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14. ABSTRACT: We have shown that the <i>Sonic hedgehog</i> (<i>Shh</i>) signaling pathway is absolutely required for normal prostate development and our studies performed with the support of the DOD New Investigator award indicate that <i>Shh</i> signaling promotes tumor growth. This proposal addresses the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression . To address this hypothesis, we have developed the DLPlacZ-LNCaP xenograft, a model that provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We will use this model to: (1) determine whether <i>Shh</i> promotes tumor growth by activating expression of <i>Gli-1</i> in the tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of a specific stromal <i>Shh</i> target genes in tumor growth.					
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INTRODUCTION:

To address the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression, we planned to use a xenograft tumor model in which LNCaP cells are co-injected with cloned, immortalized lacZ expressing stromal cells. The value of this tumor model is that it would provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We planned to use this model to: (1) determine whether Shh promotes tumor growth by activating expression of Gli1 in tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of specific stromal Shh target genes in tumor growth.

BODY:

Studies performed before this proposal was funded compared xenograft tumors made with co-injection of stromal cells from the urogenital sinus or from the different lobes of the adult mouse prostate. Based on those studies, we elected to use a mesenchymal cell line in the experiments proposed. A paper describing the isolation and characterization of the UGSM-2 cell line has been accepted for publication in The Prostate. A copy of that paper is attached.

As proposed in Specific Aim1, we have characterized the growth and androgen dependence of the LNCaP-UGSM2 xenograft and shown that Shh overexpression increases growth of the xenograft tumor (Figure 1).

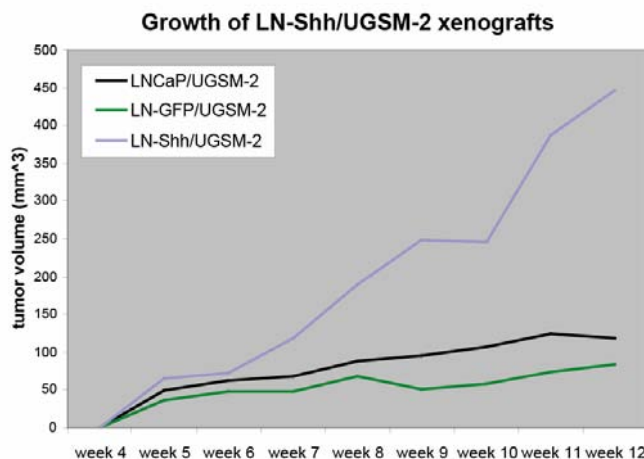


Figure 1. Shh-induced growth of LNCaP-UGSM2 tumors.

To verify that paracrine Hh signaling underlies the Shh growth effect, we analyzed the ability of Shh to induce growth of parent LNCaP in tumors composed of an equal mixture of parent LNCaP and LN-Shh cells. The tumors composed of a mixture of parent LNCaP and LN-Shh cells grew at a

faster rate than parent LNCaP tumors, validating the Shh effect in these tumors. Surprisingly, tumors generated by injecting a mixture of LNCaP and LN-Shh cells were composed primarily of parent LNCaP cells after 8 weeks of growth in vivo, confirming that Shh does not have a specific effect on LN-Shh cells, but rather induces paracrine factors that induce growth of parent LNCaP (Figure 2).

