present in human prostate tumors. Finally, the contribution of each of these target genes to Shh-induced tumor growth will be evaluated by gain-of-function and loss-of-function studies using our bi-clonal xenograft model.

Relevance: The strength of this proposal is that it is the first proposed model to identify stromal genes that regulate prostate tumor growth. We have known for decades that prostate tumor growth is dependent upon stromal influences, but we have as yet been unable to identify these influences due to the lack of a suitable model system. The stromal gene products identified in this study will provide a new class of therapeutic targets for preventing or slowing prostate cancer progression.

BODY

Task 1. Determine the mechanism by which Shh signaling accelerates tumor growth. (Months 1-12)

a. Determine whether paracrine effect is mediated by a soluble factor(s) (Months 1-6)

In vitro: LNCaP/LNShh were co-cultured with UGSM-2 cells and pulse labeled with BrdU to examine growth acceleration in LNCaP/LNShh cells. Shh does not accelerate growth of LNShh cells when they are co-cultured with UGSM-2 prostate stromal cells.

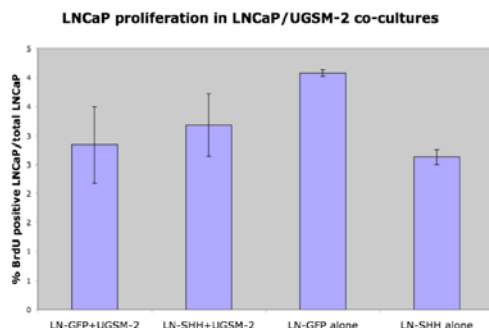


Figure 1. LNCaP/LNShh were plated on top of UGSM-2 monolayers for 48 hours, and then treated with BrdU for 30 minutes. Cells were immunocytochemically labeled for BrdU and analyzed using flow cytometry. The number of BrdU+ cells divided by total number of LNCaP is illustrated. Student's t-test revealed no significant differences.

We have provided many different models for this crucial interaction to occur. We provided different numbers of cells for different periods of time and in different culture medium. We included 3D co-cultures by culturing the cells on or within Matrigel or collagen gels. We analyzed LNCaP proliferation on cultures separated by porous membranes. We assayed LNCaP proliferation using methods other than BrdU incorporation. We analyzed LNCaP proliferation when treated with Shh treated UGSM-2 conditioned media. DHT alters the growth response of LNCaP and we

altered the DHT concentration in our co-cultures. Under all of these conditions, Shh does not induce proliferation of LNCaP cells.

This data is significant because stromal cells respond to Shh, but LNCaP do not have a growth response to stromal products. There are many reasons for this. There may be many paracrine products that alter the growth response of LNCaP and these products may come from several cell types. We may not be able to co-culture LNCaP and UGSM-2 cells long enough to recognize the growth response (cultures: up to 1 week, in vivo: 2-3 months). UGSM-2 cells may not provide the correct or the only paracrine response to Shh to induce LNCaP proliferation. Other stromal cells may contribute to the Shh growth response; i.e. endothelial cells, immune cells.

This model shows that culture conditions do not re-model tumor conditions that are required for Shh tumor growth. Therefore, co-culture model tasks for this research proposal must be limited to using xenograft or other in vivo techniques.

In vivo: We examined LNCaP proliferation in tumors composed of a 1:1 ratio of LN-Shh and parent LNCaP cells. We found that LNCaP proliferate faster than LNShh cells when included in the same tumor.

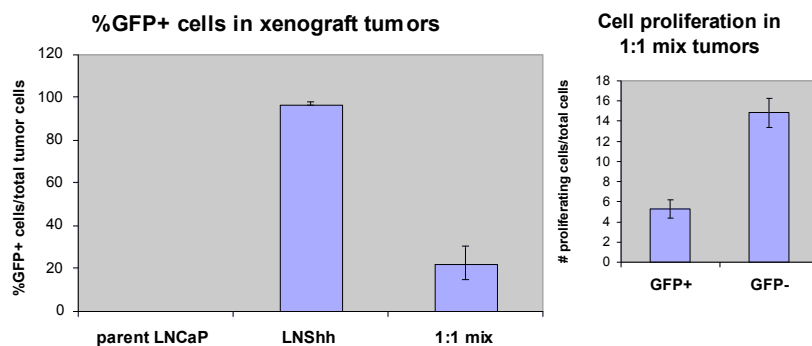


Figure 2. Equal numbers of LNCaP (GFP-) were mixed with LNShh (GFP+) cells and tumors were grown for 8 weeks. Proliferation of LNCaP/LNShh each was evaluated using GFP + Ki67 co-immunohistochemistry.

This identifies that Shh induces growth effects in LNCaP and LNShh cells in the same tumor. There are 2 options for this to occur: (1) LNCaP acquire a non-Shh dependent growth stimulus from LNShh cells (since neither LNShh nor LNCaP achieve Shh signaling in vitro), or (2) a secreted stromal factor must mediate the effect. Data to support option 2 is included in part 1b to conclude that stromal cells provide the Shh growth effect.

b. Demonstrate that growth acceleration can be achieved by stromal cell SmoM2, Gli1 or Gli2 OE. (Months 1-12)

In vitro: will not be done (see 1a, in vitro)

In vivo: We overexpressed SmoM2, Gli1 and Gli2 in UGSM-2 cells. These cells showed increased transcription of Shh-induced gene products Gli1

and Ptc1. In culture, UGSM2-SmoM2 and UGSM2-Gli1 showed normal phenotype, but UGSM2-Gli2 showed an increased intensity to form anchorage independent structures. Anchorage-independence suggests that these cells may be tumorigenic.

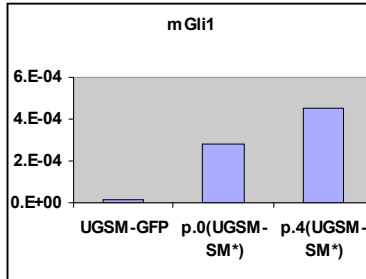
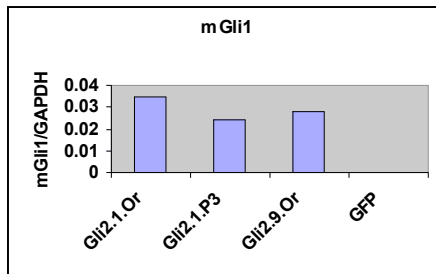
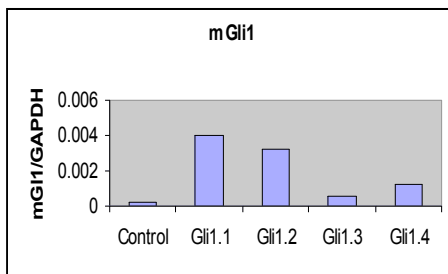


Figure 3. UGSM-2 cells were treated with human SmoM2 (top), Gli1 (middle) or Gli2 (bottom) retrovirus. Shh signaling in different passages of cells was determined by examining mouse Gli1 expression by RT-PCR.



LNCaP were co-injected with UGSM2-WT, UGSM2-Gli1 or UGSM2-Gli2 cells into nude mice and examined tumor growth rates. LNCaP + UGSM2-Gli1 tumors did not show any increased growth rate. LNCaP + UGSM2-Gli2 tumors showed a strong increase in growth rates, but this is attributed to UGSM2-Gli2 cells ability to form sarcomas (stromal-based tumors).

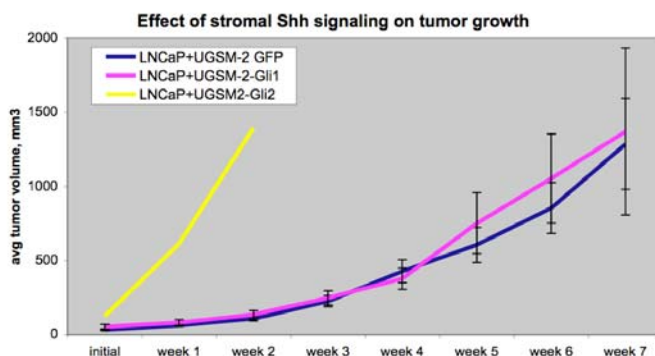


Figure 4. LNCaP were co-injected with UGSM2-SmoM2.Gli1/Gli2 cells. Tumor growth was measured as is illustrated as the average for each tumor type. LNCaP + UGSM2-Gli2 tumors formed rapidly and mice had to be sacrificed early.

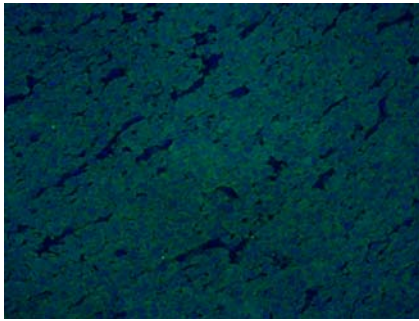


Figure 5. LNCaP + UGSM2-Gli2 tumors were immunohistochemically stained for GFP (green) and counterstained with DAPI (blue) to mark nuclei. UGSM2-Gli2 cells express GFP in addition to Gli2. Tumors that formed are composed of UGSM2-Gli2 stromal cells, and no LNCaP cells (no GFP). Therefore these tumors are sarcomas and not typical LNCaP carcinomas.

Since Gli2 induces UGSM2 cells to become tumorigenic, we cannot determine if UGSM2-Gli2 cells induce LNCaP carcinoma growth. LNCaP tumors take 3-4 weeks to begin forming and UGSM2-Gli2 sarcomas grew to a large size so that all of these mice had to be sacrificed within 2 weeks. Since these methods did not work, we used another methods of increasing Shh signaling in UGSM cells to determine if growth acceleration can be achieved by stromal Shh signaling.

Gli3^{-/-}: Gli3 is a negative regulator of Shh signaling and loss of Gli3 in stromal cells increases Shh signaling independent of Shh ligand. We isolated prostate stromal cells from mice with a genetic defect that produces a null Gli3 allele (Gli3^{xt} mice). These cells were isolated in the same fashion as UGSM-2 cells (see Shaw 2006). UGSM-Gli3^{-/-} cells have an increase in the Shh transcriptional product Gli1 and a further increase if treated with Shh ligand.

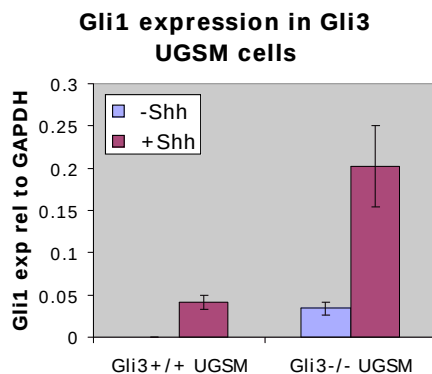


Figure 6. UGSM-Gli3^{+/+} or UGSM-Gli3^{-/-} cells were treated with or without Shh ligand for 48 hrs. We analyzed Gli1 expression by real-time RT-PCR. Gli1 expression and Shh signaling is similar in Gli3^{-/-} cells as to Gli3^{+/+} cells treated with Shh, revealing that Shh signaling in Gli3^{-/-} cells is activated independent of Shh ligand.

We co-injected LNCaP + UGSM-Gli3^{+/+} (WT tumors) or LNCaP + UGSM-Gli3^{-/-} and examined growth rates of tumors. LNCaP + UGSM-Gli3^{-/-} tumors grow at a faster rate than LNCaP + UGSM-Gli3^{+/+} tumors. This indicates that stromal Shh signaling can induce tumor growth rate in the absence of Shh ligand. This supports that Shh tumor growth is due to a paracrine response from tumor stromal cells.

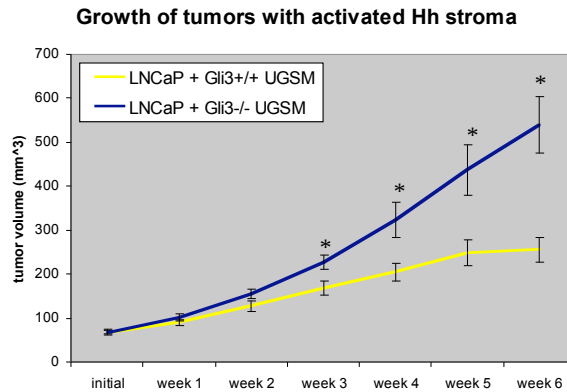


Figure 7. LNCaP were co-injected with UGSM-Gli3+/+ or UGSM-Gli3-/- cells and tumors were measured. Average tumor sizes per week after tumors were noted are shown. Student's t-test revealed significant differences at weeks 3-6. Analysis of slopes of individual tumors also showed a difference in the growth rate of tumors between the two groups.

c. Determine if stromal Gli1 or Gli2 is necessary for Shh-accelerated tumor growth. (Months 1-12)

In vitro: will not be done (see 1a, in vitro)

In vivo: This experiment is not completed.

Task 2. Identify Shh stromal target genes in prostate cancer. (Months 8-24)

a. Validate stromal target genes by cell-based assay. (Months 8-12)

We have previously completed a microarray study to identify Shh target genes in UGSM-2 cells. This task will validate the identified targets by RT-PCR analysis.

Gene	UGSM-2 Cells		
	Array w/ Shh	Validation w/ Shh	w/ Shh & Cyclopamine
Gli1	17.1	621	2.31
Ptc1	8.6	34	0.74
BRAK	27.6	18.4	0.48
Dner	24.9	15.87	0.26
Fgf5	9.1	22.56	1.56
Map3k12	3.97	0.96	1.06
Timp3	3.81	3.26	0.45
Angpt4	3.51	9.44	0.91
Hsd11b1	3.48	12.58	0.48
Tnmd	3.34	2.58	0.62
Artn	2.67	1.45	0.59
Fbn2	2.55	3.08	0.63
Igfbp-3	2.51	3.02	0.24
Ntrk3	2.4	0.9	0.95
Tiam1	2.3	3.16	0.18
Igfbp-6	2.28	2.83	1.31
Sod3	2.26	3.5	0.46
Plxna2	2.2	2.69	0.86
Sos1	2.04	0.9	0.9
Inhbb	1.03	2.08	0.84
Spdy	0.5	1.01	0.39
Rgs4	0.44	0.49	0.6
Fkbp1a	0.41	1.14	0.97
Sufu	0.37	0.97	0.64
Mmp13	0.21	0.28	0.23
Dmp1	0.16	0.4	2.42

Table 1. Shh target genes were analyzed by microarray analysis, RT-PCR validation and inhibited with cyclopamine. We identified 24 targets by array, validated 17 by RT-PCR and 16 of these are inhibited by cyclopamine.

Microarray analysis identified 24 target genes. 17 of these targets were validated using RT-PCR. We furthered this analysis by determining which of these genes is altered when cells are treated with the Shh specific antagonist cyclopamine. 16 of the 17 genes is reversed by cyclopamine treatment, indicating that these targets are specific to Shh.

b. Identify stromal target genes up-regulated by Shh signaling in LNCaP xenograft tumors. (Months 8-16)

The 16 validated target genes from 2a were analyzed in LNShh tumor stroma by species-specific RT-PCR. We also analyzed Hes1, a Shh target gene we have recently identified in prostate stroma.

Gene	LNShh tumors	Gli3-/- cells	Table 2. 17 Shh target genes and 2 canonical genes were analyzed in LNShh tumor stroma and in UGSM-Gli3 cells. Data illustrated shows if genes are changed in LNShh tumors relative to LNCaP tumors (middle column) or are changed in UGSM-Gli3-/- cells relative to UGSM-Gli3+/+ cells in culture (last column).
Gli1	increase	increase	
Ptc1	increase	increase	8 of the 17 target genes are modified in LNShh tumor stroma. Of these genes, 3 are also changed in Gli3-/- cells that accelerate tumor growth. Some of these genes may be identified as paracrine targets that increase tumor growth.
Angpt4	increase	decrease	
Artn	no change	ND	
BRAK	increase	no change	
Dmp1	no change	ND	
Dner	no change	increase	
Fbn2	increase	ND	
Fgf5	increase	no change	
Hes1	increase	increase	
Hsd11b1	increase	decrease	
Igfbp-3	no change	ND	
Igfbp-6	increase	increase	
Ntrk3	no change	ND	
Plxna2	no change	ND	
Sod3	no change	ND	
Tiam1	no change	ND	
Timp3	increase	increase	
Tnmd	no change	ND	

c. Determine whether identified Shh stromal target genes are expressed in human prostate cancers and determine whether expression correlates with level of Shh signaling. (Months 12-24)

We have begun to analyze expression of the target genes from 2b in human prostate cancer and histologically benign tissue from the same patient by RT-PCR. We have analyzed expression in 5 patients and have 18 more patients left to analyze. We have not analyzed enough tissues yet to determine statistical significance. For most samples, target gene expression in the cancer sample is similar to benign issue from the same patient. We are also immunohistochemically determining if stroma in the tissue is considered reactive stroma, since reactive stroma correlates with cancer and may distinguish stroma that secretes the growth promoting target genes. We will correlate target gene expression in cancer vs benign and in reactive vs non-reactive stroma.

Noggin: Before the microarray analysis was completed, I analyzed expression of Noggin, a gene whose expression correlates with Gli1 in

embryonic prostate stroma. Noggin expression is increased in LNShh tumor stroma compared to LNCaP tumor stroma, and Noggin expression correlates with Gli1 in LNShh tumor stroma.

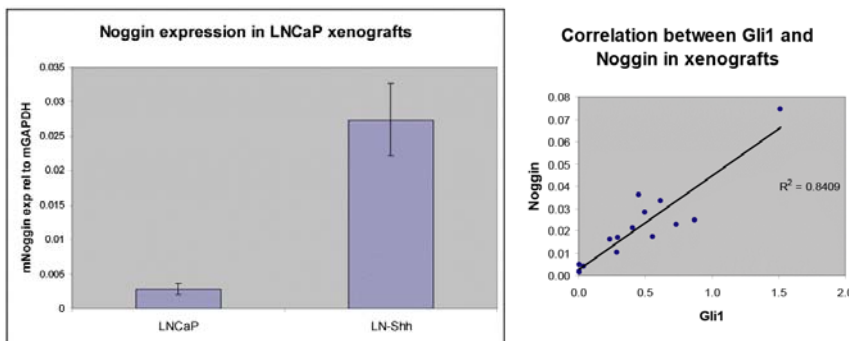


Figure 8. (left) Expression of Noggin mRNA was quantitated by real-time RT-PCR in LNCaP and LNShh tumors. (right) Expression of Noggin correlates with Gli1.

The function of Noggin is to antagonize BMP ligand effects. I examined BMP signaling by measuring the BMP transcriptional target Id-1 in LNCaP and LNShh tumors and found that BMP signaling is reduced in LNShh tumors.

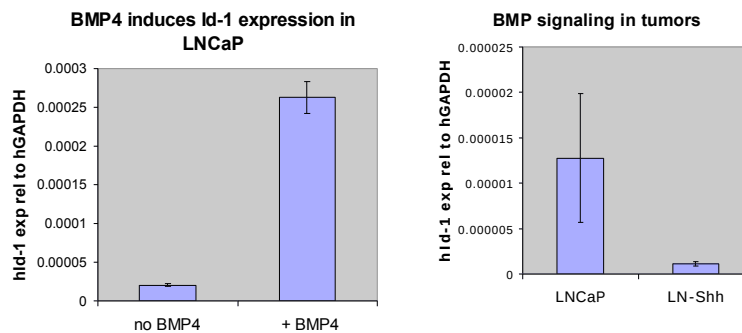


Figure 9. Id-1, a transcriptional product of BMP signaling was analyzed in LNCaP in culture and in tumors. (left) LNCaP were treated with BMP-4 for 48 hours and human Id-1 was measured by RT-PCR. (right) LNCaP and LNShh tumors were analyzed for Id-1 mRNA.

BMP is cited to inhibit proliferation of prostate cell lines and BMP inhibits LNCaP. Noggin addition reverses the inhibitory activity of BMP.

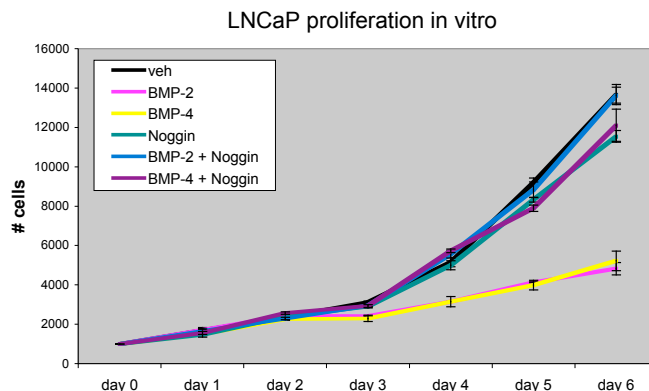


Figure 10. LNCaP in culture were treated with BMP-2, BMP-4, Noggin or combinations of BMP + Noggin. Cell growth was measured using a Coulter counter.

Since Noggin inhibits BMP signaling and BMP signaling inhibits LNCaP proliferation, Shh-induced Noggin may increase LNShh tumor growth.

Noggin is a likely Shh-induced target gene in tumor stroma that may regulate prostate tumor growth.

Task 3. Test the effect of individual stromal target genes on tumor growth. (Months 18-36)

a. Achieve OE of selected stromal target genes in UGSM-2 cells. (Months 18-26)

Noggin cDNA was obtained and cloned into a retroviral vector. Noggin was stably overexpressed in UGSM-2 cells. Flow cytometry was used to isolate cells expressing the Noggin-IRES-GFP genes.

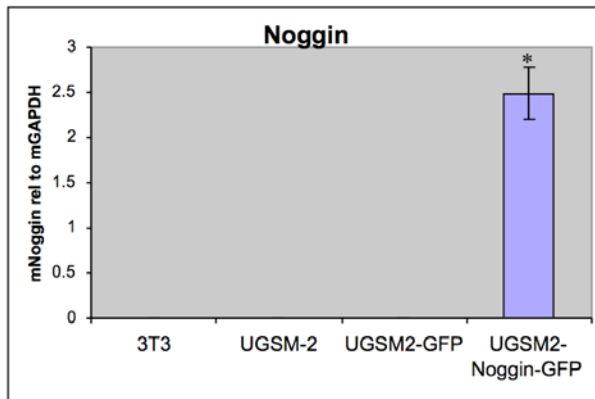


Figure 11. Noggin expression in 4 different cell types by RT-PCR. Noggin expression is increased >1000-fold in UGSM2-Noggin cells.

Conditioned media from UGSM2-Noggin cell line inhibits BMP inhibition of LNCaP growth. This indicates that Noggin is secreted by UGSM2-Noggin cells and is

functional in blocking BMP activity.