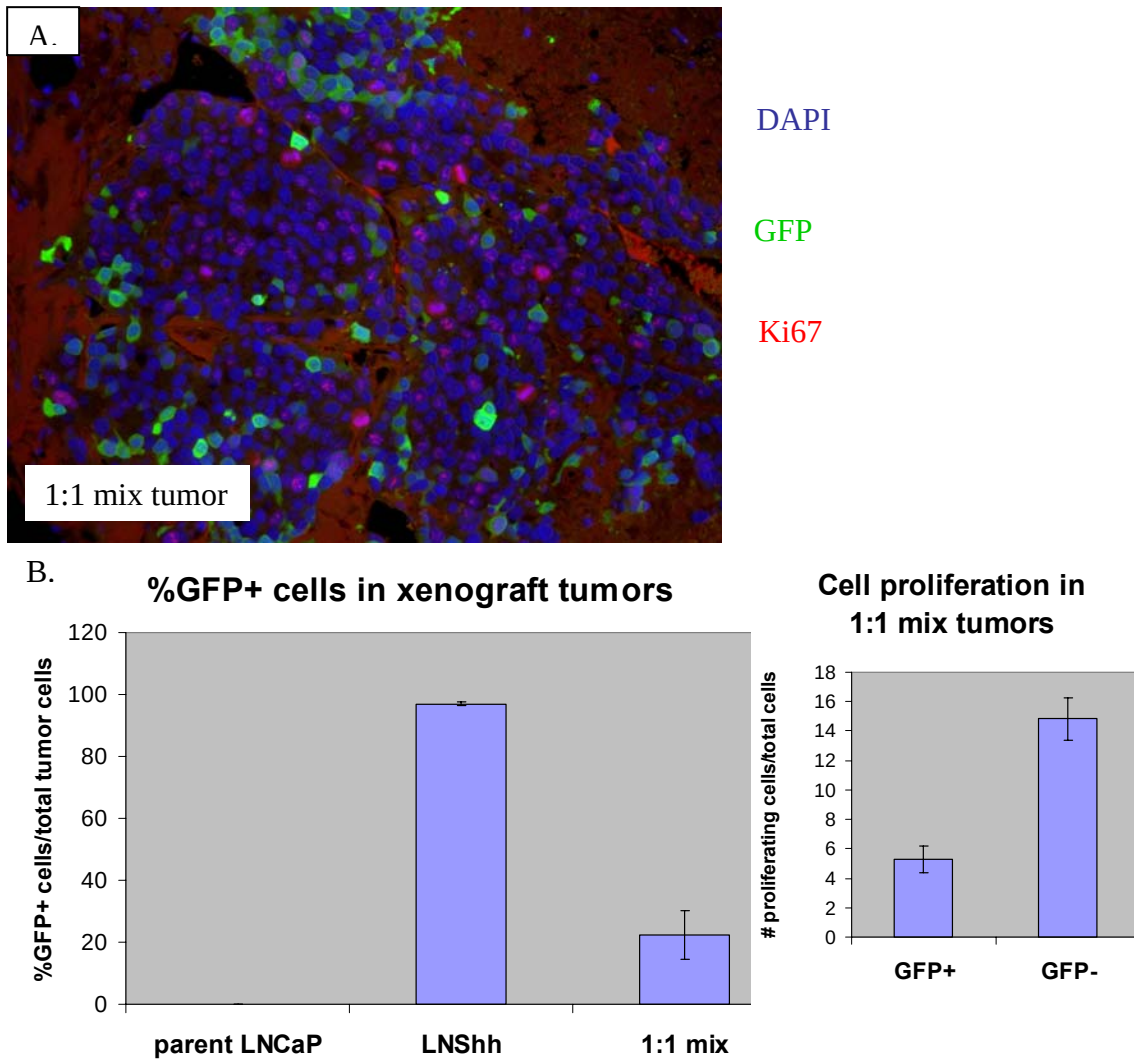


Figure 2. Shh signaling induces paracrine signaling that stimulates growth of LNCaP. (A) immunohistochemical staining for GFP and Ki67 shows that parent LNCaP are the main component of LNCaP + LN-Shh-GFP 1:1 mix tumors. (B) Enumeration of GFP+ LN-Shh and GFP- parent LNCaP in tumors and corresponding proliferation rates for each in 1:1 mix tumors. Tumors are composed mainly of parent LNCaP, supporting that Shh does not preferentially induce proliferation of cells that secrete it and that the Shh growth effect occurs by activating Hh signaling in cells other than LN-Shh.

To determine if stromal Hh signaling activation mimics the Shh growth effect, we generated a stromal cell line that lacks Gli3, a repressor of Hh signaling. Gli3 null stromal cells exhibit active Hh signaling in the absence of Shh. Co-injection of Gli3 null stromal cells resulted in rapid growth of tumors when compared with tumors containing wild-type stromal cells (Figure 3).

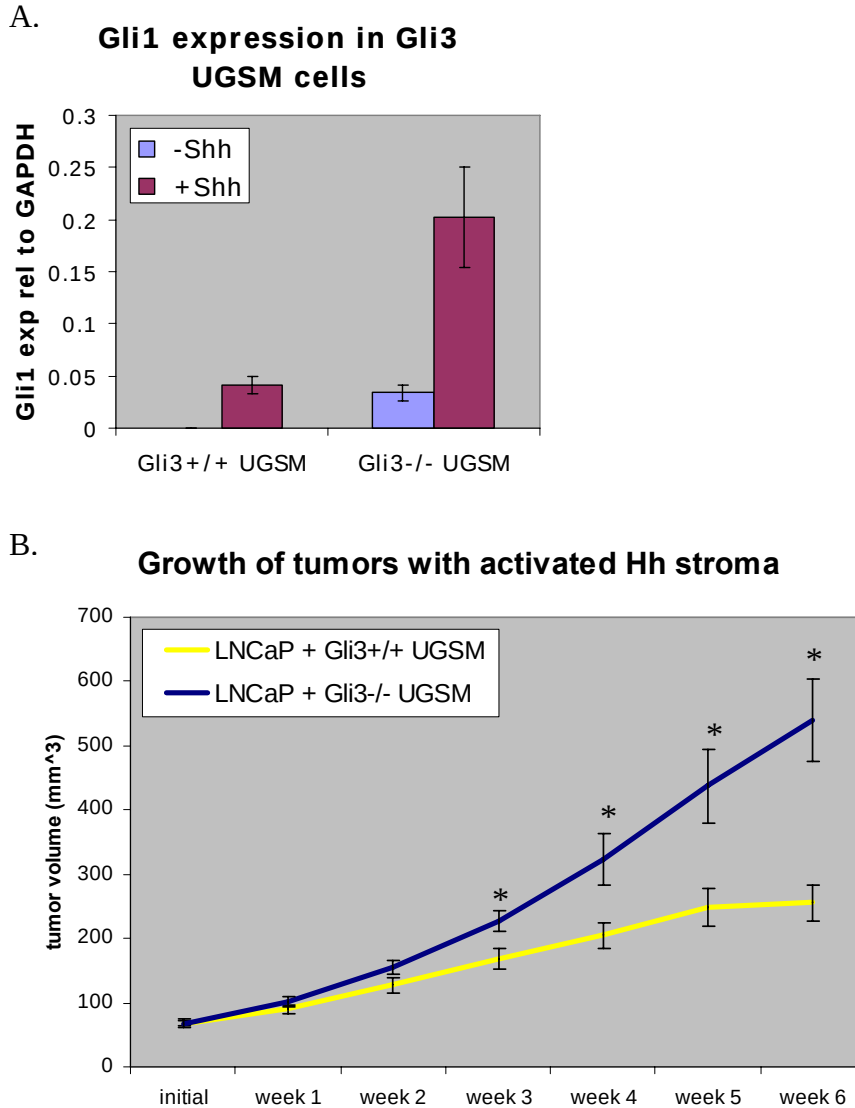


Figure 3. Stromal Hh signaling induces rapid tumor growth. (A) Gli3^{-/-} cells have activated Hh signaling in the absence of Hh ligand. (B) Tumors containing Gli3^{-/-} stromal cells grow faster than tumors containing wild-type stromal cells.