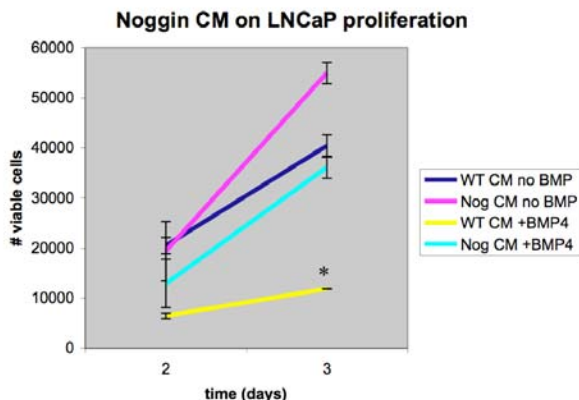


Figure 12. Conditioned media was prepared from UGSM2-Noggin or UGSM2-GFP cells by incubation with medium containing 1% FCS for 48 hours. CM and/or BMP-4 was added to LNCaP cells, and cell growth was monitored daily using a ViCell counter that counts viable cells.

b. Assay effect on tumor cell proliferation. (Months 26-36)

In vitro: will not be done (see 1a, in vitro)

In vivo: LNCaP/LNShh cells were co-injected with UGSM2-Noggin or UGSM2-WT cells. Tumor growth rates were monitored.

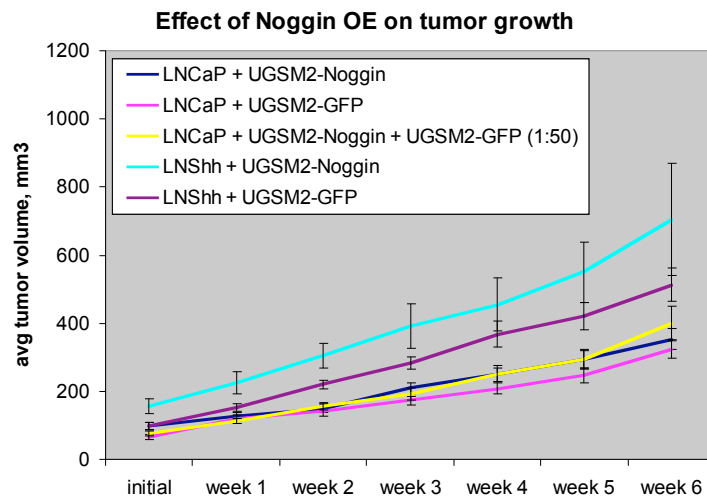


Figure 13. Tumor growth effects of stromal Noggin. LNShh tumors grow faster than LNCaP tumors. Noggin expression does not effect growth rates of tumors, regardless of Shh being expressed.

Noggin overexpression did not alter tumor growth rates in the presence or absence of Shh. UGSM2 cells form a minority of the total

stroma in tumors, so we examined Noggin expression in tumors. The level of Noggin expressed here is 20-fold more than the level seen in LNShh tumor stroma without UGSM2-Noggin cells. Therefore Noggin is upregulated above the level seen in LNShh tumor stroma when UGSM2 stroma is present.

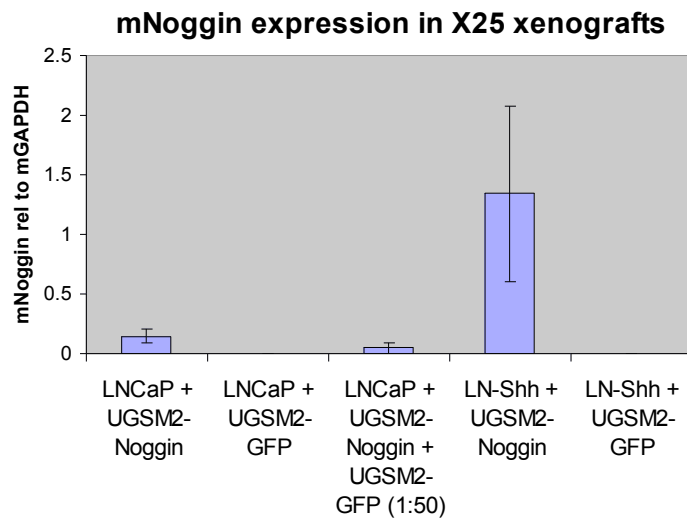


Figure 14. Expression of mouse Noggin in tumors by RT-PCR.

BMP signaling was reduced by Noggin OE in tumors, verifying that Noggin was functional. Despite the loss of BMP signaling in Noggin OE tumors, this did not modify tumor growth rate.

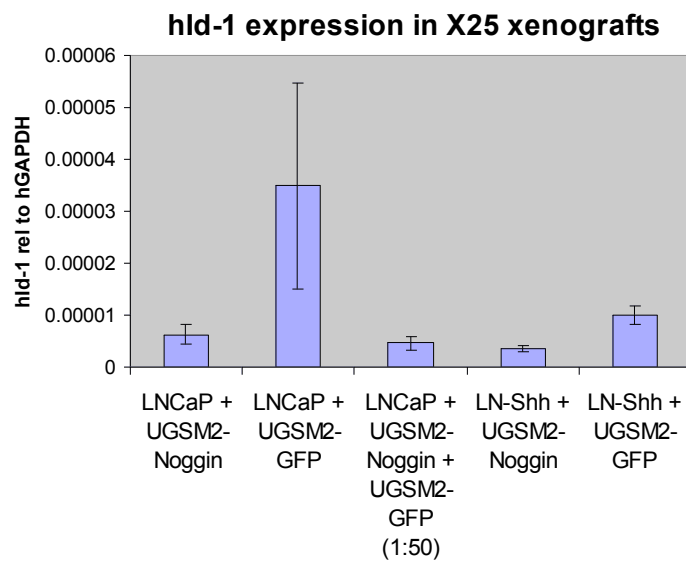


Figure 15. Id-1 expression (BMP signaling) in tumors by RT-PCR.

c. Examine effect of loss-of-function. (Months 18-36)

In vitro: will not be done (see 1a, in vitro)

In vivo: This experiment is not completed. Stable expression of RNAi construct for long enough times to examine effects months later in xenografts is not successful and in vivo toxicity is common (Snove 2006). Also, co-injection of UGSM2 with LNCaP results in a minority of UGSM2 cells amongst stromal cells in the tumor. This is a large complication for loss-of-function (LOF) studies since wild-type stromal cells will compensate the LOF UGSM2 cells. Instead, I have chosen to analyse the requirement for Noggin by deleting the response to Noggin in LNCaP. I have obtained a dominant negative BMP receptor to delete BMP signaling in LNCaP. This will help me to determine if BMP signaling loss (i.e Noggin) is required for Shh tumor growth.

KEY RESEARCH ACCOMPLISHMENTS:

- Shh growth effect is mediated by paracrine signals
- Cannot demonstrate if Shh growth effect is mediated by either soluble or insoluble secreted factors from tumor stromal cells (co-cultures)
- Gli1 or Gli2 overexpression in tumor stroma accelerates does not alter tumor growth in the absence of Shh
- Gli3 loss of expression in tumor stroma accelerates tumor growth in the

absence of Shh. Stromal Shh signaling increases tumor growth.

- Noggin is upregulated in stroma of LN-Shh tumors
- Noggin induces growth of LNCaP by blocking the inhibitory actions of BMP2/4
- Recently completed microarray to identify genes whose expression is altered by Shh treatment of UGSM-2 cells.
- Validated 16 genes that are inhibited by cyclopamine
- 8 target genes are modified in LNShh tumor stroma
- Noggin overexpression in tumor stroma cannot accelerate tumor growth in the absence of Shh
- Noggin overexpression in tumor stroma does not increase tumor growth in addition to Shh
- Noggin overexpression reduces BMP signaling in LNCaP, but does not modify tumor growth rate

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

Manuscripts

Zhang J, Lipinski R, Shaw A, Gipp J, Bushman W (2006) Lack of Demonstrable Autocrine Hedgehog Signaling in Human Prostate Cancer Cell Lines. J Urol, in press.

Shaw A, Bushman W (2006) Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line. The Prostate, in press.

Invited Review

Shaw A, Bushman W (2006) Hedgehog Signaling in the Prostate. J Urol, in press

Abstracts

Shaw A, Bushman W (2006) Effect of Hedgehog Signaling on Tumor Growth is Influenced by Stromal Composition. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

Presentations

American Association for Cancer Research Annual Meeting, April 1-5, 2006 "Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth" Washington, DC

American Association for Cancer Research Annual Meeting, April 1-5, 2006 "Hedgehog Signaling and Androgen Independent Prostate Tumor Growth" Washington, DC

UW Comprehensive Cancer Center Prostate Research Group, February 3, 2006 "Hedgehog and Bone Morphogenetic Protein Signaling in Prostate Cancer" Madison, WI

Licenses

We have been issued licenses for UGSM cell lines through Wisconsin Alumni Research Foundation

Development of cell lines

UGSM-2 cell line and derivatives of the cell lines: UGSM2-SmoM2, UGSM2-Gli1, UGSM2-Gli2, UGSM2-Noggin

UGSM-Gli3^{-/-} and UGSM-Gli3^{+/+} cell lines

Animal Models

I have developed the bi-clonal xenograft model by co-injection of LNCaP cells with UGSM cells. Both components of the bi-clonal model can be genetically modified to analyze tumor-stromal interactions.

CONCLUSION:

This research is the first molecular analysis of a biphasic stromal-epithelial route driving prostate tumor growth. We have established that Sonic hedgehog drives tumor growth by a paracrine mechanism in xenograft tumors. We have isolated several stromal paracrine products that may be involved in inducing tumors to grow.

Clinically, it is important to recognize the molecular pathways that encourage tumors to grow at a faster rate since these tumors have a more aggressive phenotype that often is untreatable by typical anti-androgen therapies. We hope that these studies will diagram new methods to trace aggressive cancers by seeking tumor microenvironment mechanisms that support tumor growth.

Next year's studies will examine tumor growth after Shh loss of function in LNCaP or stromal cells. This will determine if autocrine effects occur in addition to the paracrine effect. This will also determine if Shh tumor growth occurs only in the presence of paracrine signaling. We will analyze the effect of stroma target genes we have identified as Shh targets. We will determine if Shh target genes that are modified in LNShh tumors are present in human prostate tumors and if the presence of a reactive stroma correlates with expression of these target genes. We will examine the necessity of Noggin over-expression in LNShh tumor growth effect by deleting BMP responses in LNCaP. This experiment will determine if loss of BMP signaling is essential for Shh tumor growth effect.

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APPENDICES:

Manuscripts

Zhang J, Lipinski R, Shaw A, Gipp J, Bushman W (2006) Lack of Demonstrable Autocrine Hedgehog Signaling in Human Prostate Cancer Cell Lines. J Urol, Mar 2007; 177: in press.

Shaw A, Bushman W (2006) Hedgehog Signaling in the Prostate. J Urol, Mar 2007; 177: in press.

Shaw A, Bushman W (2006) Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line. The Prostate, 2006 Sep 15;66(13):1347-58.

Abstracts

Shaw A, Bushman W (2006) Effect of Hedgehog Signaling on Tumor Growth is Influenced by Stromal Composition. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Urological Association Annual Meeting in Atlanta, GA

Shaw A, Gipp J, Bushman W (2006) Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

Shaw A, Fan L, Wellner M, Gipp J, Bushman W (2006) Hedgehog Signaling and Androgen Independent Prostate Tumor Growth. American Association for Cancer Research Annual Meeting, Washington, DC

LACK OF DEMONSTRABLE AUTOCRINE HEDGEHOG SIGNALING IN HUMAN PROSTATE CANCER CELL LINES

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Running title: Lack of autocrine Hh signaling in prostate cancer cell lines

Key words: hedgehog pathway, prostate cancer, cell lines, cyclopamine

Abbreviations: Hedgehog; Hh, Patched; Ptc, Sonic Hedgehog; Shh, Indian Hedgehog; Ihh, Smoothened; Smo, Hedgehog-Interacting Protein ; Hip, Suppressor of Fused; SuFu, m; mouse gene, h; human gene

Word count: 3169

ABSTRACT

Introduction: Several recent reports have highlighted the role of Hedgehog (Hh) signaling in prostate cancer. However, the relative contributions of autocrine and paracrine Hh signaling to tumor growth and progression is unclear and efforts to model autocrine signaling for drug development have been hampered by conflicting reports of the presence or absence of autocrine signaling in established human prostate cancer cell lines.

Materials and Methods: We comprehensively characterized the expression of Hh pathway genes in three prostate cancer cell lines (LNCaP, PC3 and 22RV1) and examined their response to Shh ligand and to the Hh pathway inhibitor cyclopamine.

Results: Expression of Hh ligand, Ptc and Gli1 in all three cell lines is lower than the level of expression in normal human prostate tissue. All three cell lines exhibited Hh target gene activation when transfected with an activated form of Gli2, but none showed a detectable transcriptional response to Hh ligand or to transfection with an activated form of Smo. Further, treatment with the Hh pathway inhibitor cyclopamine did not inhibit Hh target gene expression in any of the three prostate cancer cell lines even though cyclopamine did inhibit proliferation in culture.

Conclusions: LNCaP, PC3 and 22RV1 show no evidence of autocrine signaling by ligand dependent mechanisms, and cyclopamine-mediated inhibition of growth in culture occurs without of any discernable effect on canonical Hh pathway activity.

INTRODUCTION

Hedgehog (Hh) signaling is required for normal prostate development¹⁻⁷. The Hh ligands Shh and Ihh are expressed in the epithelium of the urogenital sinus and the tips of the developing ducts. Expression of the Hh target genes Ptc and Gli1 primarily in the adjacent mesenchyme reflects a major component of paracrine signaling from the epithelium to the mesenchyme, but focal expression of Ptc and Gli1 in the epithelium at the tips of the growing ducts has been interpreted as evidence of localized autocrine signaling^{6, 8}.

Several studies have shown active Hh signaling in human prostate cancer and provided evidence that Hh signaling accelerates tumor growth⁹⁻¹². Xenograft studies have shown that paracrine Hh signaling alone can accelerate tumor growth, however, other studies suggest that autocrine signaling may also play a central role. Some studies suggest the operation of ligand-dependent autocrine signaling while others suggest the operation of ligand-independent mechanisms of pathway activation resulting from mutation. The development of pharmacologic inhibitors of Hh signaling for use in treating prostate cancer depends upon further studies to define the relative contribution of autocrine and paracrine signaling in human prostate cancer and development of in vitro models for drug development and testing. Divergent reports on the presence or absence of autocrine signaling in several prostate cancer cell lines have slowed research and development. We report here a comprehensive, mechanistic study of autocrine signaling in commonly used prostate cancer cell lines.

MATERIALS AND METHODS

Cell Lines. Prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the recommended medium. BPH1 cells were a generous gift from Simon Hayward (Vanderbilt University, Nashville, TN) and were grown in RPMI 1640 medium with 5% fetal calf serum (FCS). UGSM-2 cells¹³ and MEFs were isolated

in our laboratory. Four cDNA samples from independent human prostate epithelial cultures were kindly provided by David Jarrard (University of Wisconsin, Madison, WI). Human prostate total RNA and fetal brain total RNA were purchased from BD Biosciences (Palo Alto, CA). Human prostate total RNA was pooled from normal prostates of 32 Caucasian males ages 21-50. Human fetal brain total RNA is from normal fetal brains pooled from 21 spontaneously aborted male/female Caucasian fetuses, ages 26-40 weeks. Cells were plated in a 24-well plate at 1×10^5 cells/well. To assay gene expression after Shh/cyclopamine treatment, serum concentration was reduced to 1% after 1 day attachment, and either 1nM, 10nM octylated N-Shh (Curis, Inc., Cambridge, MA) or 5 μ M cyclopamine (Toronto Research Chemicals, Ontario, Canada) was added to the medium and RNA was harvested after 48 hours. A 1nM concentration of octylated N-Shh equates with a 400nM dose of unmodified N-Shh.

Co-culture. UGSM-2 cells were plated at 1.6×10^5 cells/well in a 12 well plate. After 24 hours, cancer cells were added on top of UGSM-2 cells at the same density. 5 μ M cyclopamine or 1nM octylated N-Shh was added to the medium and RNA was harvested after 24 hours.

Gli-luciferase assay. Shh LIGHTII cells expressing Gli-responsive Firefly luciferase and TK-Renilla were generously provided by Dr. Philip Beachy. Cells were plated in 10% FBS at 90% confluence in Primaria multiwell plates and attached overnight. Media was replaced with 1% FBS +/- Shh peptide at given concentrations. After 48 hrs, Firefly and Renilla luciferase activity was assayed using the Dual Luciferase Assay System (Promega).

Cell proliferation assay. Cells were set in a 24-well plate at a density of 20,000 cells/well and allowed to attach overnight. The concentration of FCS in the media was changed to 2%, and various concentrations of cyclopamine were added. Cells were grown for 4 days, harvested for RNA or trypsinized and counted by Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA).

Adenovirus infection. Adenovirus constructs carrying Δ NmGli2-GFP, hSmo*-GFP or GFP alone¹⁴ were kindly provided by Chen-Ming Fan (Carnegie Inst, Baltimore, MD). Cells were plated in a 24-well plate at 1×10^5 cells/well. After 24 hours attachment, media was replaced with 1% FCS +/- adenovirus at a multiplicity of infection of 25-100 PFU/cell +/- Shh peptide. Under these conditions, >90% of cells were infected according to GFP fluorescence analysis by flow cytometry.

RNA isolation and real time RT-PCR. RNA was isolated using Qiagen (Valencia, CA) RNeasy RNA isolation Kits and subjected to on-column DNase digestion. cDNA was generated following standard protocols. Gene expression was assayed by real time RT-PCR on BioRad iCycler instrument (Hercules, CA) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard gene. Primer sequences used in this experiment are listed in Table I.

Statistical analysis. Each experiment was repeated 3 times independently. An unpaired *t*-test was used to determine if statistically significant differences exist between treatment groups.

RESULTS

Hh pathway activity in prostate cancer cell lines. Comparison of Hh ligand expression in four prostate cancer cell lines showed that ligand expression was highest in PC3 and lowest in LNCaP (Figure 1a). Shh and Ihh expression in PC3 was of the same order of magnitude as in the fetal brain, but well below what is found in the normal adult prostate (Figure 1b). Four primary epithelial cell lines isolated from human benign prostate tissue as well as BPH1 immortalized prostate epithelial cells exhibited expression that is intermediate between LNCaP and PC3 (Figure 1c). Ptc and Gli1 are primary targets of Hh transcriptional activation. Ptc expression is highest in LNCaP and 22RV1, intermediate in PC3 and lowest in DU145 cells (Figure 2a). Gli1 expression was similar in all cell lines (Figure 2a). Ptc and Gli1 expression in these cell lines was generally comparable to expression in the four primary epithelial cell lines and BPH-1, but much lower than normal prostate tissue (Figure 2b). These studies reveal that the level of Hh ligand expression in all four cell lines is lower than that observed in pooled normal prostate specimens. Further, pathway activity in the four cell lines, as judged by Ptc and Gli1 expression, is also considerably lower than that observed in the pooled normal prostate specimens. Together these data do not suggest elevated Hh pathway activity in these cell lines.

We noted that Ptc and Gli1 expression in the cell lines does not track the level of endogenous Hh ligand expression, suggesting that target gene expression may not be linked to ligand-dependent pathway activation. We therefore examined responsiveness of the tumor cell lines to exogenous Hh ligand. Using 1nM and 10nM concentrations of octylated Shh peptide which elicit 75% and 100% of maximal induction of Gli-luciferase reporter activity in NIH 3T3 cells, respectively (Figure 3a), we observed no detectable increase in the expression of either Ptc or Gli1 in any of the tumor cell lines tested (Figure 3b and not shown). Since serum levels are known to affect Hh responsiveness in vitro (unpublished observation), we treated cells with 1nM Shh under a range of serum conditions. 1nM Shh was unable to induce expression of either Ptc or Gli1 under 10%, 1% or 0.1% FCS conditions (Figure 3c). To verify activity of Shh in the same assays, we treated a urogenital sinus mesenchyme cell line, UGSM2, in medium containing 1% FCS with 1nM Shh (Figure 3b, 3c insert). These observations are consistent with our previous observation that LNCaP stably overexpressing Shh (LN-Shh) exhibited no evidence of pathway activation⁹.

Intracellular Hh signaling in PCa cell lines. Each prostate cancer cell line expresses mRNA for the major components of the Hh signal transduction pathway (Figure 4a) although the relative abundance of each factor shows considerable variation (Figure 4b). Lack of responsiveness to Shh ligand could result from 3 different mechanisms: a block in ligand binding and transmembrane signal transduction, a defect in the intracellular signal transduction mechanism or a specific block in the transcription of Ptc and Gli1 in response to Hh pathway activation. To distinguish between these, we transiently expressed activated forms of Smo and Gli2 that have been shown to activate expression of Hh target genes in many cell types^{9, 15, 16}. The activated form of hSmo (Smo*) activates the intracellular signal transduction pathway and indirectly activates target gene transcription, whereas, the activated form of mGli2 (Δ NmGli2) is considered a direct transcriptional activator of Hh target genes. Expression of Smo* in PC3 and 22RV1 cells did not induce expression of Ptc and Gli1 in either cell line, whereas it induced robust Ptc and Gli1 expression in both mouse embryonic fibroblasts (MEFs) and UGSM-2 cells (Figure 5a insert and not shown). In contrast, expression of Δ NmGli2 induced Hh target gene expression in both cell lines. It induced robust expression of both Ptc and Gli1 in 22RV1, and induced robust expression of Gli1 but not Ptc in PC3 (Figure 5b). The simplest explanation for

the increase in Gli1 but not Ptc expression in PC3 cells is that Gli1 is a more sensitive marker of induction because of its lower basal level of expression. These studies suggest that the failure of PC3 and 22RV1 to respond to Hh ligand with induction of Ptc and Gli1 results from a defect in the intracellular signal transduction mechanism in these cell lines.

Effect of cyclopamine on Hh signaling. The plant steroidal alkaloid cyclopamine inhibits Hh signaling by preventing activation of Smo¹⁷. To examine endogenous, Smo-dependent Hh signaling in the cancer cells, we examined the ability of 5 μ M cyclopamine to block transcription of Hh target genes Ptc and Gli1 in the cell lines. Regardless of whether the assay was performed in 10%, 1% or 0.1% FCS, we observed no significant effect on Ptc or Gli1 expression in any prostate cancer cell line (Figure 6). We also observed no effect of cyclopamine when the assay was performed in the presence of 1nM exogenous Shh peptide (data not shown). In contrast, 5 μ M cyclopamine completely blocked Hh pathway activity in UGSM-2 cells stimulated with 1 nM Shh (Figure 6 insert). These findings, which demonstrate a lack of effect of the Smo antagonist cyclopamine, complement the lack of target gene activation by transfection with Smo* and further suggest the absence of Smo-dependent autocrine signaling.

Effect of cyclopamine on tumor cell proliferation. Hh pathway activity has been implicated as a stimulus of prostate cancer cell proliferation, and inhibition of tumor cell proliferation in vitro by cyclopamine has been attributed to specific inhibition of the Hh pathway¹⁰⁻¹². We examined the effect of cyclopamine on growth of cancer cell lines in culture and correlated effects on proliferation with expression of the Hh target genes Ptc and Gli1. Treatment with 5 μ M cyclopamine resulted in a decreased number of LNCaP cells after four days in culture, a slight decrease in the number of 22RV1 cells and no change in the number of PC3 cells (Figure 7). Treatment with 10 μ M cyclopamine significantly reduced the number of cells after four days in all three tumor cell lines, but this effect did not correlate with a significant inhibition of Hh pathway activity as measured by Ptc and Gli1 expression (Figure 7 insert). These observations suggest that the inhibition of tumor cell proliferation in vitro by cyclopamine does not result from a specific effect on Hh pathway activity.

Cyclopamine has been reported to inhibit growth of PC3 tumor xenografts¹⁰. This has been attributed to chemical inhibition of autocrine signaling in the xenograft, however, our studies do not demonstrate significant autocrine signaling in this cell line. To examine the possibility that cyclopamine might interfere with tumor growth by inhibiting Hh pathway activity in the tumor stroma, we examined the effect of cyclopamine on PC3 tumor cells grown in co-culture with UGSM-2 stromal cells. LNCaP cells over-expressing Shh⁹ were similarly co-cultured with UGSM-2 cells as a positive control for robust paracrine Hh pathway activation. Expression of the conserved Hh target genes Ptc and Gli1 was measured in human cancer cells and mouse stromal cells by real time RT-PCR using species-specific primers. Cyclopamine had no effect on hPtc and hGli1 transcription in the cancer cells themselves (Figure 8 top). In contrast, cyclopamine dramatically reduced mPtc and mGli1 transcription in UGSM-2 cells co-cultured with either PC3 or LN-Shh cells (Figure 8 bottom).

DISCUSSION

Our previous studies of Hh signaling in normal and neoplastic human prostate demonstrated comparable levels of expression of Hh ligand and Gli1 in specimens of benign and localized

prostate cancer, with a suggestion of higher level expression in locally advanced and/or androgen independent prostate cancer. We demonstrated expression of Shh in the tumor epithelium with localization of Gli1 predominantly in the peri-glandular tumor stroma, and used the LNCaP xenograft to show that paracrine Shh signaling accelerates tumor growth¹¹. Recently, we have shown that the paracrine effect of Shh signaling on tumor growth can be influenced by the composition of the tumor stroma (unpublished observations) and we therefore speculate that Hh signaling may exert different growth effects in the normal prostate and in prostate cancer depending on the composition and/or reactivity of the stromal compartment. Several other studies examining the expression of Shh in localized and metastatic prostate cancer suggested that increased Shh expression in localized tumors exerts a combination of autocrine and paracrine signaling activity, and dramatically increases pathway activity in metastatic disease¹⁰⁻¹². The possible contribution of autocrine signaling to tumor growth was examined by studying the effect of cyclopamine, anti-Shh antibody and Gli1 transfection on the proliferation of several human prostate cancer cell lines including LNCaP, PC3 and 22RV1¹⁰⁻¹² (Also see Shaw and Bushman, this issue, for review). The studies suggested that these cell lines were characterized by high levels of Hh pathway activity, that cyclopamine could inhibit tumor cell proliferation in culture by a Hh specific mechanism and that cyclopamine could exert a dose dependent inhibition of xenograft tumor growth. These studies clearly suggested that autocrine pathway activity promotes tumor cell proliferation and treatment with Hh inhibitors might be a promising avenue for treatment. However, the results reported in these papers are not entirely consistent. For example, Karhadkar et al¹⁰ found that anti-Shh blocking antibody inhibited PC3 proliferation, whereas Sanchez et al¹¹ found that PC3 proliferation was unaffected by either anti-Shh blocking antibody or exogenous Shh. Moreover, they conflicted with our previous studies showing an absence of Hh pathway responsiveness in LNCaP⁹. For this reason, we undertook a comprehensive analysis of autocrine Hh pathway signaling in these cell lines.

Our studies show that LNCaP, DU145, PC3 and 22RV1 all express Hh ligands and other components of Hh signal transduction. The level of ligand expression varies, with the highest level of mRNA expression present in PC3 and being comparable to the robust level of expression observed in the fetal brain. Even so, this is below the level of expression in a pooled normal prostate sample composed of 32 prostate specimens from men 21-50 years of age. The fact that expression is lower in all prostate cancer cell lines examined and in four primary prostate epithelial cell lines than in the normal prostate is intriguing and might suggest that in vitro culture conditions reduce Hh ligand expression. Similarly, the expression of Ptc and Gli1 in these cell lines is much lower than in the normal prostate and might reflect a loss of autocrine signaling in vitro or signify that the primary domain of Ptc and Gli1 expression in the intact prostate is in the glandular stroma.

Since the tumor cell lines express the Hh ligands Shh and Ihh, pathway activity could result from ligand-dependent autocrine pathway activation. However, our studies of LNCaP, PC3 and 22RV1 found no evidence for a transcriptional response to exogenous Hh ligand. While the lack of response of LNCaP was consistent with our previous studies¹¹, the unresponsiveness of PC3 and 22RV1 was unexpected and contradictory to previously reported studies. To validate these observations, we examined the effect of intracellular pathway activation in PC3 and 22RV1 cells. Infection with an adenoviral vector expressing activated Smo did not induce Ptc or Gli1 transcription in either cell line. This observation argues that the canonical Smo-mediated signal

transduction pathway is non-functional. This was confirmed by showing that transcriptional activation of Hh target genes Ptc and Gli1 could be achieved in these cells by infection with an adenoviral vector expressing an activated form of Gli2 (Δ NmGli2). These studies, which demonstrate a non-functional post-receptor signal transduction pathway in both PC3 and LNCaP, are consistent with a lack of responsiveness to Hh ligand.

Cyclopamine inhibits Hh signaling by binding to and preventing the activation by Smo¹⁷. We observed no changes in the expression of Hh target genes Ptc and Gli1 in LNCaP, PC3 or 22RV1 treated with 5 μ M cyclopamine under a range of culture conditions, a finding consistent with our transfection studies demonstrating a failure to induce Smo-mediated Hh pathway activation. These observations stand in contrast to the studies of Karhadkar et al¹⁰. However, those authors examined the effect of cyclopamine on expression of a Gli-reporter construct, rather than expression of endogenous Ptc and Gli1. It is possible that they observed an effect of cyclopamine on reporter gene expression that does not accurately reflect the effect of cyclopamine on the expression of endogenous target genes.

We observed that treatment of cells in culture with 10 μ M cyclopamine decreased cell number without any discernable effect on Hh pathway activity. These findings strongly suggest that inhibition of cell proliferation is not the result of canonical Smo-mediated Hh pathway inhibition but rather a non-specific or toxic effect. But, how can we reconcile these observations with previously published studies showing a dramatic effect of cyclopamine on PC3 and 22RV1 xenograft tumors? One explanation is that PC3 and 22RV1 cells growing in vivo exhibit a different phenotype and are susceptible to cyclopamine-mediated inhibition of canonical pathway activity. Another is that the effect of cyclopamine on xenograft tumor growth is mediated through an effect on stromal cells responding to Hh ligand produced by the tumor cells. This putative mechanism is supported by our co-culture studies and suggests that the effect of Hh inhibitors on tumor growth may include effects on paracrine as well as autocrine pathway activity.

Efforts are currently underway to develop Hh pathway inhibitors for clinical use. A critical step in this process is the development and use of appropriate cell lines and/or tumor models that are dependent on Hh signaling for growth. It has been assumed, based on previously published studies, that human prostate cancer and commonly used prostate cancer cell lines both exhibit robust autocrine signaling. However, the experiments reported here reveal no evidence for autocrine Hh signaling in the most commonly used human prostate cancer cell lines under standard culture conditions and found no evidence that the Hh inhibitor cyclopamine could inhibit cell proliferation by a specific effect on Hh pathway activity. These findings caution against using these cell lines as an in vitro model of autocrine Hh signaling in prostate cancer. It is possible that the xenografts made with PC3 and 22RV1 might exhibit autocrine signaling that cannot be modeled in cell culture, but it is also likely that xenografts made with these Hh-expressing cell lines also involve paracrine signaling interactions. Therefore, investigators testing the effect of Hh pathway inhibitors on prostate tumor xenografts should evaluate the effects of these agents on paracrine signaling as well as autocrine pathway activity.

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Table I. Sequences of quantitative real time RT-PCR Primers

Gene	Forward Primer	Reverse Primer
mGAPDH	AGCCTCGTCCCGTAGACAAAAT	CCGTGAGTGGAGTCATACTGGA
mPatched	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
hGAPDH	CCACATCGCTCAGACACCAT	GCAACAATATCCACTTACCAGAGTTAA
hPTCH	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA
hGLI1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hGLI2	AGCCAGGAGGGCTACCAC	CTAGGCCAAAGCCTGCTGTA
hGLI3	ATCATTCAGAACCTTTCCCATAGC	TAGGGAGGTCAGCAAAGAACTCAT
hSHH	AAGGACAAGTTGAACGCTTTGG	TCGGTCACCCGCAGTTTC
hIHH	CACCCCAATTACAATCCAG	AGATAGCCAGCGAGTTCAGG
hSmoothened	ACCTATGCCTGGCACACTTC	GTGAGGACAAAGGGGAGTGA
hHIP	CATGTCGTCATGGAGGTGTC	TCACTCTGCGGATGTTTCTG
hFused	GAGGGTGTACAAGGGTCGAA	TGCAAATTCCTCAGCTCCTT
hSufu	CGGAGGGGAGAGACCATATT	CACTTGGCACTGACACCACT

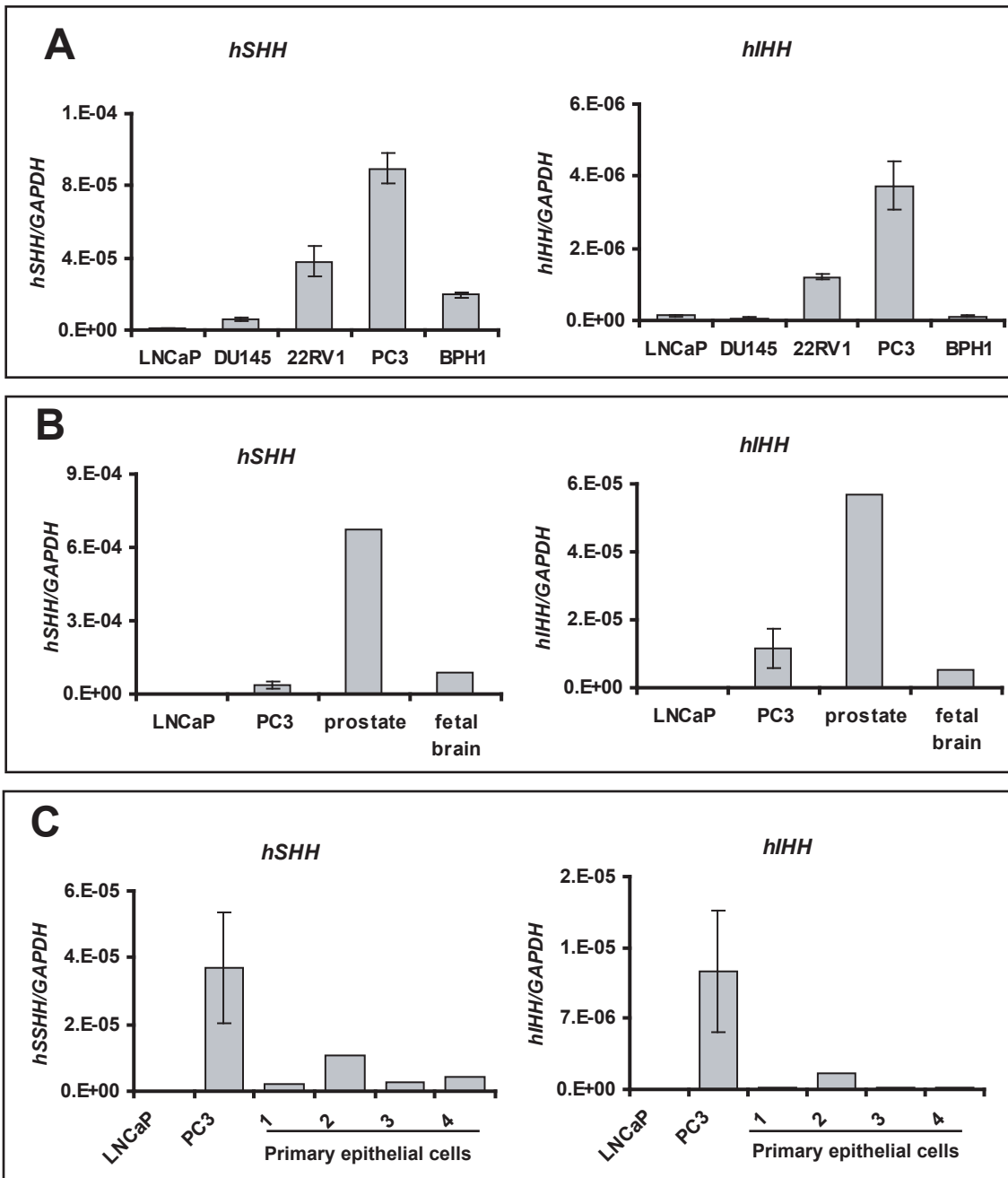


Figure 1 (A) Shh and Ihh expression in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the normal human BPH-1 cell line. (B) Comparison of expression in LNCaP and PC3 with expression in the human fetal brain and a pooled sample of normal adult prostate RNA. (C) Comparison of expression in LNCaP and PC3 with expression in four primary benign prostate epithelial cell lines.

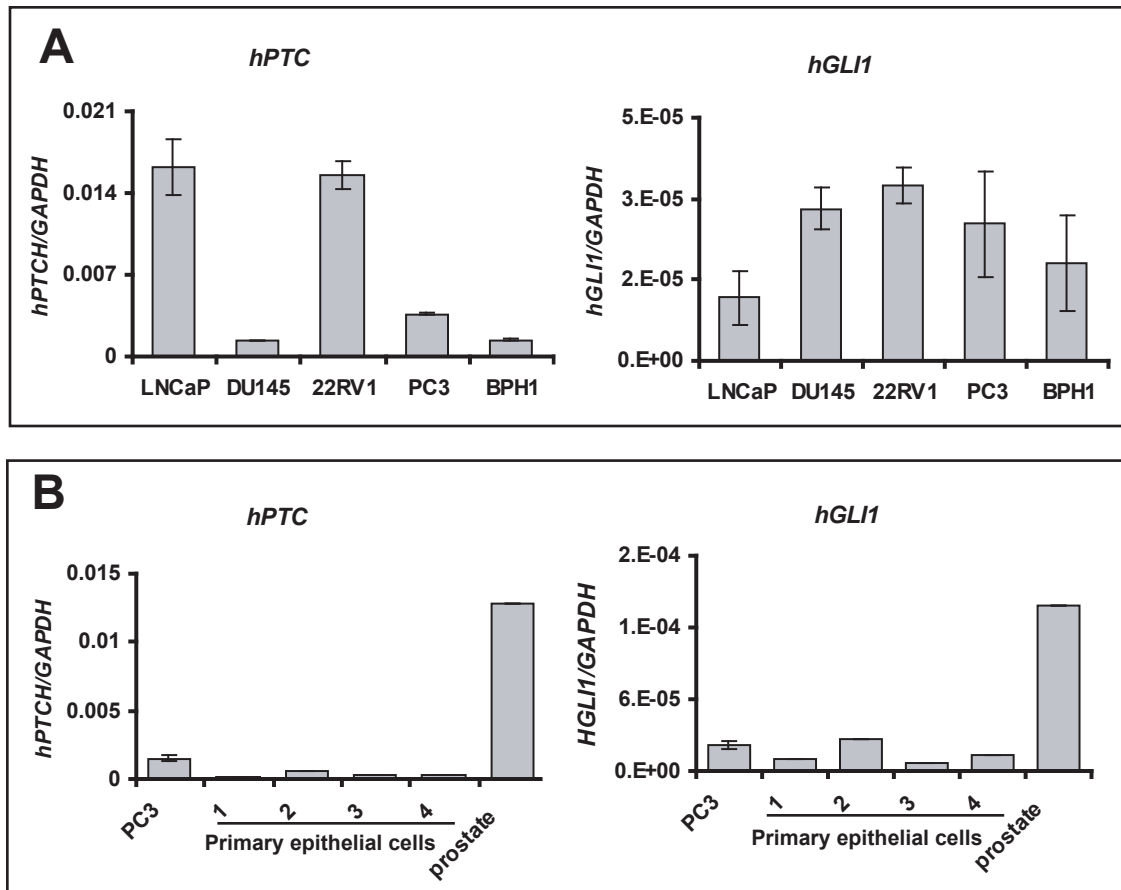


Figure 2 (A) Expression of the conserved Hh target genes *Ptc* and *Gli1* in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the normal human BPH-1 cell line. (B) Comparison of *Ptc* and *Gli1* expression in PC3 and four primary benign prostate epithelial cell lines.

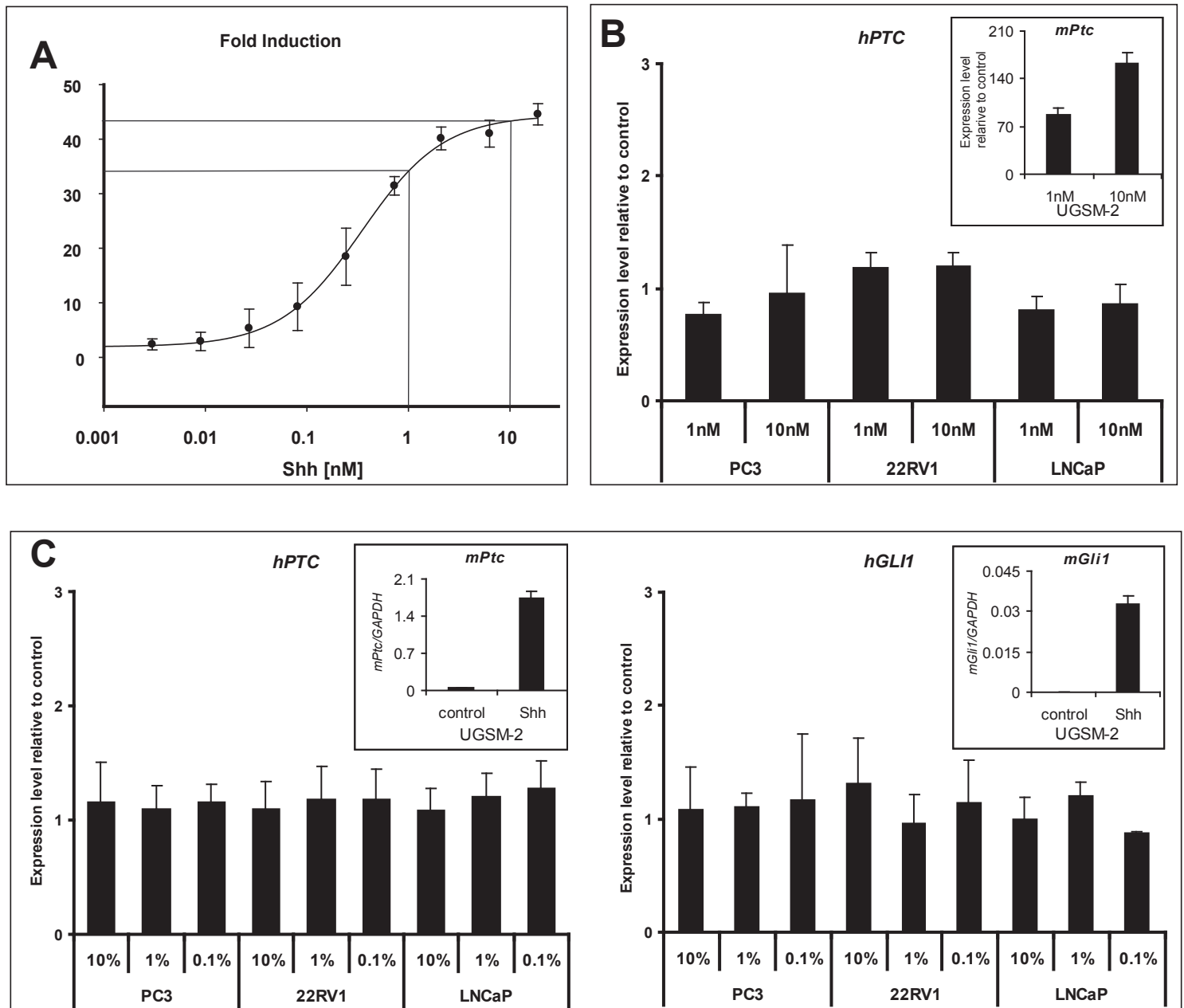


Figure 3 (A) Dose-response curve for Shh responsive Gli-luciferase reporter activity in NIH 3T3 cells. 1nM = EC75, 10nM = EC100. (B) Treatment of PC3, 22RV1 or LNCaP with 1nM or 10nM Shh does not increase *Ptc* expression. (C) Serum concentration does not alter Shh response of PC3, 22RV1 or LNCaP. However, 1 nM Shh is sufficient to significantly induce *Ptc1* and *Gli1* in UGSM-2 cells (1% FCS), $p < 0.005$ (insert).