Detailed analysis of Hh signaling in LNCaP revealed that LNCaP do not respond to Shh by increasing expression of the canonical Hh signaling mediators Gli1 and Ptc1 (Figure 4). In fact, expression of Smo in 22RV1 or PC-3 cells does not induce pathway activation as it does in other cell lines (Figure 5). However, expression of Gli1 or Gli2 in 22RV1 or PC-3 cells does induce transcription of Hh target genes Gli1 and Ptc (Figure 6). These studies revealed that intracellular Hh signal transduction in LNCaP is functionally impaired and pathway target genes can only be induced by expression of the final mediators of the Hh transcriptional response. We have recently found that Hh signaling is similarly impaired in LNCaP (not shown), lending credence to the idea that LNCaP are not capable of Hh signal transduction and growth effects on tumors must be mediated by paracrine interactions with other cells in the tumor.

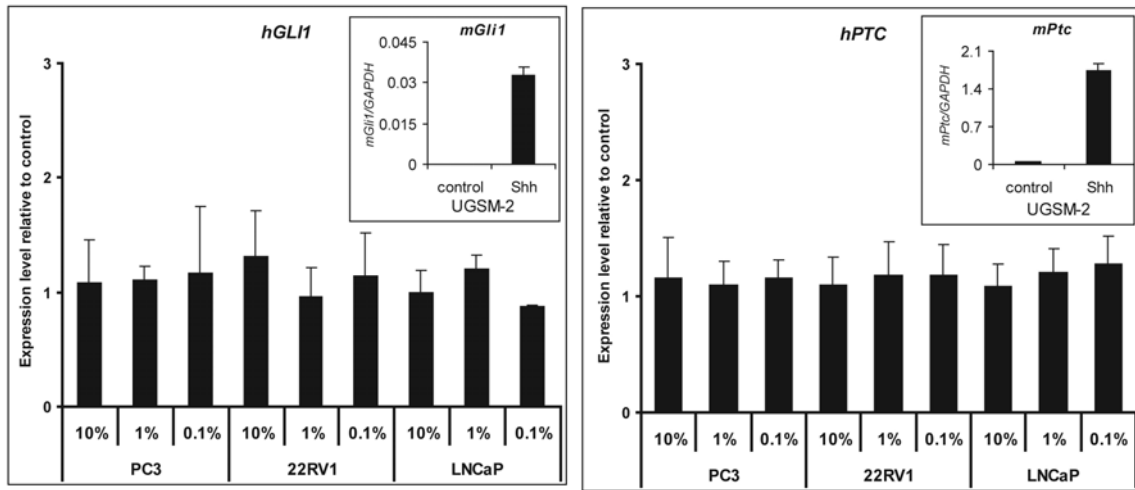


Figure 4. Treatment of Prostate cancer cell lines with 1 nM purified Shh peptide does not induce expression of Hh target genes *Ptc* and *Gli1*. (inset) Treatment of UGSM-2 mesenchymal cells with the same dose of Shh causes a ~100-fold increase in expression of Hh target genes *Ptc* and *Gli1*.

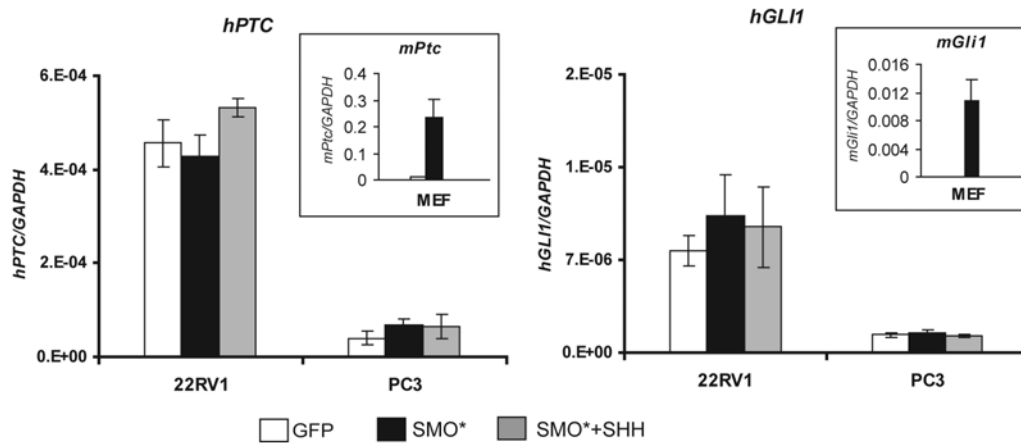


Figure 5. Expression of constitutively active Smo, an inducer of Hh signaling, fails to induce expression of Hh target genes in 22RV1 and PC-3 human prostate cancer cell lines, but the same expression construct faithfully induces Hh signaling in mouse embryonic fibroblasts (inset).

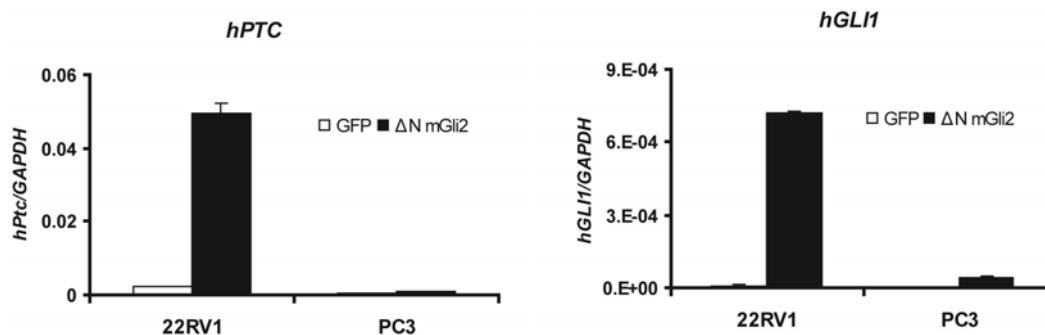


Figure 6. Expression of constitutively active Gli2 in 22RV1 or PC-3 cells induces Hh signaling.