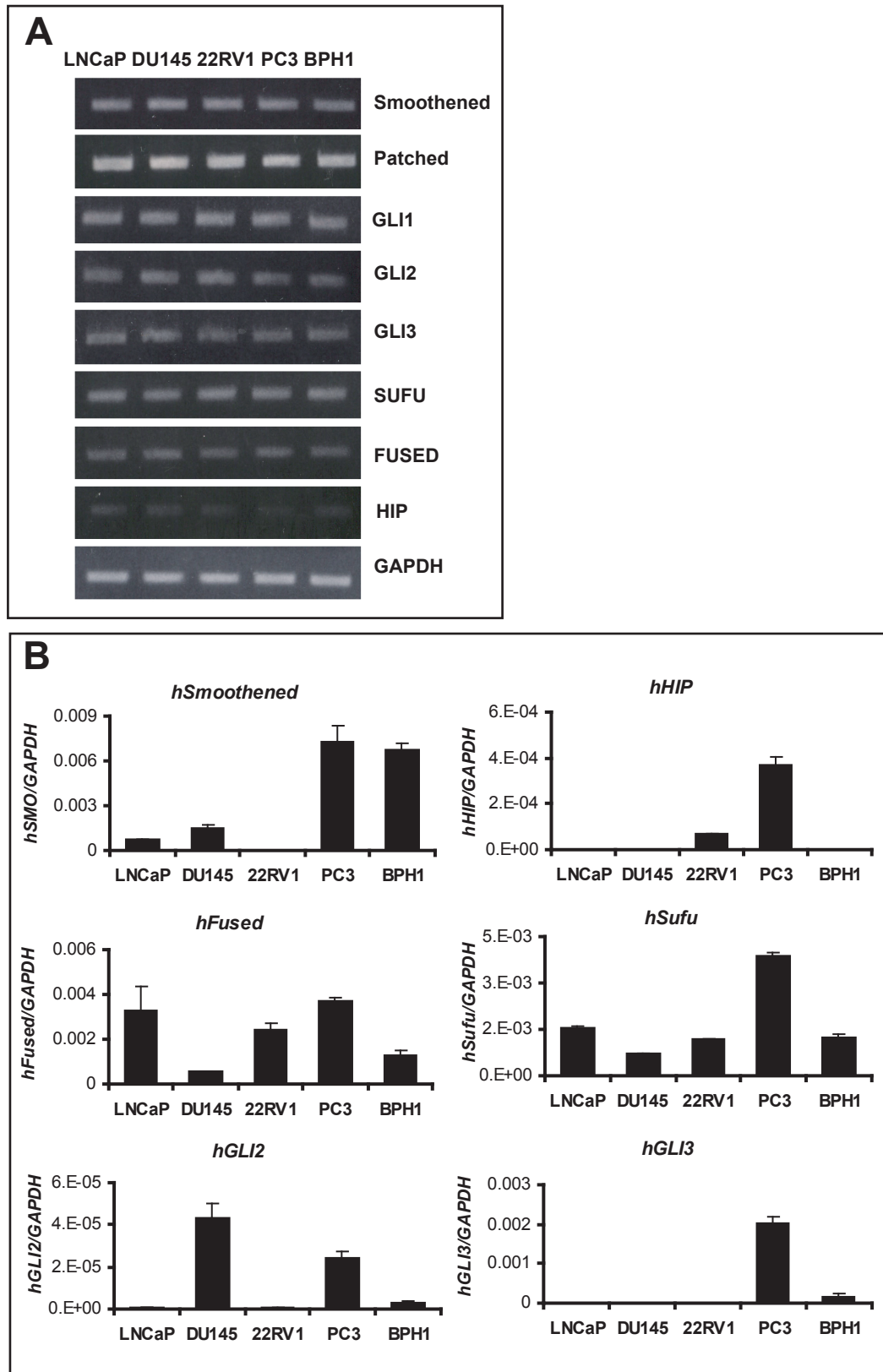


Figure 4 Expression of Hh pathway genes Smo, Ptc1, Gli1, Gli2, Gli3, SuFu, Fused and Hip in LNCaP, DU145, PC3, 22RV1 and BPH-1. (A) Resolution of RT-PCR products (40 cycles) on a 2% agarose gel using GAPDH as a loading control. (B) Quantitative real-time RT-PCR for the Hh pathway genes shows variations in the steady state levels of individual pathway components.

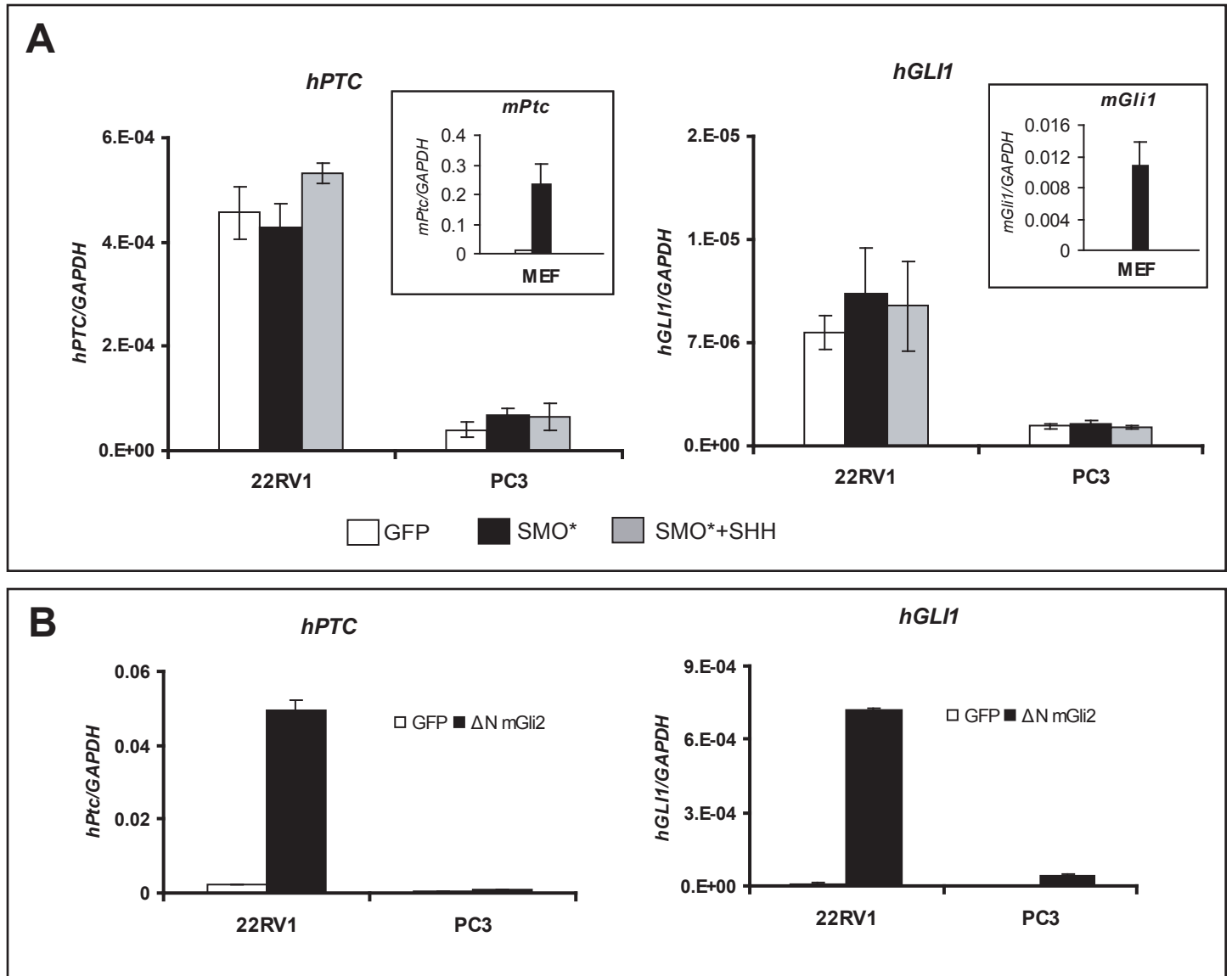


Figure 5 (A) Infection of PC3 and 22RV1 with a Smo* adenoviral vector did not activate expression of Hh target genes Ptc or Gli1, even when exogenous Shh was added. (insert A) Activation of Ptc1 and Gli1 is achieved in MEF cells under the same conditions ($p < 0.05$). (B) Infection of PC3 and 22RV1 with a Δ NmGli2 adenoviral vector induced Hh target gene expression. Both PC3 and 22RV1 exhibit significant increases in Gli1 expression ($p < 0.05$); Ptc expression was significantly increased in 22RV1 cells ($p < 0.005$) but not in PC3 cells ($p = 0.097$). Adenovirus infection rates for all constructs was ~90%.

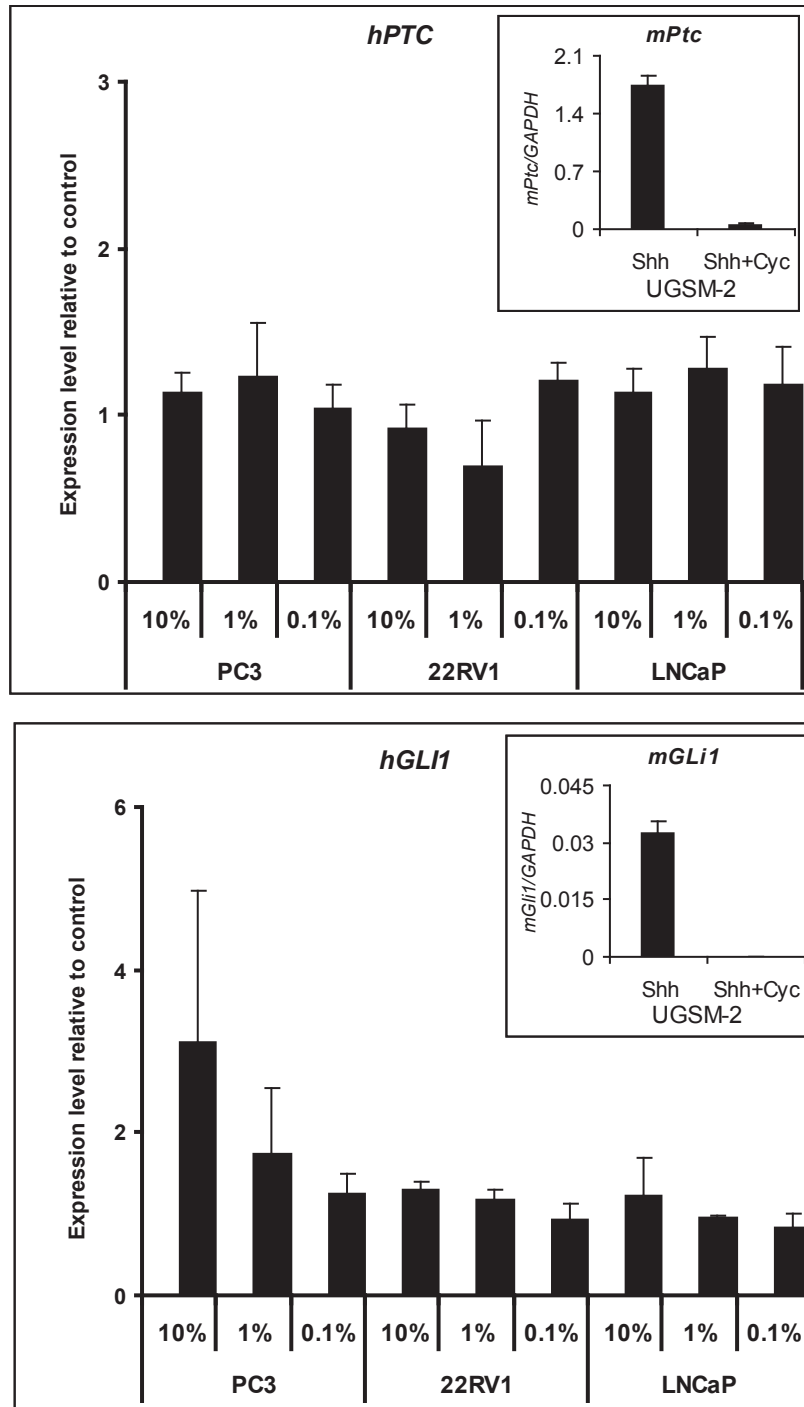


Figure 6 Cyclopamine (5 μ M) treatment of PC3, 22RV1 and LNCaP cells in media supplemented with 10, 1, 0.1% FCS did not alter expression of the Hh target genes Ptc or Gli1. Target gene expression was induced by Shh and inhibited by 5 μ M cyclopamine in UGSM-2 cells $p < 0.005$, (insert).

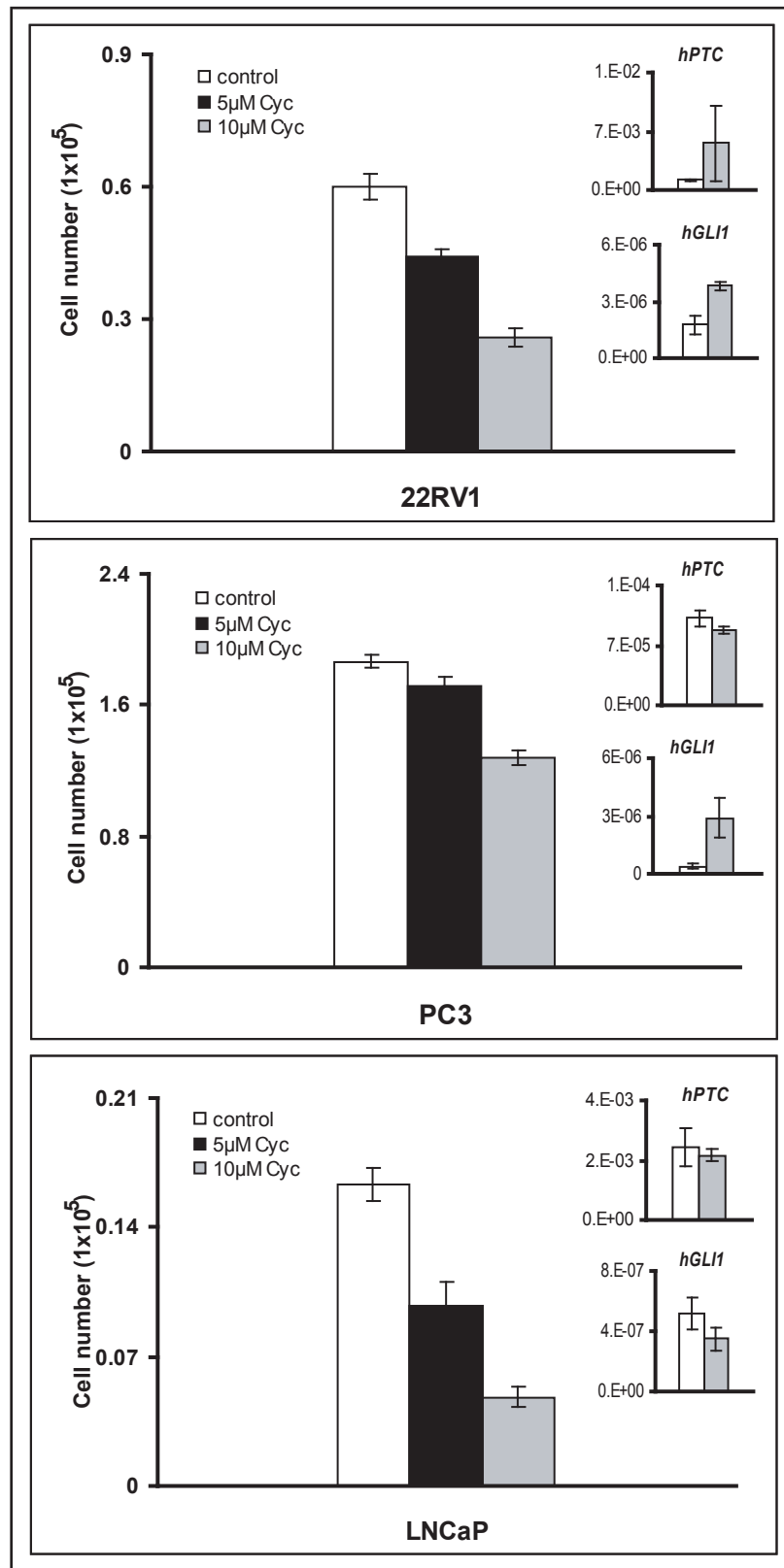


Figure 7 Proliferation of 22RV1, PC3, and LNCaP cells over 4 days was inhibited by cyclopamine in a dose dependent fashion ($p < 0.05$ at 10μM cyclopamine). In these cultures expression of the Hh target genes Ptc and Gli1 were not altered by 10μM cyclopamine suggesting that the reduction in proliferation was not through a Smo-mediated event (insert).

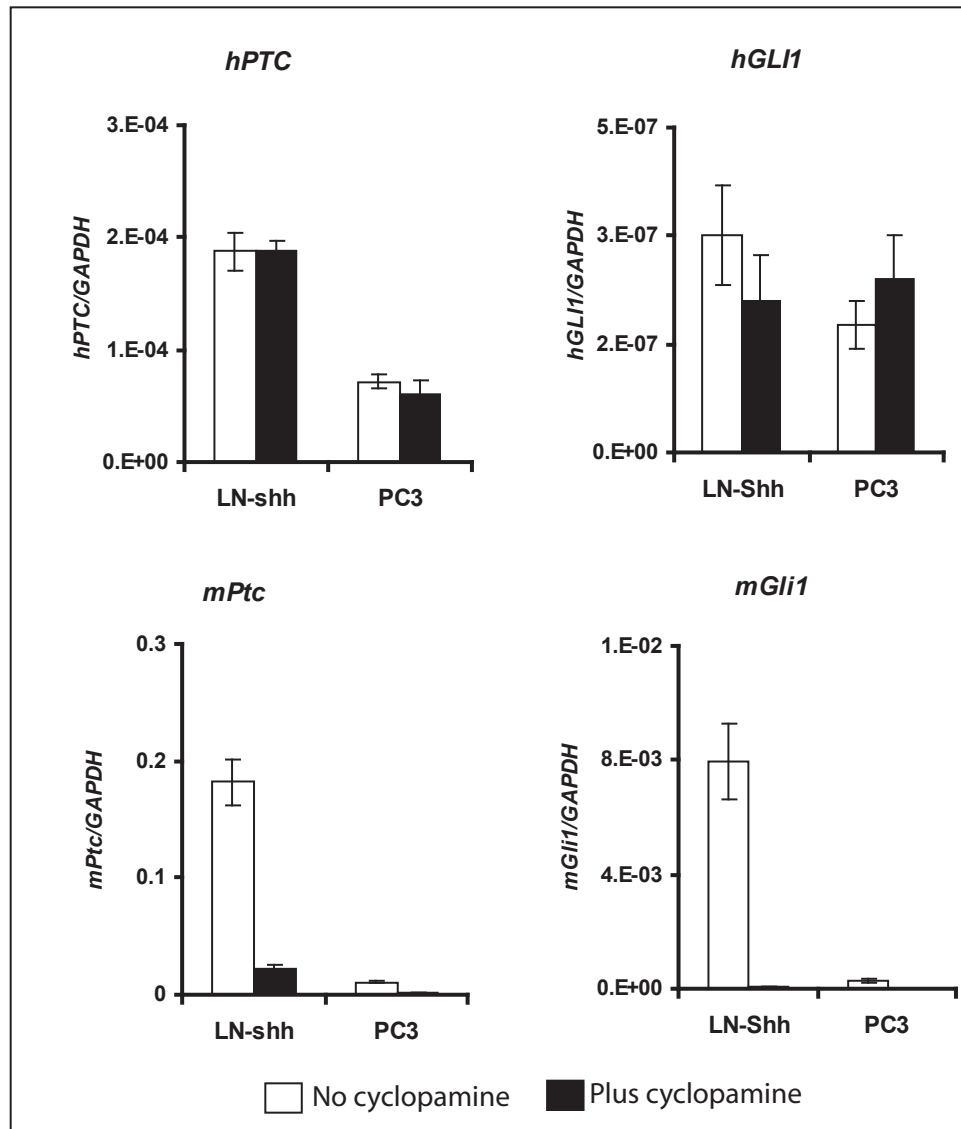


Figure 8 Effect of 10uM cyclopamine on autocrine and paracrine pathway activity in co-cultures of either LNCaP cells over-expressing Shh (LN-Shh) or PC3 cells co-cultured with mouse UGSM-2 cells. There is no effect on expression of hPtc and hGli1 (upper panel). However inhibition of paracrine signaling in UGSM-2 co-cultured with either LN-Shh or PC3 is evident from the decrease in mPtc and mGli1 expression in the presence of cyclopamine (lower panel; $p < 0.05$).

HEDGEHOG SIGNALING IN THE PROSTATE

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Key words: hedgehog pathway, prostate development, prostate cancer, tissue stem cells, inflammation

Abbreviations: Prostate cancer; PCa, Hedgehog; Hh, Patched; Ptc, Sonic Hedgehog; Shh, Indian Hedgehog; Ihh, Smoothened; Smo, Hedgehog-Interacting Protein ; Hip, Suppressor of Fused; SuFu

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ABSTRACT

Purpose. Recent discoveries have highlighted the importance of the Hedgehog (Hh) signaling pathway in prostate growth regulation. This paper reviews the role of Hh signaling in prostate development, adult prostate homeostasis and prostate cancer.

Materials and Methods. A comprehensive review of all relevant literature was conducted.

Results. Epithelial expression of Hh ligand during prostate development exerts both autocrine and paracrine signaling activities that regulate growth and differentiation. Hh signaling also occurs in the adult human prostate but the influence on epithelial proliferation and/or differentiation is unknown. Robust Hh signaling occurs frequently in prostate cancer, and both autocrine and paracrine signaling have been shown to accelerate the growth of xenograft tumors. Autocrine signaling has been implicated in stimulating stem/progenitor cells and increased Hh pathway activity may be a characteristic of advanced, androgen independent cancer. The plant alkaloid cyclopamine is a specific chemical inhibitor of Hh signaling and has produced sustained regression of established xenograft tumors.

Conclusions. Hh signaling plays an important role in prostate development and appears to be a characteristic feature of prostate cancer. It stimulates tumor growth and may exert a specific role in the proliferation of tumor stem cells. The development of Hh inhibitors based on the action of cyclopamine holds promise for novel treatments to slow or arrest tumor growth.

WHAT'S SO EXCITING ABOUT HEDGEHOG?

In a recent editorial comment in this journal, Patrick Walsh described recent findings regarding the role of Hh signaling in PCa as being among the most important basic science findings related to PCa in the past 30 years¹. Hh was first identified as an important signaling molecule in *Drosophila*. Hh signaling is conserved in vertebrates and plays an important role in fetal development of diverse structures including the prostate gland. Recent work has shown that Hh signaling promotes PCa growth and activated Hh signaling has been identified as a key feature of clinically advanced disease. Even more exciting is the possible connection of Hh signaling to proliferation of tumor stem cells, the small compartment of cells within a tumor that may be responsible for androgen-independent tumor recurrence. Specific chemical inhibitors of Hh signaling have produced sustained regression of various xenograft tumors without overt toxicity to the adult host, suggesting that they may represent an entirely new class of therapeutic agents that could target previously untreatable cancers.