Collectively, these results indicate that Shh expression by LNCaP in LN-Shh tumors induces stromal Hh signaling that leads to accelerated tumor growth.

As proposed in Specific Aim2, we have identified new Hh target genes in stromal cells and have begun correlating expression of these genes with accelerated tumor growth in LN-Shh tumors. We have identified 40 new target genes and are currently in the process of determining if these genes are modified in LN-Shh tumors with accelerated growth. On the basis of its known role in prostate development, we analyzed expression of Noggin in LN-Shh vs. parent LNCaP tumors. Noggin expression is significantly elevated in LN-Shh tumor stroma (Figure 7).

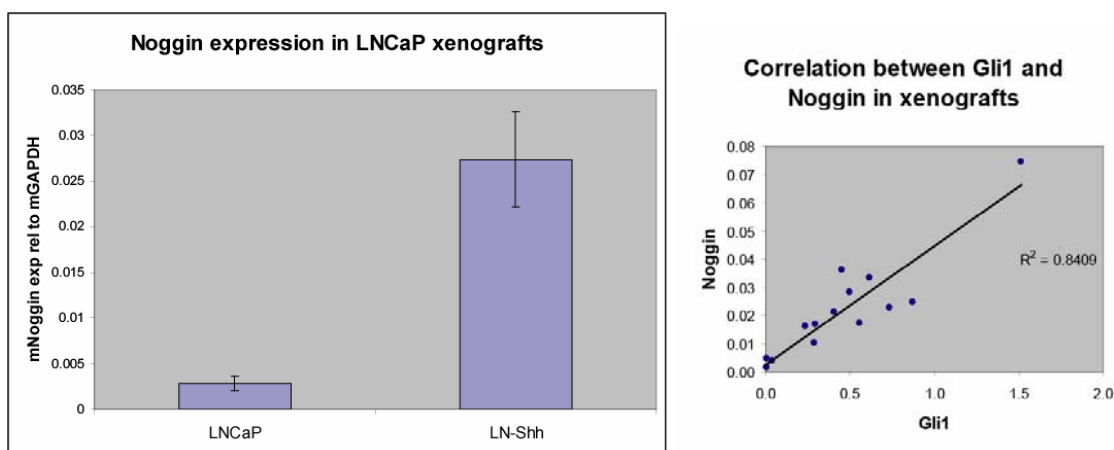


Figure 7. Noggin expression is increased in stroma of LN-Shh tumors and correlates with stromal Gli1 expression.

Noggin is a secreted BMP antagonist and blocks BMP inhibition of LNCaP proliferation in vitro (Figure 8). BMP signaling correlates with expression of Id-1 in LNCaP and LN-Shh tumors exhibit decreased BMP signaling as a result of Noggin overexpression in the stroma (Figure 9).

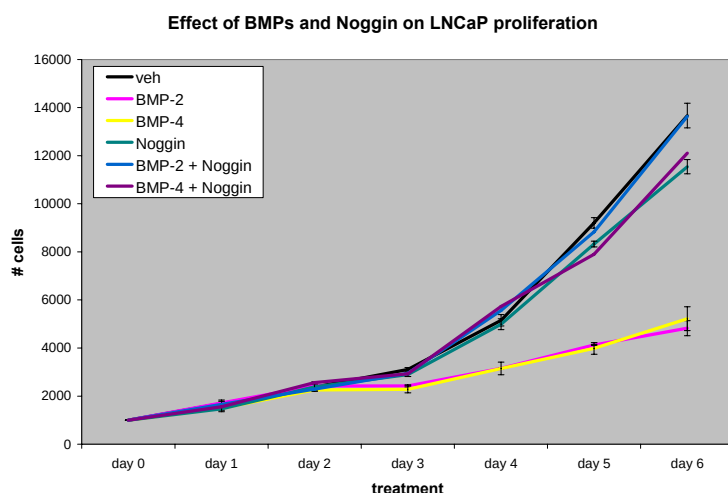


Figure 8. Growth of LNCaP in culture was measured daily after treatment with recombinant proteins. BMP-2 and BMP-4 inhibited cell growth while Noggin had no effect. However treatment with BMP together with Noggin reversed the BMP inhibitory effect.

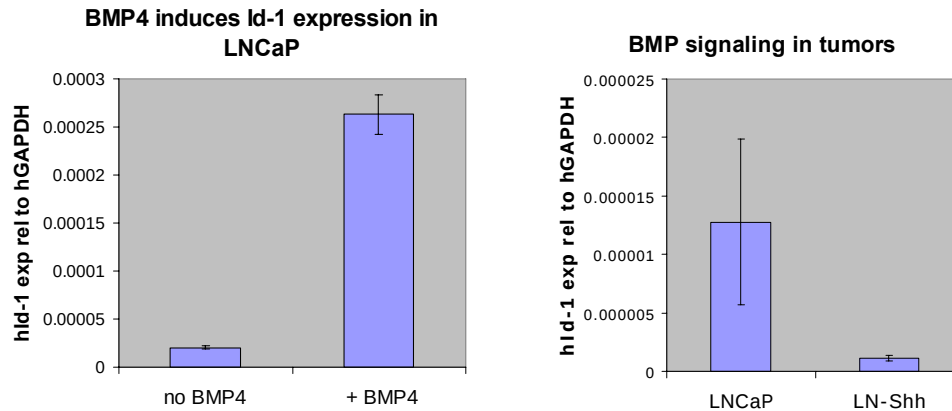


Figure 9. Reduced BMP signaling in LN-Shh tumors. BMP4 treatment in vitro induces expression of Id-1 in LNCaP. LN-Shh tumors that express high levels of the BMP antagonist Noggin have reduced BMP signaling, as evidenced by Id-1 expression.

We are currently in the process of identifying other Shh target genes whose expression is modified in LN-Shh tumor stroma. As these genes are identified we will determine appropriate ways to examine the effect of these factors on tumor growth.

To perform the studies described in Specific Aim 3, we have generated UGSM-2 cells which overexpress Smo, Gli1 or Gli2 and UGSM-2 cells which lack Gli1 and Gli2 function. A newly identified dominant negative Gli2 mutant holds promise as a means of repressing Hh signaling in UGSM-2 cells. We have determined that Gli1 and Gli2 overexpression is toxic and lethal to LNCaP, but LNCaP stably overexpressing Smo have been obtained.

The second portion of Specific Aim 3 is to examine the effect of stromal Hh target expression on tumor growth. To this end, we have stably overexpressed Noggin in UGSM-2 stromal cells and generated tumors by combining LNCaP + UGSM2-Noggin cells. Analysis of the growth of these tumors showed that Noggin overexpression in tumor stroma does not accelerate tumor growth, nor is Noggin overexpression additive with Shh for inducing tumor growth (Figure 10). Thus, Noggin does not act alone to stimulate tumor growth.

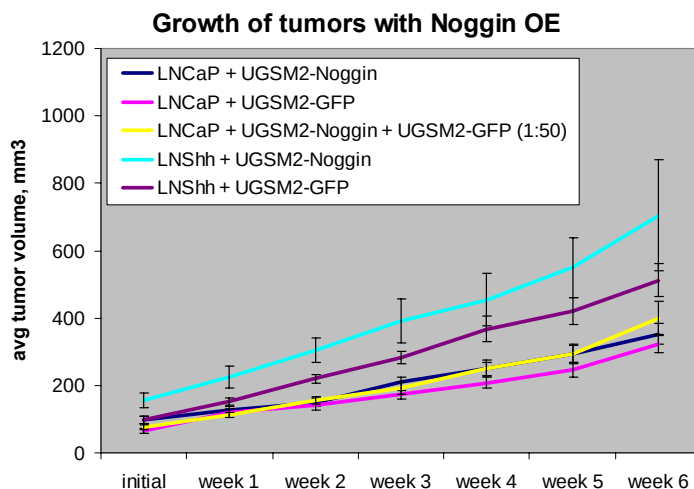


Figure 10. Noggin over-expression in tumor stroma does not stimulate tumor growth. Stromal Noggin overexpression does not induce tumor growth in addition to Shh.

We are currently in the process of generating stromal cells lines that lack Noggin to examine the requirement for stromal Noggin in the Shh growth effect. As new Shh target genes are identified in Specific Aim 2, we will examine the contribution of these genes to Shh-induced tumor growth.

KEY RESEARCH ACCOMPLISHMENTS:

We have developed and characterized a new mesenchymal cell line that will be used to genetically manipulate gene expression in tumor cells, tumor stromal cells or both.

We have confirmed that Shh-induced rapid tumor growth also occurs in bi-clonal xenograft tumors created by co-injecting LNCaP cells with UGSM-2 cells.

We have shown that paracrine Hh signaling is the primary mechanism of Shh-induced tumor growth.

We have generated a set of retroviral expression constructs containing Shh pathway genes for use in determining the role of each of these genes in Shh accelerated tumor growth. We have generated UGSM and LNCaP cell lines stably expressing these genes.

We have generated stromal cell lines lacking Gli1 and Gli2 for the purpose of identifying stromal Gli1 and Gli2 roles in Shh-induced tumor growth.

We have identified Noggin as a gene overexpressed in stroma of LN-Shh tumors. Stromal Noggin overexpression alone is not sufficient to accelerate tumor growth.

REPORTABLE OUTCOMES:

A manuscript which describes the isolation and characterization of the UGSM-2 cell line is in press for publication in Prostate (manuscript attached).

A manuscript examining hedgehog signaling in human prostate cancer cell lines has been accepted for publication in the Journal of Urology (manuscript attached).

A review of hedgehog signaling in prostate cancer has been accepted for publication in the Journal of Urology (manuscript attached).

Invited Speaker – SBUR December, 2005 Miami Beach, Florida

SBUR Keynote Speaker Award, 2005 Miami Beach, Florida

CONCLUSIONS:

We are making good progress on the approved aims of the grant and I anticipate no significant changes in our research plan.

REFERENCES:

None

APPENDIX:

Manuscripts attached

Author Proof

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* 9999: 1–12, 2005. © 2005 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 (Lipinski et al., [submitted](#)^{Q1}), 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 mesenchyme. 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

TABLE I. Sequences of RT-PCR Primers

Target gene	Forward primer	Reverse primer
mGAPDH	AGCCTCGTCCCGTAGACAAAT	CCGTGAGTGGAGTCATACTGGA
mSMA	ATCATGCGTCTGGACTTGG	AATAGCCACGCTCAGTCAGG
mVim	CCCCCTTCCTCACTTCTTTC	AAGAGTGGCAGAGGACTGGA
mDesmin	GTGAAGATGGCCTTGGATGT	TTGAGAGCAGAGAAGGTCTGG
mHCM	GCAGCTTCTACAGGCAAACC	CAAAGCGAGAGGAGTTGTCTG
mAR	GTGAAGCAGGTAGCTCTGGG	GAGCCAGCGGAAAGTTGTAG
mCD31	CTGAGCCTAGTGTGGAAGCC	TACATCCATGTTCTGGGGGT
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
mPtc1	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
mIGFBP6	AGCTCCAGACTGAGGTCTTCC	GAACGACACTGCTGCTTGC
mHIP	CCTGTCGAGGCTACTTTTCG	TCCATTGTGAGTCTGGGTCA
hGAPDH	CCACATCGCTCAGACACAT	GCAACAATATCCACTTTACCAGAGTTAA
hGli1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hPtc1	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA

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 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.