RELEVANCE OF DEVELOPMENTAL STUDIES TO CANCER

The Hh transcriptional activator Gli1 was first identified as an oncogene in glioblastoma². Inactivating mutations in the Hh receptor Ptc were found in medulloblastomas³ and Gorlin/nevoid basal cell carcinoma syndrome^{4,5}. More recently, aberrant Hh signaling has been found to be a consistent feature of a variety of tumors originating in organs where Hh signaling plays an important developmental role, including sporadic basal cell carcinoma of skin, pancreatic cancer, small cell lung cancer, gastric cancer and PCa, prompting widespread speculation that reactivation of developmental signaling pathways is a critical step in tumor development.

A STEM CELL CONNECTION

An important facet of Hh signaling is its connection to stem cell proliferation⁶. Recent studies have shown a role for Hh signaling in stem/progenitor cell proliferation in the CNS, mammary gland⁷, skin⁸, gut⁹ and pancreas^{10, 11}. Hh signaling localizes to germinal cell populations in the developing CNS and is required for maintenance and expansion of progenitors^{12, 13}. Disruption of Hh signaling in the fetal brain reduces the number of neural progenitors while Hh pathway activation in the mature brain increases proliferation of telencephalic progenitors¹² and sustained pathway activation produces medulloblastomas^{14, 15}. These findings have ignited speculation that Hh signaling is a key factor in sustaining proliferation of tumor stem cells.

A THERAPEUTIC OPPORTUNITY

Craniofacial birth defects in lambs born in Idaho in the 1950's were ultimately traced to the teratogenic effects of an alkaloid, cyclopamine, in the plant *Veratrum californicum*. The similarity to defects observed in the *Shh* null mouse¹⁶ led to the discovery that cyclopamine is a specific chemical inhibitor of Hh signal transduction¹⁷. Cyclopamine has been used to examine the effect of Hh pathway inhibition on tumor growth and has shown dramatic treatment efficacy in animal models of basal cell carcinoma, medulloblastoma, pancreatic cancer and PCa. Recently, topically applied cyclopamine showed remarkable efficacy against basal cell carcinoma of skin in humans¹⁸.

OVERVIEW OF HH SIGNALING

Of the three mammalian Hh genes Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), Shh is the most widely expressed during development. Shh binds to a specific receptor Ptc on the target cell surface and activates an intracellular signal transduction pathway involving the Gli family of transcription factors that activates transcription of specific genes in the target cell (summarized in Figure 1).

Hh signaling is regulated at several levels. The transmembrane Ptc receptor constitutively represses Hh pathway activity through its interaction with a second transmembrane protein, Smo. Binding of Hh ligand to Ptc disrupts this interaction and de-represses pathway activity. Induction of Ptc expression by Hh signaling creates a negative feedback loop that re-asserts repression at the level of the membrane. A second mechanism for negative feedback is provided by Hh-induced expression of Hip, a cell surface glycoprotein that sequesters Hh ligand. Three Gli genes (Gli1, Gli2, and Gli3) encode transcriptional regulators which share a conserved DNA-binding domain and bind the same 9bp recognition sequence. Gli1 is a transcriptional activator of Hh target genes. Gli2 provides redundancy in the transcriptional activating functions of Gli1. Gli3 functions primarily as a transcriptional repressor that balances and refines transcriptional activation by Gli1 and Gli2. A third domain of Hh pathway regulation depends upon a complex network of regulatory elements in the cytoplasm, involving PKA and several other proteins including Fused (Fu), Suppressor of Fused (SuFu) and Costal 2 (Cos2) which regulate the location and activity of Gli proteins¹⁹.

HH SIGNALING IN PROSTATE DEVELOPMENT

During prostate ductal morphogenesis, Shh expression localizes to sites of active growth. During ductal budding, Shh expression in the epithelium is up-regulated and condenses at sites of epithelial evagination. During ductal outgrowth, Shh expression is strongest at the duct tip. Shh expression in the urogenital sinus (UGS) is not dependent upon testosterone, but testosterone does modestly increase the level of expression and Shh redistribution during budding is certainly tied to an androgen-induced morphogenetic event²⁰. Blockade of Hh signaling by antibody blockade or chemical inhibition of Hh signaling disrupts ductal budding and glandular morphogenesis, respectively^{20, 21}. However, Berman et al²² and Freestone et al²³ both observed budding of the Shh transgenic null UGS and glandular morphogenesis in subcapsular renal grafts. The apparent discrepancy between these observations was resolved by our recent finding that Ihh provides functional redundancy for Shh. This conclusion is based on the observation that Shh null urogenital sinuses grown as renal grafts maintain expression of Hh targets Gli1, Ptc1 and Hip, and this correlates with increased expression of Ihh in the Shh null grafts²⁴. Impairment of Hh signaling by transgenic Gli2 loss of function results in decreased Hh target gene expression, disruption of ductal budding, diminished expression of the stem cell marker Nestin and hyperplasia of p63⁺ basal cells²⁴. These studies show that Hh signaling and Gli-mediated transactivation of Hh target genes is required for normal ductal budding, and to balance progenitor cell proliferation and differentiation.

Hh signaling can occur between tissue layers (paracrine signaling) or among cells within the same tissue layer (autocrine signaling). Ptc and Gli1, targets of Hh signaling, are tightly localized in the mesenchyme surrounding the nascent buds of the developing prostate. Localization of Shh expression to the tip of the elongating ducts is mirrored by Gli1 and Ptc expression in the surrounding mesenchyme^{20, 25}. Paracrine signaling directly affects mesenchymal proliferation^{23, 26} but also influences epithelial proliferation and differentiation by paracrine feedback mechanisms^{20, 26-28}. In addition, there is concentrated epithelial expression of Ptc and Gli1, an indication of autocrine signaling, in the nascent buds and at the tips of the growing ducts^{25, 29}. Given that autocrine signaling stimulates progenitor cell proliferation in other organs, it is tempting to speculate that autocrine signaling at the tips of growing buds plays a role in progenitor epithelial cell expansion (Figure 2).

Several recent observations are consistent with a role for Hh signaling in the maintenance and/or proliferation of prostatic progenitor cells. Gli2 loss of function and impaired Hh signaling are associated with decreased expression of the stem cell marker Nestin²⁴ in the prostate. Castration-induced regression of the ventral prostate is associated with increased expression of Hh ligand, Smo, Gli1 (indicating increased Hh signaling) and this is paralleled by increased Nestin expression. These changes are all reversed during testosterone induced re-growth (unpublished observations) and, remarkably, chemical blockade of Hh signaling prevents testosterone-induced re-growth³⁰.

INFLAMMATION IN PROSTATE CANCER

Chronic inflammation and oxidative stress have been identified as key factors in predisposing to the development of PCa³¹ and, indeed, lesions in the human prostate characterized by proliferating epithelial cells and activated inflammatory cells

(proliferative inflammatory atrophy) are considered likely precursors of PIN and PCa³²⁻³⁴. An emerging paradigm postulates that epithelial injury and inflammation activates proliferation of stem cells as part of the repair process. These proliferating progenitor cells are exposed to oncogenic forces such as oxidative stress that can induce genetic or epigenetic changes leading to a persistent state of activation. The interaction between the persistently activated progenitor cell and the reactive stroma associated with inflammation and healing results in tumor formation and unregulated growth. The Hh and Wnt signaling pathways have been identified as the two critical pathways regulating stem cell activation. In some tissues, such as the colon, activated Wnt signaling appears to be the dominant actor. In other tissues, such as the brain, skin, lung, pancreas and prostate, Hh signaling appears to play a key role in regulating stem cell activation and tumor development⁶.

HH SIGNALING IN THE DEVELOPING AND ADULT PROSTATE

Shh is abundantly expressed in the human fetal prostate and is down-regulated before birth^{27, 35}. A highly quantitative analysis of Hh signaling in the adult prostate by Fan et al²⁷ utilized real-time RT-PCR to compare Shh, Gli1, Gli2 and Gli3 expression in normal prostate tissue from organ donors, BPH tissue obtained by prostatectomy, and both tumor and zone-homologous normal tissue from radical prostatectomy specimens. The human fetal brain and fetal prostate were included as controls to compare expression of these genes in tissues where HH pathway activity is known to be high. These studies showed that expression of Shh and Gli1 in specimens of normal prostate and BPH varied over several orders of magnitude but was generally comparable to the robust level of expression observed in the fetal brain and in the fetal prostate. A tight correlation between Shh and Gli1 expression was observed, consistent with a dependence of Gli1 expression on Shh signaling. Karhadkar and colleagues³⁰ did not examine expression in the normal prostate per se, but performed RT-PCR analysis for presence or absence of Shh, Ihh and pathway gene expression in primary epithelial (PrE) cells, benign prostate tissue adjacent to tumors, localized PCa, and PCa metastases. In their assays, they observed that Shh and Ihh were expressed in PrE cells, tumor associated benign tissue and localized PCa, but the conserved Hh target genes Ptc and Gli1 were not. Ptc and Gli1 were only expressed in metastatic tumors. Sanchez et al³⁶ examined expression in normal human prostate tissue using real-time RT-PCR to compare expression of Shh, Ptc and the Gli genes in six specimens of human PCa and tumor associated benign tissue. These studies, combined with immunostaining of a tissue microarray containing both tumor and tumor-associated benign tissue, suggested a basal level of Shh, Ptc and Gli1 expression in the benign tissue that is variably increased in tumor. Neither the Sanchez nor Karhadkar studies included a reference control such as fetal brain to establish the relative level of expression in their specimens. This led to the widely shared perception that the level of expression of Shh and Ptc and Gli1 is “low” in benign prostate tissue, but this interpretation is incorrect. The quantitative comparisons provided by Fan et al clearly show that expression of Shh and Gli1 in normal adult and benign prostate tissues rivals the robust level of expression seen in the fetal brain. This is reinforced by a recent comparison of expression in a pooled specimen of 30 normal prostate tissues and the fetal brain, showing high levels of expression of Shh, Ptc, Gli1 and Smo in the normal prostate³⁷.

In situ hybridization studies using a highly specific radiolabeled probe localized Shh expression to the prostatic epithelium and Gli1 expression almost exclusively to the periglandular stroma. Ptc, which is expressed at a basal level in the absence of Hh pathway activity, was expressed in both compartments²⁷. Sanchez et al³⁶ performed in situ hybridization with a digoxigenin-labeled probe and immunostaining to demonstrate relatively weak co-expression of Shh, Ptc and Gli1 in the prostatic epithelium. These studies suggest that Hh signaling in the normal/benign adult prostate may involve a combination of autocrine and paracrine signaling. While the role of Hh signaling in the adult prostate are not yet known, studies of Hh signaling during prostate development suggest a diverse repertoire of potential activities. Studies of Hh signaling in early prostate development highlighted a role for Hh signaling in stimulating epithelial proliferation. In contrast, studies examining the effect of Hh signaling in the postnatal prostate suggested that Hh signaling inhibits proliferation and stimulates terminal epithelial differentiation. These studies make clear that Hh signaling exerts multiple effects, both growth stimulatory and growth inhibitory^{20, 22, 23, 25, 26, 29}. These activities may be distinguished by autocrine versus paracrine signaling mechanisms and/or by an evolving response of the mesenchyme to paracrine signaling as it differentiates. Whatever the case, it is clear that Hh signaling evokes a variety of effects that might underpin homeostatic growth regulation in the normal adult prostate as well as in response to epithelial injury and inflammation.

HH SIGNALING IN PROSTATE CANCER

Studies of Hh signaling in human PCa suggest that (1) both autocrine and paracrine signaling contribute to tumor growth, (2) the effect of paracrine signaling may be influenced by the reactive character of the tumor stroma, and (3) ligand dependent and ligand independent autocrine pathway activation is a feature of advanced disease. Fan et al²⁷ compared Shh and Gli gene expression in tumor specimens obtained by radical prostatectomy to expression in specimens of BPH and normal prostate. Mean expression in tumors was nearly an order of magnitude higher than in the benign specimens, though the difference was not statistically significant because of the wide range of expression in benign specimens. In a separate analysis, tumor and zone matched benign tissue from the same patients was examined and this showed generally comparable levels of robust Shh expression in both tissues from the same patient. Karhadkar et al³⁰ used RT-PCR analysis to compare Hh ligand expression and Hh pathway activity in specimens of localized and metastatic PCa. They showed that Hh ligand was expressed abundantly in both localized and metastatic PCa, but that Hh pathway activity, as evidenced by Ptc and Gli1 expression, was dramatically increased in metastatic lesions. They attributed this to an increased responsiveness to Hh ligand conferred by renewed expression of Smo. An alternative explanation is that the Hh response in metastases is due to increased Hh sensitivity of stroma at metastatic sites. Sheng³⁸ also demonstrated an increase in Ptc expression in advanced PCa, and attributed some of the increase to mutations in SuFu leading to dysregulated autocrine pathway activity. Sanchez³⁶ used RT-PCR analysis to demonstrate a variable increase in Shh expression and pathway activity in tumor tissue as compared to matched benign tissue from the same specimen and used immunostaining for Shh to show that increased Shh expression occurred in nearly 33% of tumor

specimens as compared to <1% in benign tissues. Together, these studies suggest that high levels of Shh and Gli1 expression is found in localized prostate tumors as well as benign, zone homologous tissue in the same gland and that a further increase in Shh expression and Hh signaling occurs in advanced PCa.

Localization studies performed by Fan et al²⁷ showed Shh expression in the tumor and glandular epithelium and Gli1 expression primarily in the periductal stroma. Sanchez et al³⁶ performed both in situ hybridization and immunostaining and showed Shh, Ptc and Gli1 expression co-localizing to the tumor epithelium. The apparent discrepancy in the location of Gli1 expression - and therefore the cell type exhibiting pathway activation - could be a product of different methods of assay and/or might reflect heterogeneity of autocrine and paracrine signaling in PCa.

The commonly used PCa cell lines LNCaP, PC3, 22RV1 and DU145 all express Shh and Ihh as well as the major components of the Hh pathway. The levels of expression vary considerably and the secretion of functional ligand has not been confirmed in most cases. Work presented in three different papers has suggested that autocrine signaling in tumor cell lines stimulates cell proliferation, however, there are significant discrepancies in the findings in different laboratories. Karhadkar et al³⁰ found that anti-Shh blocking antibody inhibited PC3 proliferation in culture, suggesting that ligand-dependent autocrine signaling stimulates cell proliferation. However, Sanchez et al³⁶ found that PC3 proliferation was unaffected by either anti-Shh blocking antibody or recombinant Shh. The discrepancy in these results has not been resolved. The Hh pathway inhibitor cyclopamine was found to inhibit proliferation of PC3 and LNCaP cells in culture^{30, 36, 38}. Cyclopamine inhibited Gli1 expression in LNCaP cells, arguing that the effect is pathway specific, however, the unresponsiveness of LNCaP cells to exogenous Shh³⁶ argues against operation of a ligand-dependent pathway. The potential of chemical blockade of Hh signaling to inhibit tumor growth was examined by administering cyclopamine to mice with human PCa xenografts³⁰. Both PC3 and 22RV1 tumors showed a dose dependent inhibition of tumor growth, and complete and sustained regression at the highest dose tested. The specificity of this effect was confirmed by showing that xenografts made with tumor cells overexpressing Gli1 were resistant to the anti-tumor effect of cyclopamine. These studies were interpreted as evidence that autocrine signaling in the PC3 and 22RV1 tumors promotes tumor growth and can be inhibited by cyclopamine blockade. Additional experiments performed with rodent tumor cell lines showed that cyclopamine could inhibit growth and metastasis of the aggressive AT6.3 cell line and that Gli1 overexpression conferred a highly aggressive and metastatic phenotype to the normally less aggressive AT2.1 cell line. While these observations are all consistent with the notion that cyclopamine inhibited tumor growth by blocking Hh signaling, it is important to point out that the effect of cyclopamine on growth of PC3, 22RV1 and AT6.3 tumors was not correlated with an inhibition of Hh signaling. Detailed studies under a variety of conditions in our laboratory showed that LNCaP, PC3 and 22RV1 do not exhibit the canonical transcriptional response to Hh ligand (see Zhang et al, this issue). In addition, cyclopamine treatment did not produce an inhibition of Ptc and Gli1 expression even at concentrations that inhibited cell growth in culture. These observations, clearly at odds with previously published observations, were complemented

by transfection-based studies showing that the Hh signal transduction pathway is non-functional in both PC3 and 22RV1. These findings are important for three reasons. First, they show that PC3 and 22RV1 cannot be used to model ligand-dependent autocrine signaling in human PCa. Second, they demonstrate that expression of Ptc and Gli1 in PC3 and 22RV1 is independent of the canonical Hh signal transduction mechanism and therefore may be an inappropriate model for studying ligand-independent pathway activation that results from dysregulation of signal transduction. Third, these cell lines are not appropriate models for testing Hh pathway inhibitors based on cyclopamine's mechanism of action.

The effect of paracrine signaling on tumor growth was examined using the LNCaP xenograft. Overexpression of Shh by LNCaP tumor cells increased expression of Ptc and Gli1 in the tumor stroma, without any evidence of autocrine pathway activation, and accelerated tumor growth²⁷. This suggested that Shh expressed by the tumor cells acted on adjacent stromal cells to elicit paracrine signals that promoted tumor growth. Recently, we have shown that Hh pathway activation in the tumor stroma alone is sufficient to accelerate tumor growth (unpublished observations). Other recent studies show that the effect of tumor cell Shh expression on tumor growth is determined by the phenotype of the tumor stroma (unpublished observations). The dominant effect of the stromal phenotype on the growth response to paracrine signaling may explain the differing effects of Hh expression in the growth quiescent normal prostate and in prostate cancer where a reactive stroma is generally present³⁹ (Figure 3).

HH SIGNALING, ANGIOGENESIS AND METASTASIS

Vascular endothelium is a well established target of Hh signaling. Shh induces expression of pro-angiogenic molecules including vascular endothelial growth factors (VEGF) and angiopoietins (Angpt) by stromal cells. VEGF and Angpt stimulate endothelial proliferation and growth of vessels into tumors. Hedgehog-interacting protein, an inhibitor of Hh signaling, is abundantly expressed in resting endothelial cells and is downregulated in PCa xenografts undergoing angiogenesis⁴⁰. The pro-angiogenic effects of Hh may provide a growth stimulus for tumors and also a means to metastasize.

Hh signaling correlates with metastatic potential and Gli1 overexpression can render a non-metastatic cell line metastatic (Karhadkar 2004). Hh signaling is implicated in mediating epithelial-mesenchymal transition (EMT), an event that is postulated to facilitate carcinoma invasion. Overexpression of Gli1 in a non-metastatic PCa cell line stimulated expression of Snail, a marker of EMT, to levels seen in metastatic lines and increased cell invasion in vitro. In addition, Hh signaling may contribute to the predilection of PCa for bony metastasis since bone marrow stromal cells are responsive to Hh ligands and both Shh and Ihh stimulate bone remodeling^{41, 42}.

CONCLUSIONS

Robust Hh signaling is characteristic of the adult human prostate and may play a variety of roles in homeostatic growth regulation and the response to injury or inflammation. Hh ligand expression and pathway activity is common in localized PCa and may promote tumor cell proliferation by a combination of autocrine and paracrine signaling. Some of

this may occur by canonical ligand dependent mechanisms and some may involve, as suggested by Sheng et al³⁸, mutations affecting the regulation of Hh pathway activity in the tumor cells. Hh pathway activity is dramatically increased in advanced, metastatic PCa but whether this represents mutational activation or an increased responsiveness of the tumor cell or ectopic stroma to Hh ligand is not known.

Hh signaling is a unique target for therapy both because of the apparently limited toxicity associated with chemical inhibition and the potential of this pathway to attack the postulated stem cell core of PCa. Recognizing that success in animal xenograft studies frequently doesn't translate to success in treating human cancers, what can we realistically expect? The first point to make is that Hh signaling occupies a unique niche in the signaling realm. There is some level of functional redundancy at both the level of the ligand (Shh, Ihh, Dhh) and at the level of target gene regulation (Gli1, Gli2, Gli3), but the signal transduction pathway appears to funnel specifically through the Ptc/Smo complex at the membrane level. There is little known cross-talk involving the Ptc/Smo receptor and it is therefore likely that inhibitors targeted to Ptc/Smo will allow little room for escape by physiologic mechanisms. Paracrine or autocrine signaling which occurs by a ligand dependent mechanism are therefore promising targets for therapy. The stromal response involved in paracrine signaling is especially likely to depend upon the canonical ligand-dependent pathway and is therefore a prime target for therapy to slow or arrest tumor progression. The relative contributions ligand-dependent autocrine signaling and mutational activation of the pathway in localized and metastatic tumor growth is as yet unknown. Autocrine signaling that proceeds through an intact signal transduction pathway and regulatory mechanisms is likely to be responsive to Hh blockade by analogs of cyclopamine, however, autocrine pathway activity that occurs downstream from Ptc/Smo, through inactivation of SuFu for example, can be expected to escape the action of cyclopamine-like inhibitors. Thus, it is possible that as tumors progress and acquire an increasing number of mutations they could acquire changes that result in autocrine pathway activation that is un-responsive to the Hh inhibitors based on the action of cyclopamine.

What is needed? The overly simplistic conclusion that Hh signaling is increased in PCa and that tumor growth can be stopped by treatment with Hh inhibitors like cyclopamine needs to be refined. We now know that Hh signaling is present in the normal prostate as well as in cancer and in order to really understand what's going on, we have to understand how the roles of Hh signaling are similar and different in the normal prostate and in PCa. This will entail further studies to define the relative abundance of autocrine and paracrine signaling in the normal prostate, localized cancer and metastatic cancer and mechanistic studies to examine how these activities are related to stem cell proliferation, amplifying or transit cell proliferation/differentiation, and androgen regulation of growth and invasion. Moreover, we must identify what proportion of autocrine signaling in PCa is ligand dependent and what proportion results from intracellular pathway mutations. This information will enable us to select the tumor cell lines, xenograft models, and animal models that most accurately represent the human tumor and use these for drug development and testing. A novel and minimally toxic intervention that can cut to the

root of a tumor is an exciting prospect for the treatment of PCa. Realization of the goal will require a great deal of work but it may not be so far away.