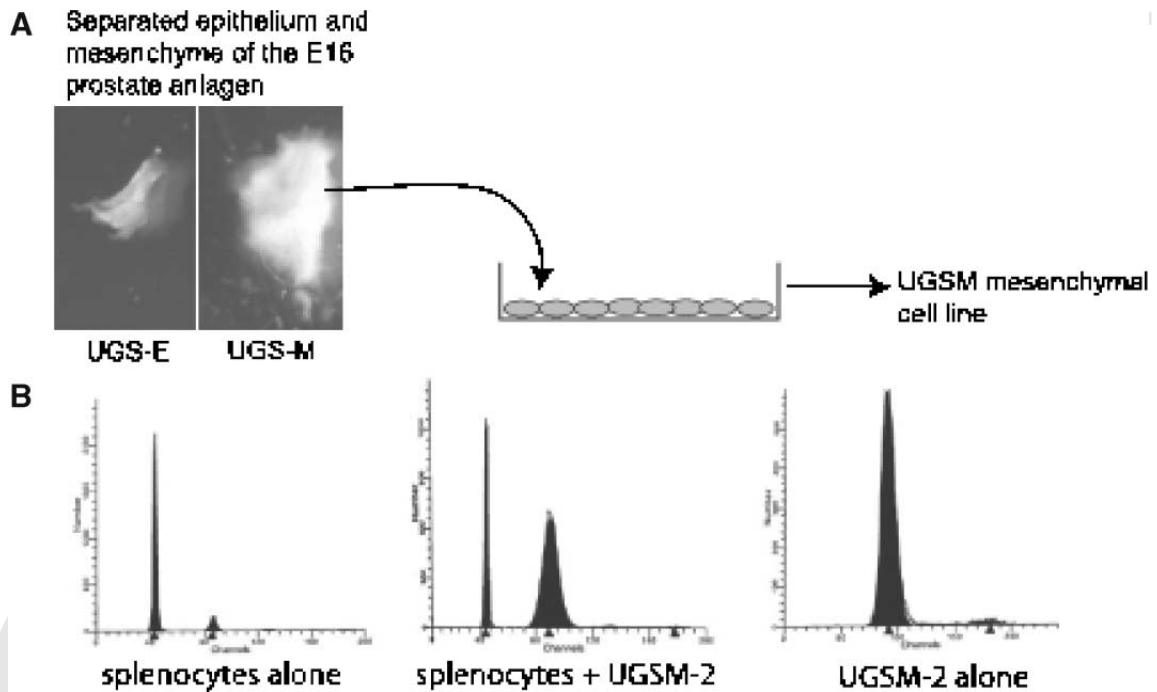


Fig. 1. [XXXXX^{Q2}](#).

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 immunocytochemistry (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).

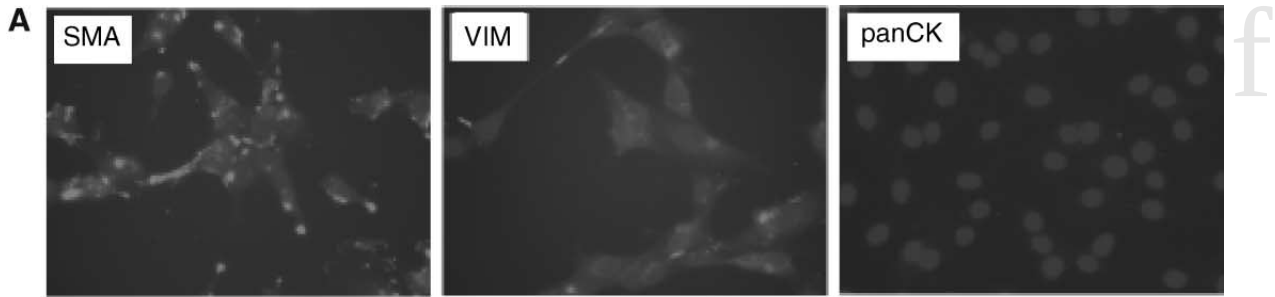
The presence of a plateau phase shows that UGSM-2 cells are contact inhibited and, indeed, the cells at confluence adopt a tight monolayer appearance (Fig. 5A).