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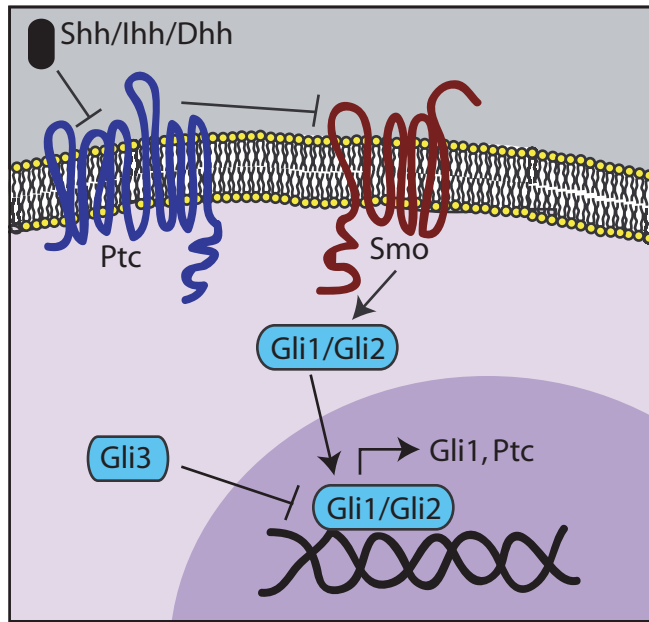
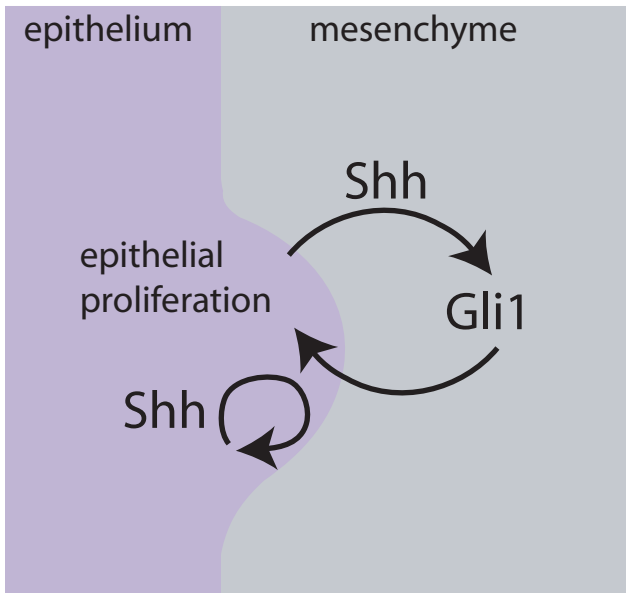


Figure 1. The mammalian Hh signaling pathway. Hedgehog ligands Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) bind to the transmembrane receptor Patched (Ptc) and relieve constitutive repression of Smoothened (Smo). Smo activation curtails transcriptional repression by Gli3 and promotes activation/translocation of Gli1 and Gli2 to the nucleus, resulting in transcriptional activation of Hedgehog target genes. Gli1 and Ptc are primary targets of Hh pathway activation and serve as reliable indicators of Hh signaling.

ductal budding



ductal outgrowth

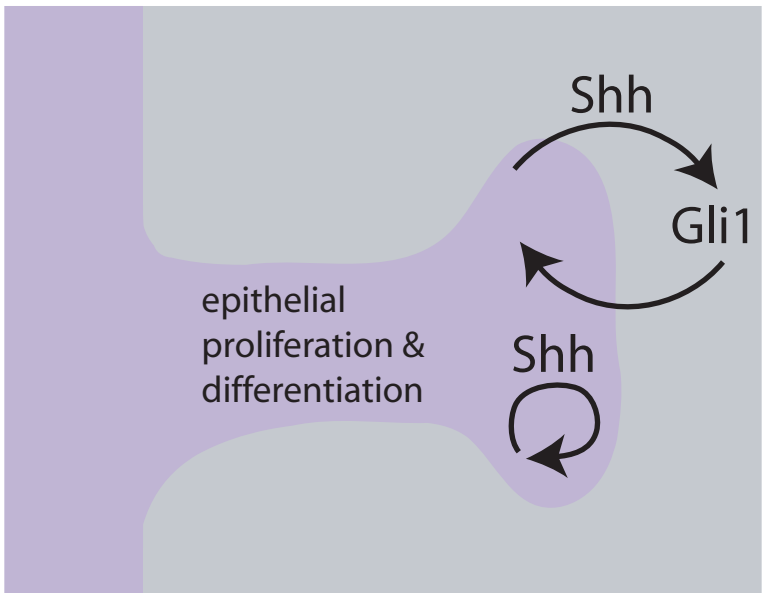
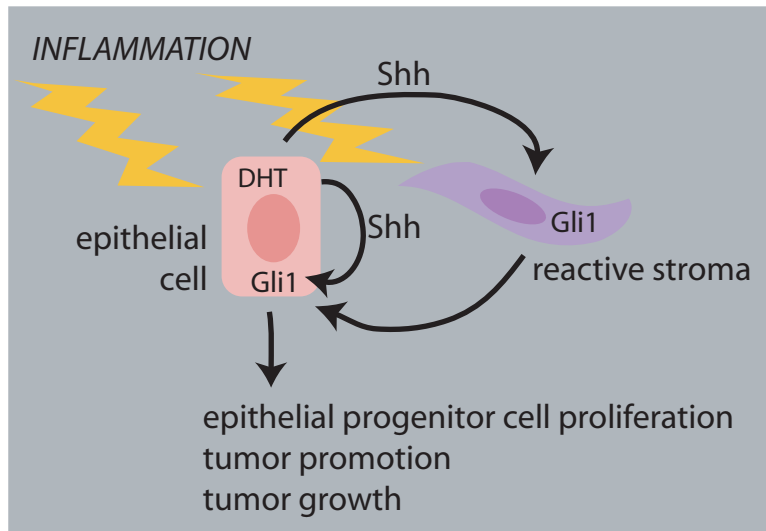


Figure 2. Postulated actions of Hh in prostate development. (Left) Androgen-dependent ductal budding is associated with focal expression of Shh in the epithelium of nascent buds. Shh acts on adjacent mesenchyme to activate expression of Hh target genes and increases epithelial proliferation (paracrine signaling). Autocrine signaling at the tip of the bud may stimulate progenitor cell proliferation. (Right) During ductal morphogenesis, autocrine signaling at the duct tip stimulates continued progenitor cell proliferation while paracrine signaling regulates epithelial differentiation.

ligand dependent signaling



combined ligand dependent/independent signaling

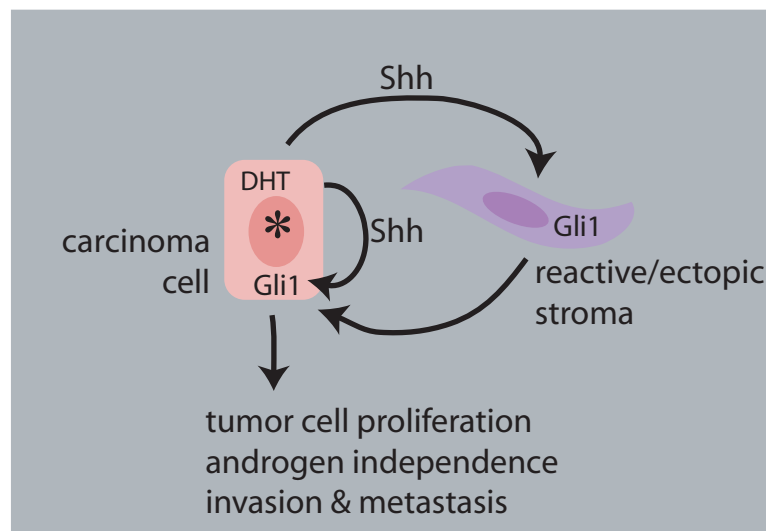


Figure 3. Postulated actions of Hh in prostate cancer. (Top) Injury and inflammation induce ligand-dependent autocrine stem cell activation and proliferation while paracrine signaling elicits growth stimulating responses from the reactive stroma. This creates an environment that promotes tumor formation and growth. (Bottom) Tumor growth is accelerated by ligand-dependent autocrine and paracrine signaling mechanisms and by mutations (*) that produce ligand-independent pathway activation. These activities promote invasion, metastasis and androgen independent tumor growth.

Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line

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BACKGROUND. Stromal-epithelial signaling plays an important role in prostate development and cancer progression. Study of these interactions will be facilitated by the use of suitable prostate cell lines in appropriate model systems.

METHODS. We have isolated an immortalized prostate mesenchymal cell line from the mouse E16 urogenital sinus (UGS). We characterized its expression of stromal differentiation markers, response to androgen stimulation, ability to induce and participate in prostate morphogenesis, response to Shh stimulation, and interaction with prostate epithelial cells.

RESULTS. UGSM-2 cells express vimentin and smooth muscle actin, but not the mature smooth muscle markers myosin and desmin. This expression profile is consistent with a myofibroblast phenotype. Unlike other fibroblasts such as 3T3, UGSM-2 cells express androgen receptor mRNA and androgen stimulation increases proliferation. UGSM-2 cells are viable when grafted with embryonic UGS under the renal capsule and participate in glandular morphogenesis, but are not capable of inducing prostate morphogenesis of isolated UGS epithelium. Co-culture of UGSM-2 cells with human BPH-1 cells or co-grafting in vivo results in organized clusters of BPH-1 cells surrounded by a mantle of UGSM-2 cells. UGSM-2 cells are responsive to Sonic hedgehog (Shh), an important signaling factor in prostate development, and mimic the transcriptional response of the intact UGS mesenchyme. In co-cultures with BPH-1, UGSM-2 cells exhibit a robust transcriptional response to Shh secreted by BPH-1.

CONCLUSIONS. UGSM-2 is a urogenital sinus mesenchyme cell line that can be used to study stromal-epithelial interactions that are important in prostate biology. *Prostate* 66: 1347–1358, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: stromal-epithelial interactions; androgen; sonic Hedgehog; prostate development; mesenchyme

INTRODUCTION

The prostate develops from a specific region of the endodermal urogenital sinus (UGS) termed the prostatic anlagen. Formation of the prostatic ducts begins at embryonic day 17 (E17) in the mouse when epithelial buds evaginate into the surrounding mesenchymal sheath. Discrete groups of buds define the origins of the anterior, dorsal and ventral lobes of the prostate. At the time of ductal budding, the UGS mesenchyme is composed of undifferentiated fibroblasts and myofibroblasts. As the buds elongate, they lumenalize to form true secretory ducts connected to the urethral lumen and branch to form a highly complex ductal tree. As the ducts grow, they are surrounded by a sheath of mesenchyme, which differentiates to a periductal stroma comprised of smooth muscle cells and fibroblasts [1].

The embryonic mesenchyme and its adult descendent stroma have emerged as key regulators of prostatic growth and differentiation. In the UGS, mesenchymal cells express androgen receptors and act under the influence of androgens to induce prostatic differentiation of the endodermal epithelium [2,3].

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Tissue recombination experiments have shown that the mesenchyme is the primary determinant of epithelial growth and differentiation [4]. In the adult prostate, there is regional heterogeneity within the ducts: the distal tips are encased in a delicate fibroblastic sheath, while the more proximal segments are surrounded by thicker sheaths rich in smooth muscle [5]. Androgen receptor expression is localized to the dense smooth muscle sheath surrounding epithelial ducts, whereas fibroblasts rarely express androgen receptors [6]. Smooth muscle is required for maintenance of epithelial secretory function [7] and loss of smooth muscle in the adult prostate is associated with cancer lesions and de-differentiation of epithelium [8].

Primary stromal cells from human prostate tissue have been used to discover factors that regulate smooth muscle differentiation and proliferation of prostate stroma, and to identify stromal-derived factors that regulate epithelial functions. Several prostate stromal cell lines have been generated, including rat NbF-I, mouse PSMC1, rat PS-1, human WPMY-1, human DuK50, and human PS30 cells [9–14]. Two rat UGS mesenchymal cell lines have been generated: rUGM and U4F1 [15,16]. To our knowledge, none of these cell lines is able to induce or participate in prostate morphogenesis.

The signaling interactions that regulate prostate ductal budding and branching morphogenesis have received considerable attention as the paradigm for understanding normal prostate growth regulation. These studies have demonstrated that the UGS mesenchyme is the target of several key signals, including testosterone, estrogen, and sonic hedgehog [17–19]. UGS mesenchyme is also the origin of several key morphogens including BMP-4, FGF-10, TGF β [20–23], and Shh target genes such as IGFBP-6 [31], which may regulate both epithelial and mesenchymal proliferation and differentiation. The complexity of these interactions is daunting. For the Shh pathway alone, there are three different Gli genes expressed in the UGS mesenchyme and each of these plays a unique role in the transcriptional response to Hh signaling [18]. Similar complexities exist in the multiplicity of receptor subtypes for BMP, TGF β , and FGF signaling. To elucidate the complex regulation and crosstalk between these pathways in mesenchymal cells, we have developed an immortalized UGS mesenchymal cell line and demonstrated that it phenocopies the UGS mesenchyme response to Shh stimulation.

Several unique characteristics distinguish the mesenchyme of the urogenital sinus. These include responsiveness to androgen, the ability to induce prostate differentiation of isolated urogenital sinus epithelium, and responsiveness to morphogens such as Sonic hedgehog. UGSM-2 cells were found to be

androgen responsive and to mimic the canonical response of urogenital sinus mesenchyme to Sonic Hedgehog. UGSM-2 cells did not induce morphogenesis of isolated UGS epithelium sheets, but when grafted together with the E16 UGS they did proliferate and become incorporated into the periductal stroma during glandular morphogenesis.

MATERIALS AND METHODS

Animals and Cell Lines

Balb/c 3T3 fibroblasts were obtained from ATCC and cultured according to ATCC guidelines. BPH-1 cells were obtained from Simon Hayward (Vanderbilt University, Nashville, TN) and maintained in RPMI + 25 mM HEPES + 10% FBS. UGSM-2 cells were maintained in DMEM/F12 + ITS + 10% FBS + 10^{-8} M DHT. Wild-type CD-1 and CD-1 nude mice were obtained from Charles River (Wilmington, MA). INK4a^{-/-}, β -actin-tva transgenic mice were obtained from Bart Williams (Van Andel Research Inst., Grand Rapids, MI). All animals were housed according to institutional animal use and care guidelines.

Isolation of UGSM-2 Cells

Immortalized UGSM-2 cells were derived from the urogenital sinus of an E16 male INK4a^{-/-} tva transgenic mouse embryo. INK4a^{-/-}, β -actin-tva transgenic mice were provided by Bart Williams (Van Andel Research Institute, Grand Rapids, MI). UGS epithelium was separated from mesenchyme following trypsin digestion as described previously [24]. Mesenchyme was further dissociated into single cells by digestion in 0.5% collagenase. Dissociated mesenchymal cells were grown in DMEM + 15% FBS + 1% pen/strep until they reached confluence in a 6-well plate. Thereafter cells were grown in DMEM/F12 + 10% FBS + 1% pen/strep + 1% ITS + 10^{-8} M DHT (INK4 culture medium). The UGSM-2 clone was isolated from the mixed UGSM population by dilution cloning followed by ring cloning.

Growth Curve Analysis

UGSM-2 and 3T3 cells were plated at a density of 4×10^4 cells per well in 6-well plates in normal culture media containing 10% charcoal-stripped, dextran-treated fetal bovine serum, csFBS (Hyclone, Logan, UT). After 48 hr, cells were treated with 10^{-8} M R1881 or 0.1% ethanol in normal culture media containing 10% csFBS. Each day, cells were trypsinized, diluted 1:100 in Isoton II solution (Beckman, Fullerton, CA) and counted in triplicate using a ViCell XR viable cell counter (Beckman Coulter). No significant difference in

cell viabilities between treatments was noted. Doubling time was calculated by determining the time required to double the number of cells in linear mid-log phase.

Ploidy Analysis

UGSM-2 cells were determined to be tetraploid by comparison to ploidy number of known diploid cells: freshly isolated splenocytes from the spleen of a CD-1 mouse (Charles River, Wilmington, MA). Splenocytes and UGSM-2 cells were combined in the following three ways: (1) 2×10^6 splenocytes, (2) 0.5×10^6 splenocytes + 2×10^6 UGSM-2 cells, and (3) 2×10^6 UGSM-2 cells. Cells were pelleted and fixed in ice cold 70% ethanol for 30 min. Cells were then pelleted and resuspended in 33 μ g/ml propidium iodide + 1 mg/ml RNase A + 0.2% Nonidet P-40 in PBS. DNA content of cells was determined using a FACScan cytometer and analyzed using ModFitLT V3.0 software.

Immunocytochemistry

UGSM-2 cells were grown on Lab-Tek II chamber slides (Fisher, Pittsburgh, PA) and immunostained for vimentin, smooth muscle actin (SMA) or pan-cytokeratin (pan-CK). Slides were fixed in 4% paraformaldehyde and blocked in 5% normal goat serum in PBS. Anti-vimentin clone LN-6 (Sigma, St. Louis, MO), anti-smooth muscle actin monoclonal antibody clone 1A4 (Sigma) or anti-pan-cytokeratin monoclonal antibody (Zymed, South San Francisco, CA) was applied at a dilution of 1:200. Staining was visualized by incubating with goat anti-mouse Alexa 546 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Slides were mounted with Vectashield Hardset + DAPI mounting media (Vector, Burlingame, CA) and imaged using an Olympus model BX51 fluorescent microscope and Spot Advanced software v. 3.5.2.

RT-PCR

RNA was isolated from confluent cells using RNeasy mini kit (Qiagen, Valencia, CA) with optional on-column DNase digestion to eliminate contaminating DNA. Total RNA (1 μ g) was reverse transcribed to generate cDNA using M-MLV reverse transcriptase (Invitrogen). Relative mRNA quantity was determined by real-time RT-PCR using iCycler instrumentation and software (BioRad, Hercules, CA). Primer sequences are listed in Table I. Primer sets whose name starts with 'm' are mouse-specific, while primer sets whose name starts with 'h' are human-specific. All sequences are listed in 5'-3' orientation.

Co-Cultures

UGSM-2 and BPH-1 cells were plated at equal densities (1×10^6 cells each) in 25 cm² flasks coated with neutralized rat tail collagen [25]. Morphology of cells was observed and photographed over a 1-week period using a Nikon Eclipse TS100 inverted light microscope with a Spot Insight QE digital camera. RNA was prepared from 48 hr co-cultures as described above. Expression of Shh signaling targets Gli1 and Ptc1 was examined by RT-PCR.

Renal Capsule Grafts

For UGE + UGSM-2 grafts, E16 UGSs were separated into epithelium (UGE) and mesenchyme (UGM) using the method described previously [24]. UGE + UGSM-2 were combined and allowed to adhere together overnight on 0.6% agar plates containing INK4 culture medium. For UGS + UGSM-2 grafts, UGSs were dissected from E16 male CD-1 mouse embryos and chopped into five to six pieces, combined with UGSM-2 cells, and incubated overnight on agar plates prepared

TABLE I. Sequences of RT-PCR Primers

Target gene	Forward primer	Reverse primer
mGAPDH	AGCCTCGTCCCGTAGACAAAAT	CCGTGAGTGAGTCATACTGGA
mSMA	ATCATGCGTCTGGACTTGG	AATAGCCACGCTCAGTCAGG
mVim	CCCCCTTCCTCACTTCTTTC	AAGAGTGGCAGAGGACTGGA
mDesmin	GTGAAGATGGCCTTGGATGT	TTGAGAGCAGAGAAGGTCTGG
mHCM	GCAGCTTCTACAGGCAAACC	CAAAGCGAGAGGAGTTGTCC
mAR	GTGAAGCAGGTAGCTCTGGG	GAGCCAGCGGAAAGTTGTAG
mCD31	CTGAGCCTAGTGTGGAAGCC	TACATCCATGTTCTGGGGGT
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
mPtc1	CTCTGGAGCAGATTTCGAAGG	TGCCGCAGTCTTTTGAATG
mIGFBP6	AGCTCCAGACTGAGGTCTTCC	GAACGACACTGCTGCTTGC
mHIP	CCTGTCTGAGGCTACTTTTCG	TCCATTGTGAGTCTGGGTCA
hGAPDH	CCACATCGCTCAGACACCAT	GCAACAATATCCACTTTACCAGAGTTAA
hGli1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hPtc1	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA

with INK4 culture media. For BPH-1 + UGSM-2 grafts, 500,000 UGSM-2 and 100,000 BPH-1 cells were resuspended in cold Matrigel (BD Biosciences, Becton, MA) and allowed to gel in sterile culture dishes. After 30 min, Matrigel beads containing cells were covered with INK4 culture medium and placed in a CO₂ incubator overnight. Recombinants were placed under the renal capsule of CD-1 adult male nude mice using the method outlined by Cunha et al. (<http://mammary.nih.gov/tools/mousework/Cunha001/Pages/Navigation.html>). After 1–4 weeks, grafts were harvested, fixed, and paraffin-embedded sections were prepared.

BrdU Pulse and Immunolabeling

BrdU labeling was used to trace UGSM-2 cells in renal grafts. Subconfluent UGSM-2 cells were incubated with 10 μ M bromodeoxyuridine (BrdU) in normal culture media overnight. Overnight incubation with BrdU resulted in approximately 50% of cells with BrdU incorporated. Immunolabeling of cells in formalin fixed paraffin-embedded sections was accomplished using the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN). We used goat anti-mouse-Alexa 546 conjugated antibody (Molecular Probes) to visualize BrdU stained cells. Sections were co-stained for pan-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution. Pan-CK was visualized by incubating with goat anti-rabbit Alexa 488 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Sections were mounted with Vectashield Hardset mounting media + DAPI counterstain (Vector).

Shh Treatment

UGSM-2 cells were plated in 6-well plates at a density of 4×10^5 cells/well in complete media and allowed to attach overnight. The next day, cells were treated with 1 nm octylated N-Shh peptide (Curis, Inc., Cambridge, MA). After 48 hr, cells were lysed and RNA was collected. RNA was purified and prepared for RT-PCR as described above.

Shh Overexpression

A mammalian expression vector expressing human Shh driven by CMV promoter (pIRES2-hShh-EGFP) was constructed as described previously [26]. BPH-1 cells were transfected with pIRES2-hShh-EGFP vector or pIRES2-EGFP vector control (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen). BPH-1 cells stably overexpressing Shh/GFP were derived by fluorescence-activated cell sorting for GFP for 2 months after transfection. BPH-Shh cells stably express 50,000-fold more Shh mRNA than BPH-GFP or parent BPH-1 cells.

Statistical Analysis

An unpaired *t*-test was used to determine if significant differences exist between cell growth rates for untreated, testosterone or dihydrotestosterone treated cells. The Wilcoxon Rank Sum test was used to determine if there were significant differences in the gene expression responses to Shh treatment.

RESULTS

Isolation and Characterization of UGSM-2 Cells

Immortalized UGS mesenchymal (UGSM) cells were derived from a subline of the *INK4a* mouse, a transgenic knockout that lacks p16^{INK4a} and p19^{ARF}. Both p16^{INK4a} and p19^{ARF} are specific inhibitors of cyclin-dependent kinases Cdk4 and Cdk6 that regulate cell cycle progression [27]. Loss of p16^{INK4a} and p19^{ARF} allows mouse embryonic fibroblasts (MEFs) to escape cellular senescence. *INK4a*^{-/-} MEFs spontaneously immortalize in culture [28]. UGSM cells were isolated by dissecting UGS mesenchyme from an E16 *INK4a*^{-/-} mouse embryo (Fig. 1A). UGSM cells obtained in this fashion were propagated continuously without evidence of crisis. Immortalized mouse cells are typically tetraploid and these cells remained stably tetraploid for over 100 passages (data not shown). Several ring clones were derived and characterized. All exhibited a similar growth rate and morphology in culture and all responded to treatment with Sonic Hedgehog by upregulating transcription of the conserved Hh target genes *Ptc* and *Gli1*. One representative clonal cell line, UGSM-2, was selected for use in subsequent experiments. Like the parent mixed cell population, UGSM-2 cells were found to be stably tetraploid (Fig. 1B). Recent studies revealed that *INK4a*^{-/-} MEFs can acquire chromosomal rearrangements at high passage [29]. To assess tumorigenicity, both the parent UGSM cell line and UGSM-2 cells were co-injected with Matrigel into the flanks of nude mice. No tumor formation was observed in any of 12 injections for each group of cells over 6 months observation, whereas co-injection of LNCaP cells with Matrigel at the same time yielded tumor formation at over 80% of sites injected within 6 weeks (data not shown). Sarcoma formation was observed when a mixed population of UGSM cells at high passage (>30) were injected into nude mice, however, we have never observed sarcoma formation with the UGSM-2 clone.

UGSM-2 Cells Display a Myofibroblast Phenotype in Culture

The mesenchymal identity of UGSM-2 cells was established by characterizing expression of selected differentiation markers by RT-PCR and immunocyto-

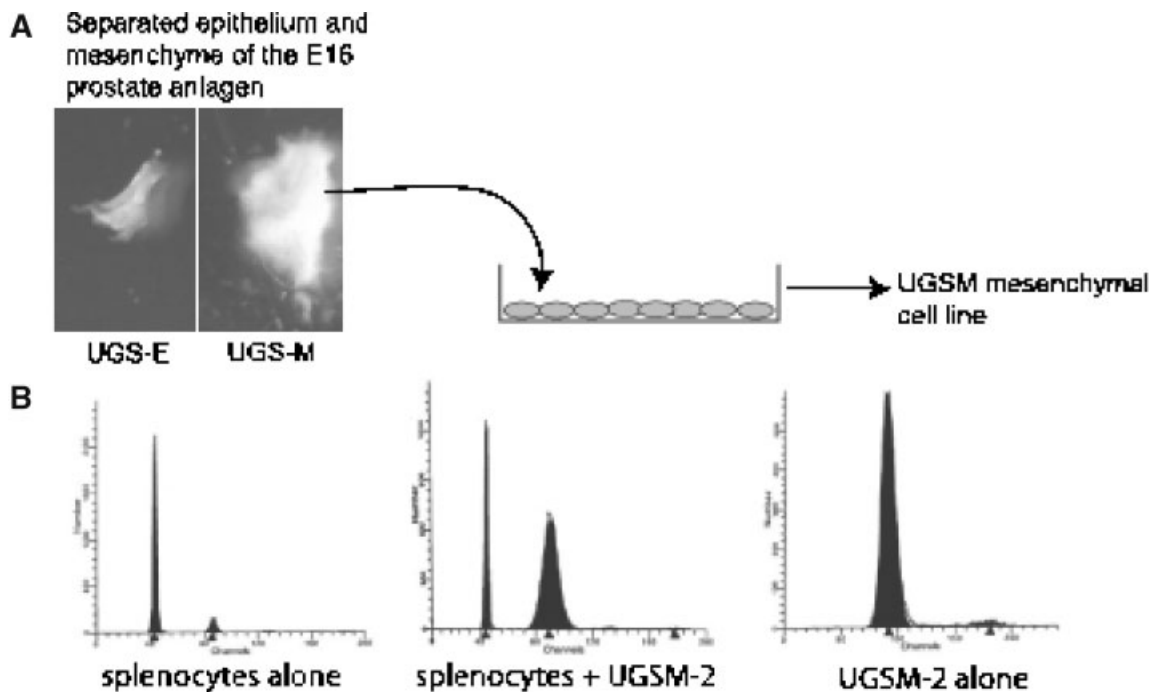


Fig. 1. Isolation of UGSM-2 cells. **(A)** Schematic of UGSM-2 isolation from the E16 UGS mesenchyme of an *INK4a*^{-/-}; *tva* transgenic mouse. **(B)** The clonal cell line UGSM-2 is tetraploid on flow cytometric analysis. Freshly isolated mouse splenocytes were used as a diploid comparison.

chemistry (Fig. 2). UGSM-2 cells express the stromal differentiation markers smooth muscle actin (SMA) and vimentin, and do not express either cytokeratins or the endothelial marker CD31/PECAM. The prostatic stroma contains cells that are classified as fibroblasts or smooth muscle, as well as cells termed myofibroblasts, which exhibit an intermediate phenotype. The profile of four stromal markers has been used to characterize cells as fibroblast (SMA⁻, vimentin⁺, desmin⁻, HCM⁻), myofibroblast (SMA⁺, vimentin⁺, desmin⁻, HCM⁻), or smooth muscle (SMA⁺, vimentin⁻, desmin⁺, HCM⁺) [30]. According to this classification UGSM-2 cells, which express SMA and vimentin, but do not express either desmin or heavy chain myosin (HCM) would be considered to exhibit a myofibroblast phenotype.

Growth Characteristics of the UGSM-2 Cell Line

Growth of many cell lines in culture is characterized by three phases: a lag phase while cells attach to the substrate; a log phase of exponential growth; and a plateau phase triggered by confluence and contact inhibition. UGSM-2 cell growth in culture exhibits all three phases of growth. The typical doubling time for UGSM-2 cells in normal culture media is 13 hr (Fig. 3A). The presence of a plateau phase shows that UGSM-2 cells are contact inhibited and, indeed, the cells at confluence adopt a tight monolayer appearance (Fig. 5A).

Androgen Response of UGSM-2 Cells

The fetal urogenital sinus mesenchyme expresses androgen receptor and the androgen response of UGS mesenchyme is an important aspect of prostate biology. We examined androgen receptor expression by RT-PCR and found that UGSM-2 cells express the androgen receptor at levels comparable to the E16 UGS. Another fibroblast cell line that is not derived from the embryonic urogenital sinus, 3T3 fibroblasts, do not express androgen receptor (Fig. 2B). UGSM-2 cells are not dependent on androgen for survival or proliferation (data not shown), however, their proliferation in culture is androgen sensitive. When we compared UGSM-2 growth in charcoal stripped serum supplemented medium without exogenous steroid hormone or with 10^{-8} M synthetic androgen R1881, we found that UGSM-2 cells cultured in the presence of androgen grow at a significantly faster rate. 3T3 fibroblasts do not increase their proliferation rate in response to androgen (Fig. 3B). The same effects were seen with either 10^{-8} M testosterone or dihydrotestosterone (data not shown).

Participation of UGSM-2 in Prostate Morphogenesis

Our goal in developing the UGSM-2 cell line was to create a genetically modifiable cell line that could be used to study specific stromal-epithelial signaling interactions in prostate development. We therefore examined the ability of UGSM-2 cells to mimic three

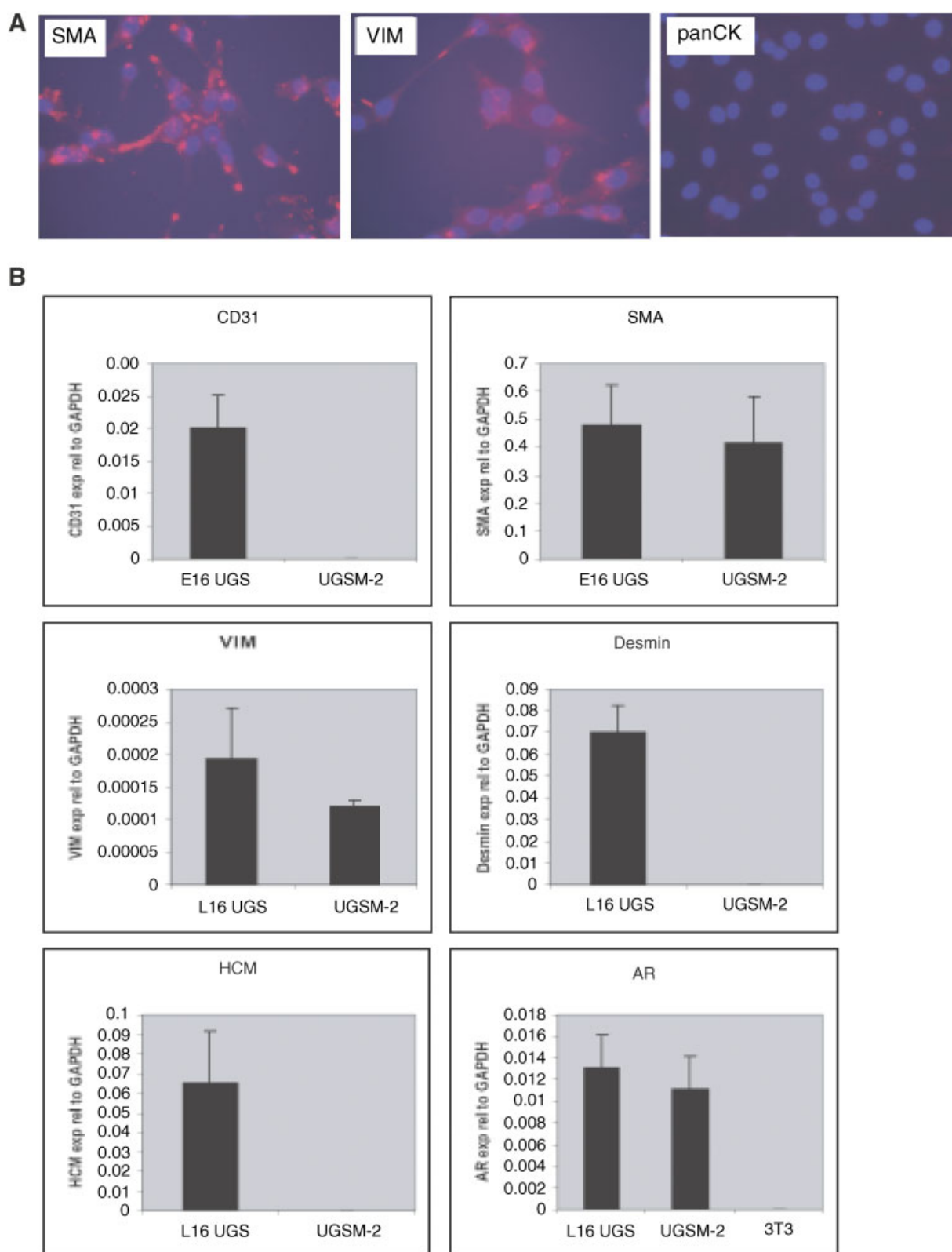


Fig. 2. (A) UGSM-2 cells immunostained for smooth muscle actin (SMA), vimentin (VIM), and high molecular weight cytokeratin (panCK). (B) Real-time RT-PCR analysis of expression of stromal differentiation markers by UGSM-2 cells and freshly isolated intact E16 urogenital sinus (UGS). Genes studied include the endothelial marker CD31, SMA, VIM, desmin, heavy chain myosin (HCM) and androgen receptor (AR). Each bar represents the mean $\pm$ sem of at least 2 independent determinations.

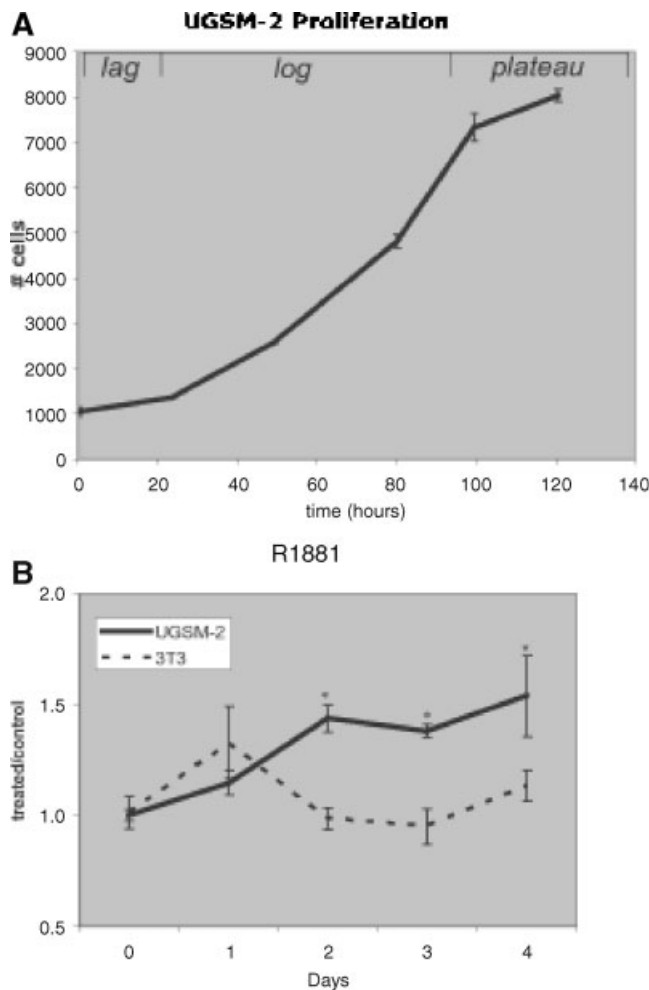


Fig. 3. Unique characteristics of UGSM cells. **(A)** Growth of UGSM-2 cells in culture. UGSM-2 cells were grown in normal INK4 medium (see Methods). Cell growth was monitored by daily Coulter counts for 1 week. Growth in culture exhibits three phases: lag, log and plateau. **(B)** Growth of UGSM-2 cells is stimulated by androgen. UGSM-2 cells were grown in charcoal-stripped serum media alone or with 10 nM synthetic androgen R1881. Cell growth was monitored by daily Coulter counts for 1 week. Data are shown as fraction of viable cells relative to untreated controls. No significant differences in cell viability were noted by Trypan blue staining (not shown). *significant increase in cell number, $P < 0.05$. All data points represent the mean of at least three independent determinations.

attributes of E16 UGS mesenchyme (UGM): the capacity to induce prostatic differentiation in the UGS epithelium (UGE), the potential to form the stromal component of prostatic glands, and the ability to mimic the signaling interactions of urogenital sinus mesenchyme. To examine the ability of UGSM-2 cells to induce prostate morphogenesis we grafted UGSM-2 cells together with isolated E16 UGE sheets under the renal capsule of adult male nude mice. When retrieved 1 month later, the resulting grafts were much smaller than grafts composed of E16 UGE and E16 UGM

(Fig. 4A) and histologic examination did not reveal any evidence of glandular morphogenesis (not shown). Therefore, UGSM-2 cells are unable to induce prostate development in this model system. To determine whether UGSM-2 cells can participate in glandular morphogenesis during prostate development, UGSM-2 cells were grafted with minced E16 UGS under the renal capsule of adult male nude mice. UGSM-2 cells were pre-labeled with BrdU to trace their fate in matured UGS/prostate. The fate of UGSM-2 cells was examined after 1, 2, and 3 weeks of growth in vivo. UGS + UGSM-2 grafts had a similar size, gross morphology (data not shown) and histology (Fig. 4B) to minced E16 UGS implanted alone. Immunohistochemical staining for BrdU showed that BrdU-labeled UGSM-2 cells were present within the periductal stroma of the mature prostate tissue (Fig. 4C). The BrdU staining in nuclei of UGSM-2 cells exhibited varying degrees of speckling that increased from 1–3 weeks (data not shown). This was interpreted as indicating active UGSM-2 proliferation during growth of the grafted tissue.

To assess the interaction of UGSM-2 cells with adult prostate epithelial cells, UGSM-2 cells were co-cultured with human prostate epithelial BPH-1 cells. After 24 hr in co-culture, BPH-1 cells became organized into tight clusters surrounded by elongated UGSM-2 cells (Fig. 5A). When UGSM-2 cells were grafted together with BPH-1 cells under the renal capsule of adult male nude mice and the grafts examined one month later, the BPH-1 cells were organized into clusters surrounded by stromal cells very similar to those observed in co-culture (Fig. 5B). Mitotic figures were common in clusters, indicating active cell proliferation. Since BPH-1 cells injected alone do not form viable grafts, these observations suggest that UGSM-2 cells and BPH-1 can participate in a rudimentary process of cellular organization and that allows BPH-1 cells to survive and proliferate.

Shh Response of UGSM-2

To determine if the UGSM-2 cell line could accurately model the mesenchymal response to Shh signaling, we assayed gene expression in UGSM-2 cells treated with Shh peptide. When treated in cell culture with purified Shh peptide, UGSM-2 cells show robust activation of the conserved Hh target genes *Gli1*, *Ptc1* and *Hip*. In addition, the Shh target gene *IGFBP6*, recently found to be upregulated in the UGS mesenchyme in response to Shh, was also induced (Fig. 6A). The three-fold increase in *IGFBP6* expression after treatment with Shh is comparable to the response of the isolated E16 UGS mesenchyme to Shh [31]. To determine whether UGSM-2 cells would respond to

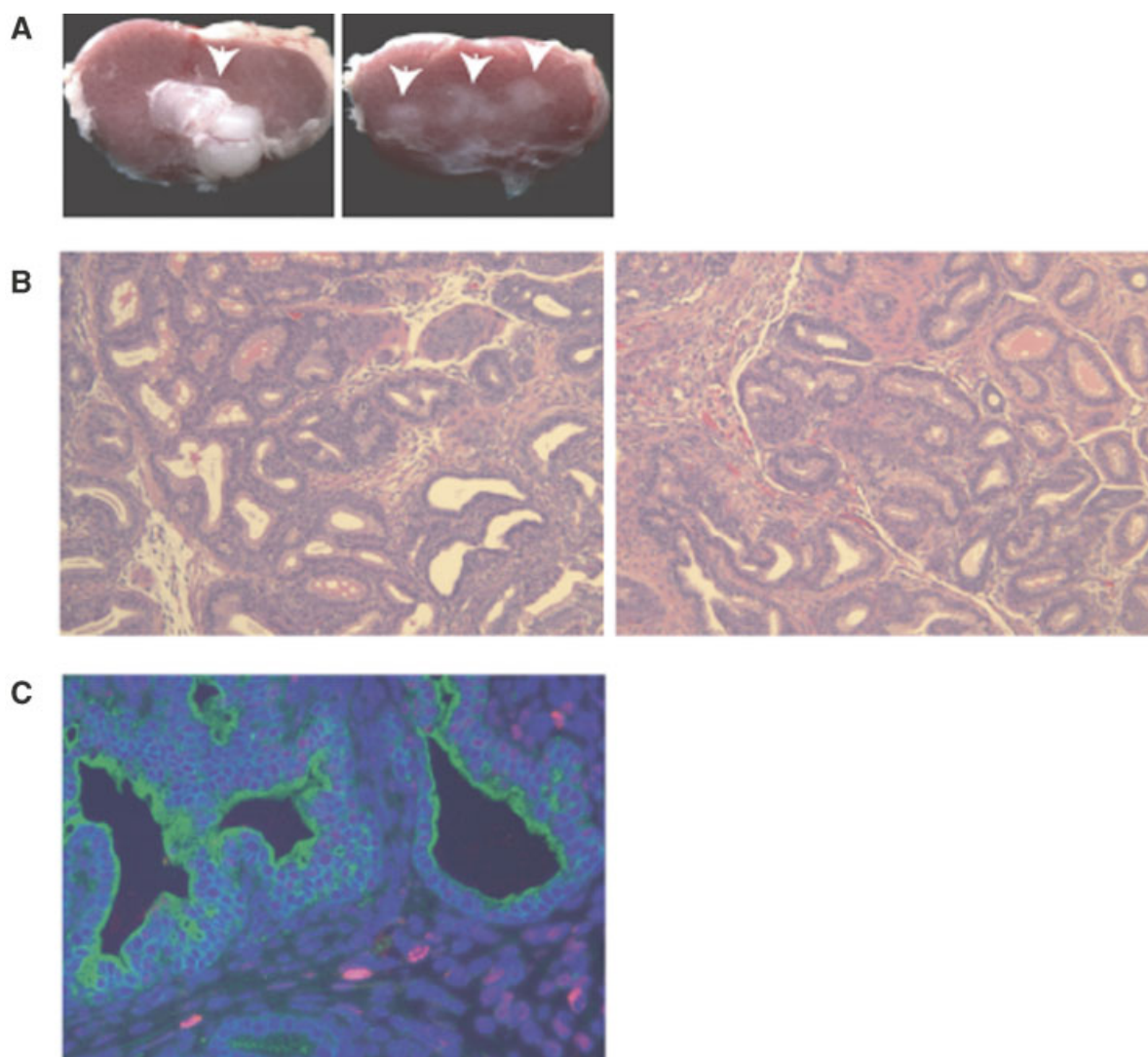


Fig. 4. Developmental fate of UGSM-2 cells in renal subcapsular grafts. **(A)** Gross morphology of 1 month renal grafts of E16 UGE combined with UGM (UGE+UGM, left) or UGSM-2 (UGE+UGSM2, right). Arrows indicate position of grafts. **(B)** H&E stained sections of UGS+UGM (left) or UGS+UGSM-2 (right) recombinants 2 weeks after grafting. **(C)** BrdU immunostaining (red) of 2 week UGS+UGSM2 renal grafts identifies UGSM-2 cells in the graft. DAPI stained nuclei are blue. Pan-cytokeratin identifies ductal epithelium in green.