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



B

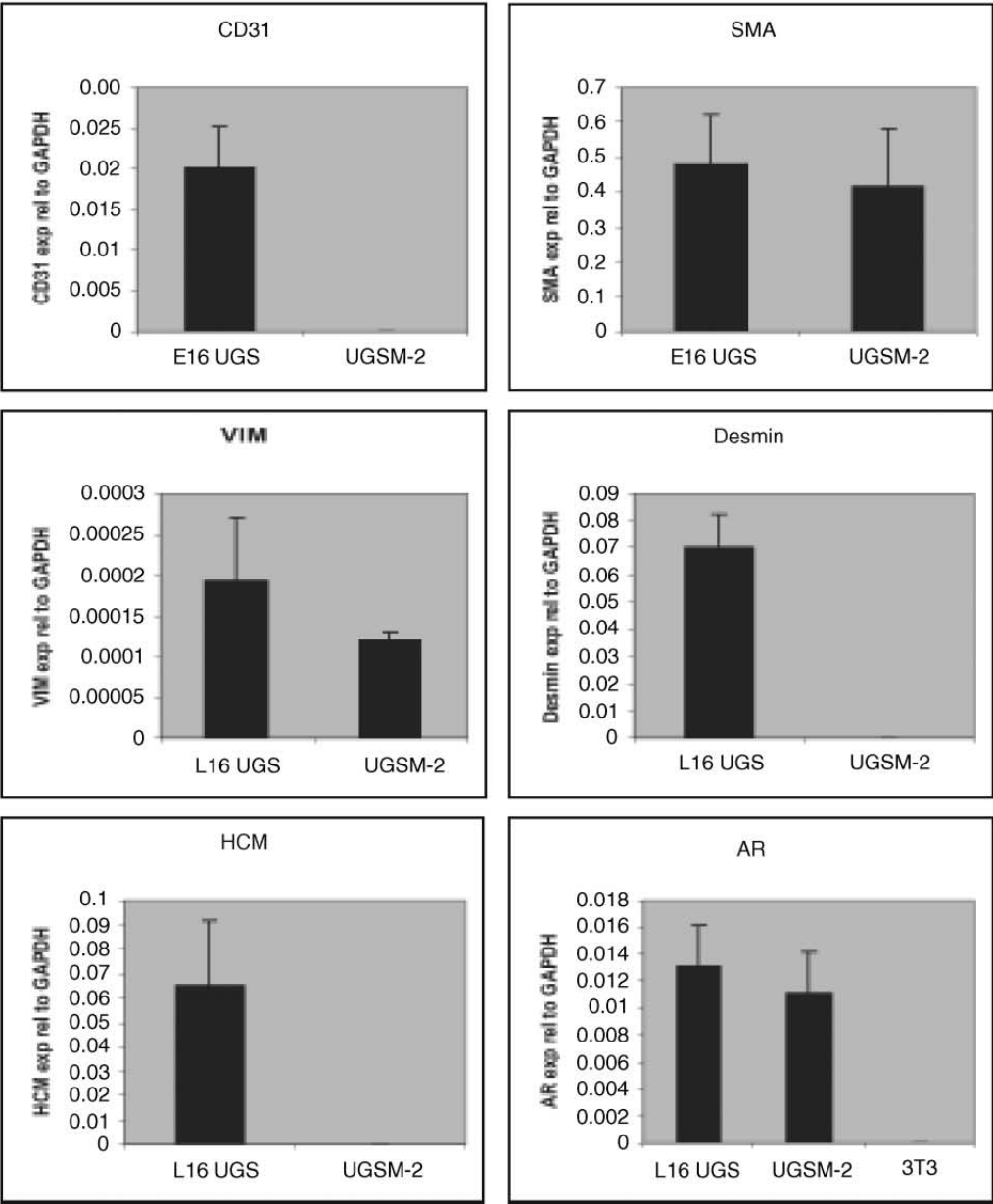


Fig. 2. Xxxxx.

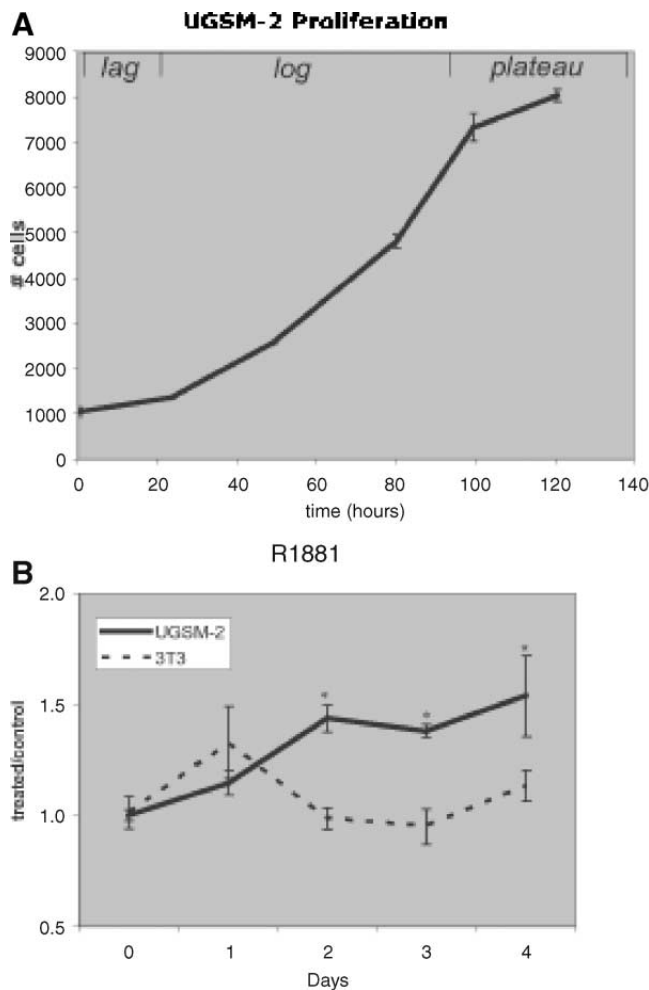


Fig. 3. Xxxxx.

interactions in prostate development. We therefore examined the ability of UGSM-2 cells to mimic three 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 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