Shh secreted by prostate epithelial cells in co-culture, we transfected BPH-1 cells with a Shh overexpression construct or GFP control vector (described in Fan et al., 2004). We cocultured UGSM-2 cells with the BPH-1 overexpressing or GFP control cells and analyzed Shh target gene expression using species-specific primers. This showed that overexpression of Shh by BPH-1 cells increased Gli1 and Ptc1 expression specifically in the UGSM-2 cells. There was no induction of Ptc and Gli1 in the BPH-1 cells (Fig. 6B). These experiments show that UGSM-2 cells in co-culture respond to a signaling ligand expressed by epithelial cells and can therefore mimic a stromal-epithelial interaction that plays an important role in prostate development.

CONCLUSIONS

Mechanistic studies of cell–cell interactions are facilitated by the use of genetically modified cell lines. Our long-term goal in developing the UGSM-2 cell line is to provide a tool for mechanistic studies of prostate development. We will use it to probe the mesenchymal signaling pathways that are important for prostate growth and differentiation. Urogenital sinus mesenchyme serves a critical role during prostate development as a medium for communication with developing epithelial glandular structures. Two of the signaling molecules involved in mesenchymal-epithelial communication during prostate development are androgen

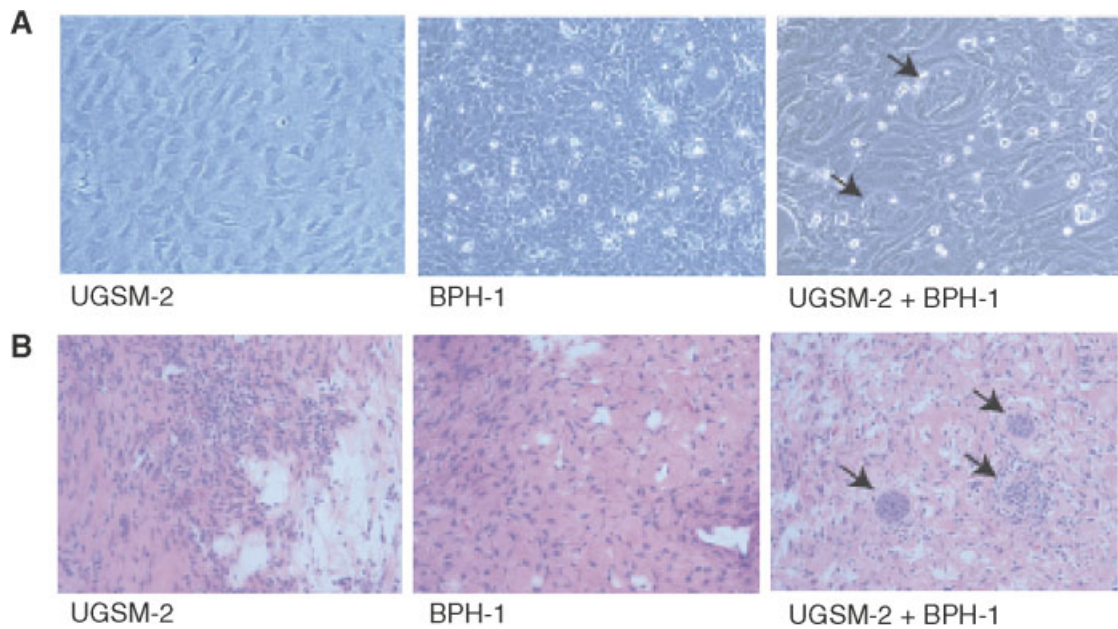


Fig. 5. Interaction of UGSM-2 and BPH-1 epithelial cells. **(A)** Photomicrograph of BPH-1, UGSM-2 co-cultures on collagen gels showing small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells. **(B)** Grafting of UGSM-2 cells alone under the renal capsule yields only stromal tissue. BPH-1 cells grafted alone do not produce identifiable viable grafts. Grafting UGSM-2 and BPH-1 cells together results in small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells.

and Sonic hedgehog. The ability of UGSM-2 cells to respond to both of these molecules makes it an appropriate tool for mechanistic studies of androgen and Sonic hedgehog activities in prostate development.

We found that these cells could not induce prostate differentiation when co-transplanted with the isolated sheets of E16 UGS epithelium tissue. However, we cannot exclude the potential of these cells to exhibit inductive potential in other assays such as one that uses dissociated UGS epithelial cells grafted under the renal capsule [32]. When UGSM-2 cells were mixed with and co-transplanted with the whole UGS, they clearly did populate the mesenchyme/stroma of the subcapsular graft. In these grafts, UGSM-2 cells took up various positions within the stroma of mature prostate. Some UGSM-2 cells were situated beside ductal epithelium, whereas others were embedded among other stromal cells in interductal stromal sheets. Although we have not analyzed stromal differentiation in these grafts, the ability of UGSM-2 cells to localize to different regions of the mature graft could indicate that they may take up both fibroblast and smooth muscle positions or functions in mature prostate tissue. Since UGSM-2 cells are able to occupy a stromal niche in developing UGS renal grafts, they may be used in *in vivo* gain and loss-of-function studies to examine the role of various gene products in early prostate development.

In addition to their ability to participate in prostate development, UGSM-2 cells form primitive acinar structures when either co-cultured or co-grafted with

human BPH-1 prostate epithelial cells. Clustering of BPH-1 cells has been observed previously when co-cultured with primary fibroblasts derived from normal human prostate, but not with primary fibroblasts derived from human prostate tumors (Simon Hayward, personal communication). Cunha has shown that the inductive relationships between epithelium and mesenchyme are preserved between human and rodents [21]. Since the interactions between human epithelial cells and rodent mesenchymal cells are preserved, recombinants composed of human epithelium and UGSM-2 cells provide a useful model system for studying the role of these interactions in prostate development. An additional strength of this model is that we can distinguish signaling in mesenchyme and epithelium using species-specific RT-PCR. This dual species cell-based model therefore allows manipulation and analysis of gene expression in both epithelial and mesenchymal components to examine mesenchymal-epithelial interactions *in vitro* and *in vivo*.

In addition to their use in co-culture and xenograft models, UGSM-2 cells can be used as a cellular model to study mesenchymal signaling pathways that are important in prostate development. The first and most obvious use is to probe the molecular mechanisms of specific pathways. For example, we have used UGSM-2 cells to examine the concentration dependence and kinetics of Gli gene activation by Shh signaling (unpublished observations). The second is to use UGSM-2 cells in microarray studies to identify specific

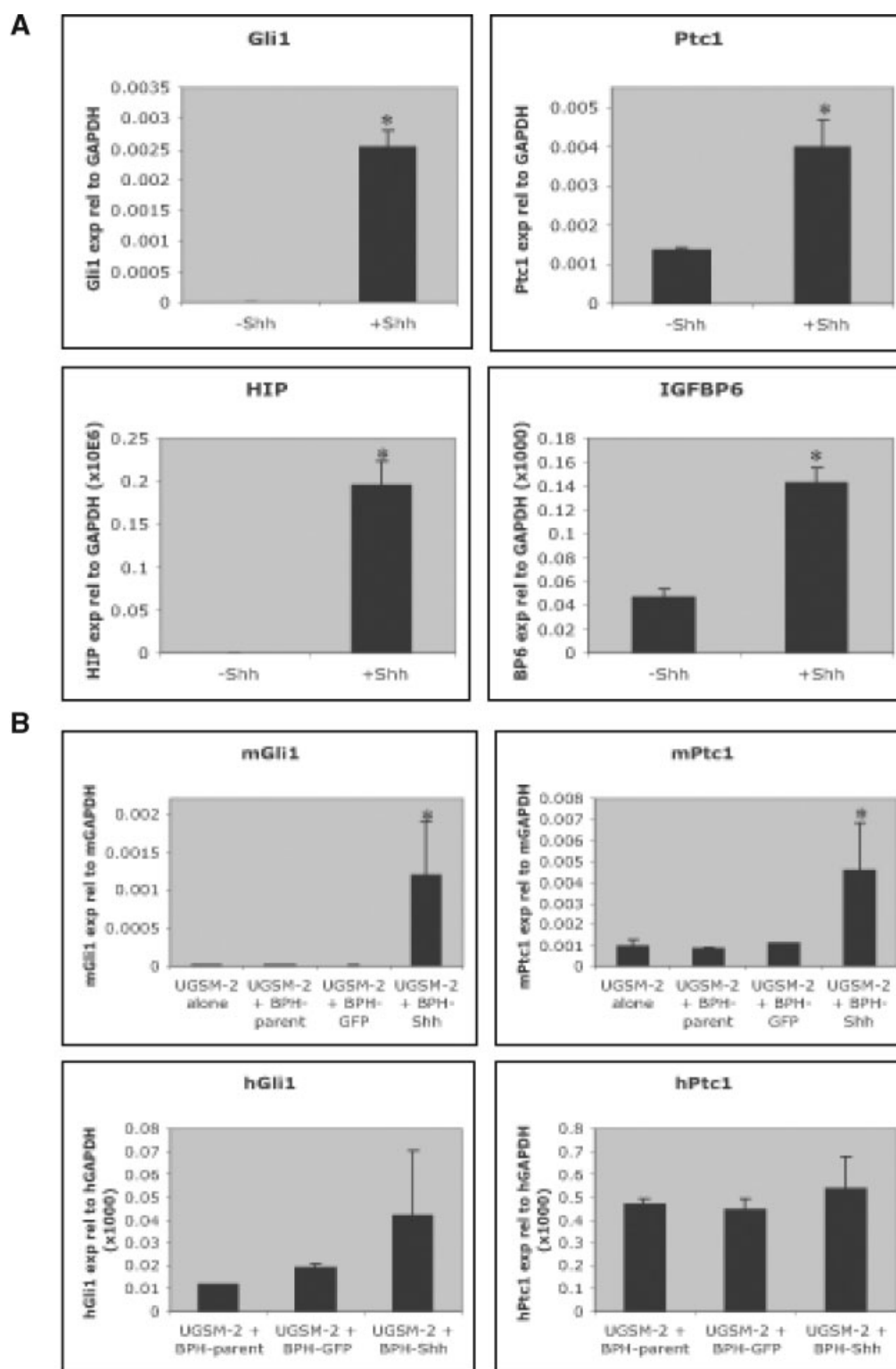


Fig. 6. Response of UGSM-2 to Shh. **(A)** Real-time RT-PCR of UGSM-2 cells treated with purified Shh peptide shows activation of Shh target genes Gli1, Ptc1, HIP and IGFBP-6. **(B)** UGSM-2 cells were co-cultured with BPH-I prostate epithelial cells stably overexpressing Shh (BPH-Shh), empty vector (BPH-GFP) or untransfected (BPH-parent). RT-PCR analysis using species-specific primers shows significant activation of Shh target genes Gli1 and Ptc1 in mouse UGSM-2 cells (top), but not in human BPH-I cells (bottom). *significantly increased from untreated or UGSM-2+BPH-GFP, $P = 0.06$. Error bar represents mean $\pm$ sem of at least 2 independent determinations.

target genes of selected inductive signals. Finally, the immortalized UGSM cells can be used for genetic gain- and loss-of-function studies. Overexpression of selected genes in UGSM-2 cells may be engineered to examine the gain-of-function effect. It should be noted that the INK4a mutant was created by insertion of a neomycin resistance gene and cell lines derived from this mouse are neomycin resistant.

Therefore, an alternative method of selection must be used when these cells are transfected. We have successfully used adenovirus, retrovirus, and plasmid vectors with hygromycin or zeocin resistance selection to express genes of interest in UGSM-2. UGSM cells are particularly useful in studying genetic changes that are lethal, since harvest of UGSM cells at E16 allows for isolation of cells even from non-viable mutants. Indeed, we have developed UGSM cell lines from INK4a^{-/-} mice bred to transgenic lines with mutations in various Shh signaling pathway components.

The potential for immortalized stromal cell lines to become tumorigenic is well recognized. The INK4a^{-/-} mutation produces impairment of G1 checkpoint control and the INK4a^{-/-} mouse is prone to develop tumors in several mesenchymal tissues [28]. A recent report shows that INK4a^{-/-} mouse embryonic fibroblasts display chromosomal rearrangements at high passage and develop the potential for sarcoma formation [29]. We have found that after 30 passages in culture, a mixed population of UGSM cells can form sarcomas when co-injected with Matrigel into nude mice. This can occur even while the cells remain contact inhibited and monolayer in culture (unpublished observations). However, we have never observed sarcoma formation with the UGSM-2 clonal cell line that was derived from the mixed UGSM population. Even so, we utilize the cells at low passage and perform sentinel grafts to monitor for sarcoma formation in all in vivo studies.

The UGSM-2 cell line and comparable cell lines derived from specific transgenic mutant mice will provide powerful tools to study signaling between prostate mesenchymal and epithelial cells. Using genetically modified UGSM cells in complementary cell-based assays, in vitro co-culture models and xenografts will allow detailed mechanistic studies of specific pathways and their influence on prostate development.

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Effect of Hedgehog Signaling on Tumor Growth is Influenced by Stromal Composition

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INTRODUCTION: We have shown previously that paracrine Hedgehog (Hh) signaling accelerates growth of the LNCaP xenograft tumor. This study was performed to determine whether the paracrine effect on tumor growth is influenced by the stromal composition.

METHODS: Immortalized mesenchymal/stromal cells were isolated from the embryonic day 16 (E16) UGS, or ventral prostate, dorsal prostate, dorsolateral prostate or coagulating gland of the adult INK4a mouse prostate. Bi-clonal xenograft tumors were created by co-injecting either LNCaP or LNCaP cells engineered to over-express Shh (LNShh) with each of the immortalized prostate stromal cell lines.

RESULTS: As compared to the canonical LNCaP xenograft tumor, all bi-clonal tumors made by co-injecting LNCaP cells with prostate stromal cells demonstrated a relative inhibition of growth, with the E16 UGS exhibiting the most potent tumor growth inhibiting effect. The effect of stromal composition on the growth response to Shh was compared in a 2x2 comparison of bi-clonal xenograft tumors made by co-injecting either LNCaP or LNShh cells with either E16 UGS mesenchymal cells or adult DLP stromal cells. Shh over-expression significantly increased growth of LNCaP xenografts made with E16 UGS mesenchymal cells but did not increase growth of xenografts made with adult DLP cells.

CONCLUSIONS: The tumor growth response to Shh is dependent upon the stromal composition of the tumor. This is an important observation which may explain why Shh stimulates growth in the developing prostate but not in the normal adult prostate. Moreover, it suggests that the paracrine effect of Shh on prostate tumor growth may depend upon the “reactive stroma” that is postulated to play a pivotal role in tumor progression.

Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth

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INTRODUCTION AND OBJECTIVES: Hedgehog (Hh) signaling is an important regulator of prostate development and we have shown previously that Shh over-expression and paracrine activation of stromal Hh pathway activity accelerates LNCaP xenograft tumor growth. In this study, we demonstrate that stromal Hh activity alone is sufficient to accelerate tumor growth.

MATERIALS AND METHODS: We examined LNCaP proliferation in xenograft tumors made by co-injecting an equal mixture of parental LNCaP and LNCaP cells over-expressing Shh (LNShh) into the flank of nude mice. We then examined the effect of constitutive Hh pathway activity in the tumor stroma. Comparison of immortalized urogenital sinus mesenchymal (UGSM) cells derived from E16 Gli3^{-/-} mice and wild type littermate demonstrated ligand-independent activation of Hh signaling in the Gli3^{-/-} cells. Bi-clonal xenografts were generated composed of LNCaP co-injected with either Gli3^{-/-} or Gli3^{+/+} UGSM cells to examine the effect of stromal pathway activation on tumor growth.

RESULTS: LNCaP/LNShh mix tumors exhibited significantly increased tumor growth rate compared to tumors made with LNCaP cells alone. Immunohistochemical staining for GFP expressed by LNShh, but not parent LNCaP showed that proliferation was significantly and selectively increased in the parent LNCaP cells rather than the LN-Shh cells. Bi-clonal xenografts containing Gli3^{-/-} UGSM cells exhibited significantly faster growth than tumors containing Gli3^{+/+} UGSM.

CONCLUSION: These studies show that activation of the Hh pathway in the stroma of LNCaP xenografts is sufficient to accelerate tumor growth and that the paracrine stimulation of growth is not preferentially directed toward cells overexpressing Hh ligand.

Hedgehog Signaling and Androgen Independent Prostate Tumor Growth

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INTRODUCTION AND OBJECTIVES: Testosterone-induced prostate regeneration is a Hedgehog (Hh)-dependent process and increased Hh pathway activity has been associated with clinically advanced, metastatic prostate cancer. To determine the role of Hh signaling in androgen independent growth, we examined the effect of hormonal manipulation on Hh pathway activity in the mouse prostate and studied the effect of Shh over-expression on androgen dependence of the LNCaP xenograft.

METHODS: Hh pathway gene expression was examined in adult mice at time zero, 14 days after castration/sham operation and 3 days after testosterone supplement. LNCaP xenograft tumors were made by injecting either parental LNCaP or LNCaP cells over-expressing Shh (LNShh) into the flank of nude mice. Mice were castrated after tumors reached 200 cubic mm. Immortalized urogenital sinus mesenchymal (UGSM) cells were derived from E16 Gli3^{-/-} mouse embryos and found to exhibit ligand-independent pathway activity. Bi-clonal tumors with constitutive stromal Hh pathway activation were generated by co-injecting LNCaP cells with Gli3^{-/-} UGSM cells

RESULTS: Expression of the Hh ligands Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) is increased 14 days post-castration and this is paralleled by increases in the Hh target genes Ptc and Gli1. Expression levels of all of these genes return to baseline levels 3 days after testosterone supplementation. Growth of established LN-Shh xenograft tumors is unabated after castration of the host mouse. However, castration of mice bearing LNCaP + Gli3^{-/-} UGSM bi-clonal xenografts resulted in tumor involution.

CONCLUSIONS: Hh pathway activity is increased in the mouse prostate following castration and LNCaP tumor cell over-expression of Shh allows androgen-independent growth of established xenograft tumors. This effect cannot be mimicked by activation of the Hh pathway in the stroma of a bi-clonal xenograft, suggesting that androgen independence may depend on a more robust level of paracrine pathway activation, non-canonical autocrine signaling or Hh pathway activation in unique elements of the host stroma (eg. endothelial cells).

Stromal Hh Pathway Activity Accelerates Prostate Tumor Growth

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Hedgehog (Hh) signaling is an important regulator of prostate development and we have shown previously that Shh over-expression and paracrine activation of stromal Hh pathway activity accelerates LNCaP xenograft tumor growth. In this study, we demonstrate that stromal Hh activity alone is sufficient to accelerate tumor growth. We examined LNCaP proliferation in xenograft tumors made by co-injecting an equal mixture of parental LNCaP and LNCaP cells over-expressing Shh (LNShh) into the flank of nude mice. LNCaP/LNShh mix tumors exhibited significantly increased tumor growth rate compared to tumors made with LNCaP cells alone. Immunohistochemical staining for GFP expressed by LNShh, but not parent LNCaP showed that proliferation was significantly and selectively increased in the parent LNCaP cells rather than the LN-Shh cells. Thus, Shh does not have an autonomous effect only on LNShh cells, but effects proliferation of all LNCaP within a tumor. We then examined the effect of constitutive Hh pathway activity in the tumor stroma. Comparison of immortalized urogenital sinus mesenchymal (UGSM) cells derived from E16 Gli3^{-/-} mice and wild type littermate demonstrated ligand-independent activation of Hh signaling in the Gli3^{-/-} cells. Bi-clonal xenografts composed of LNCaP co-injected with either Gli3^{-/-} or Gli3^{+/+} UGSM cells were generated to examine the effect of stromal pathway activation on tumor growth. Bi-clonal xenografts containing Gli3^{-/-} UGSM cells exhibited significantly faster growth than tumors containing Gli3^{+/+} UGSM. These studies show that activation of the Hh pathway in the stroma of LNCaP xenografts is sufficient to accelerate tumor growth and that the paracrine stimulation of growth is not preferentially directed toward cells over-expressing Hh ligand.

Hedgehog Signaling and Androgen Independent Prostate Tumor Growth

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Increased Hedgehog (Hh) pathway activity has been associated with clinically advanced, metastatic prostate cancer. To examine the role of Hh signaling in androgen independence, we examined the effect of hormonal manipulation on Hh pathway activity in the mouse prostate and tested the effect of Shh over-expression on growth of the LNCaP xenograft in a castrated host. Hh pathway gene expression was examined in adult mice at time zero, 14 days after castration/sham operation and 3 days after testosterone supplement. Expression of the Hh ligands Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) is increased 14 days post-castration and this is paralleled by increases in the Hh target genes Ptc and Gli1. Expression levels of all of these genes return to baseline levels 3 days after androgen supplementation. This observation, coupled with the previously reported finding that chemical blockade of Hh signaling prevents testosterone-induced re-growth, suggests that Hh signaling maintains progenitors that are required to regenerate the prostate in response to androgen supplementation. LNCaP xenograft tumors were made by injecting either parental LNCaP or LNCaP cells over-expressing Shh (LNShh) into the flank of nude mice. Mice were castrated after tumors reached 200 cubic mm. Whereas the canonical xenograft undergoes involution and growth arrest, growth of LN-Shh xenograft tumors is unabated after castration. As we have shown previously, Shh over-expression in the LNShh tumor activates paracrine signaling without any detectable activation of autocrine signaling. We therefore tested whether paracrine signaling is sufficient for androgen independent tumor growth by generating LNCaP tumors containing stromal cells with activated Hh signaling. Immortalized urogenital sinus mesenchymal (UGSM) cells were derived from E16 Gli3^{-/-} mouse embryos and found to exhibit ligand-independent pathway activity. Bi-clonal tumors with constitutive stromal Hh pathway activation were generated by co-injecting LNCaP cells with Gli3^{-/-} UGSM cells. Castration of mice bearing LNCaP + Gli3^{-/-} UGSM bi-clonal xenografts resulted in tumor involution. These studies suggest that increased Hh pathway activity in the mouse prostate following castration is involved in maintaining androgen-independent progenitors and that Shh over-expression in LNCaP tumor cells allows androgen-independent growth of established xenograft tumors. This effect cannot be mimicked by activation of the Hh pathway in the stroma of a bi-clonal xenograft, suggesting that androgen independence may depend on a more robust level of paracrine pathway activation, non-canonical autocrine signaling or Hh pathway activation in unique elements of the host stroma not represented by the UGSM cells (eg. endothelial cells).