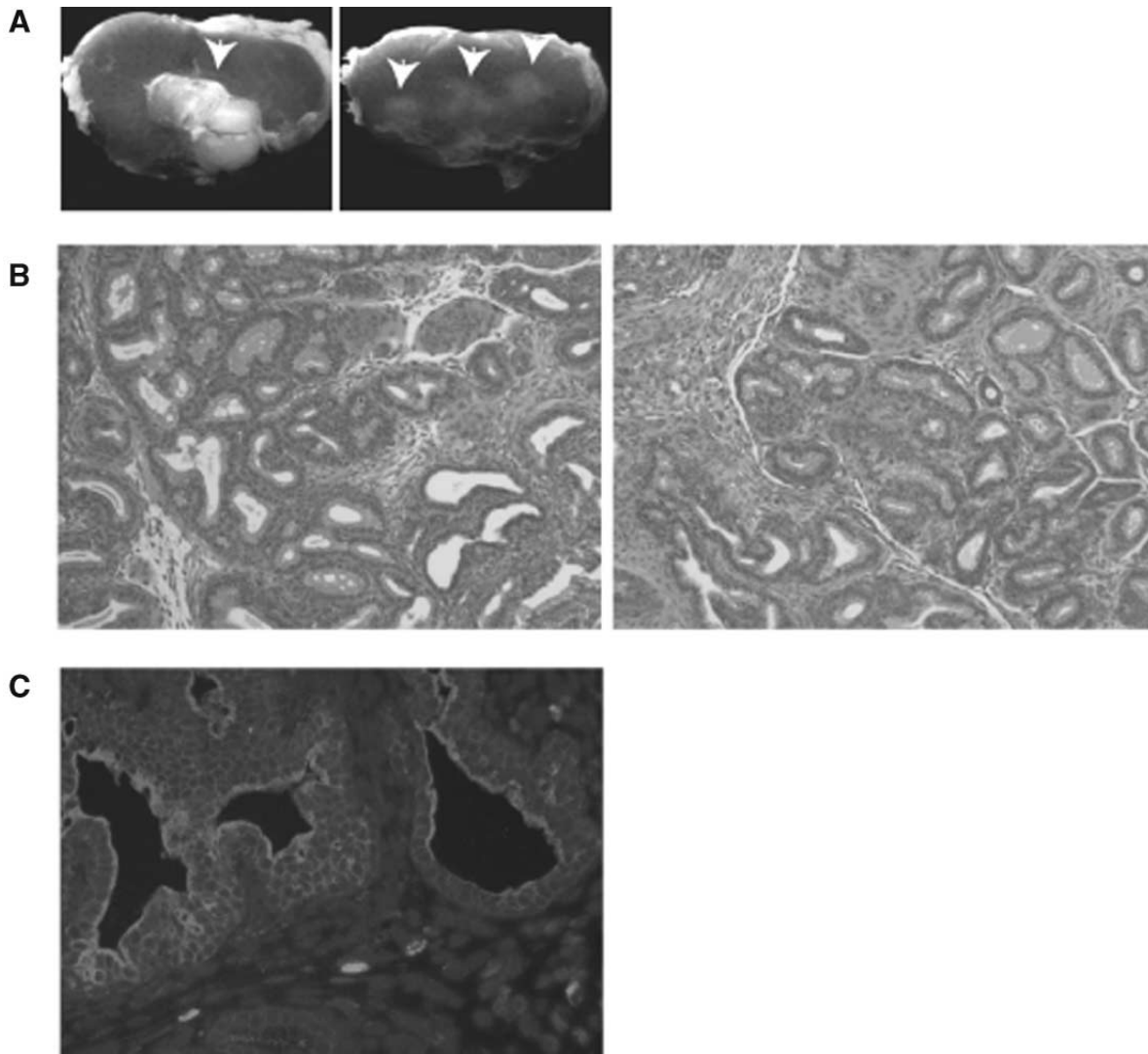


Fig. 4. XXXXX.

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 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

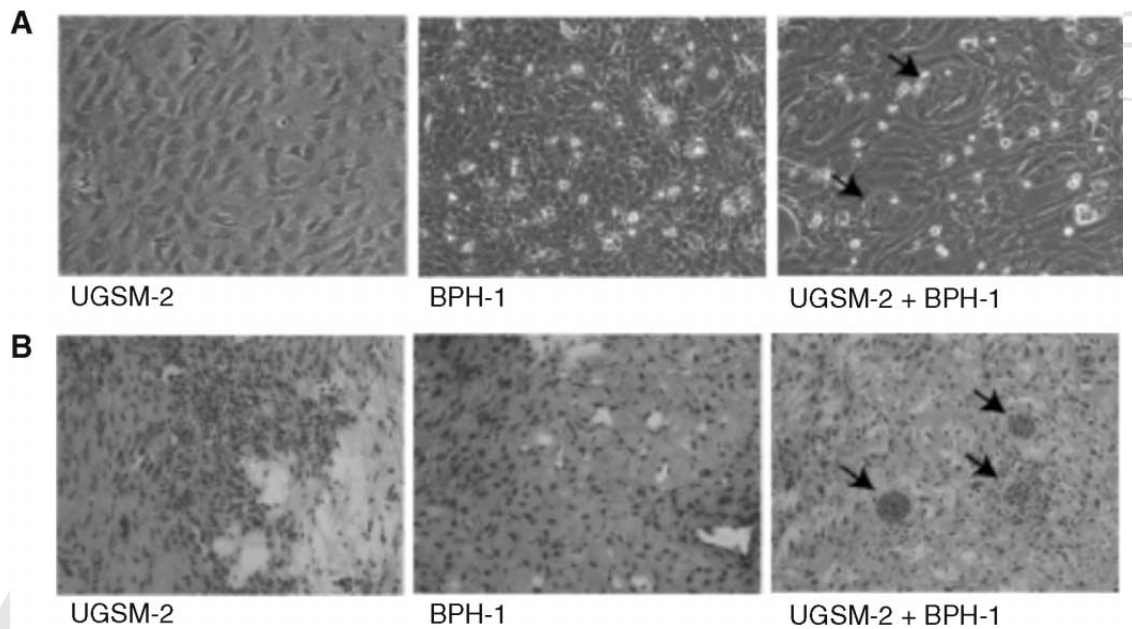


Fig. 5. Xxxxx.

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 manipula-

tion 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 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.

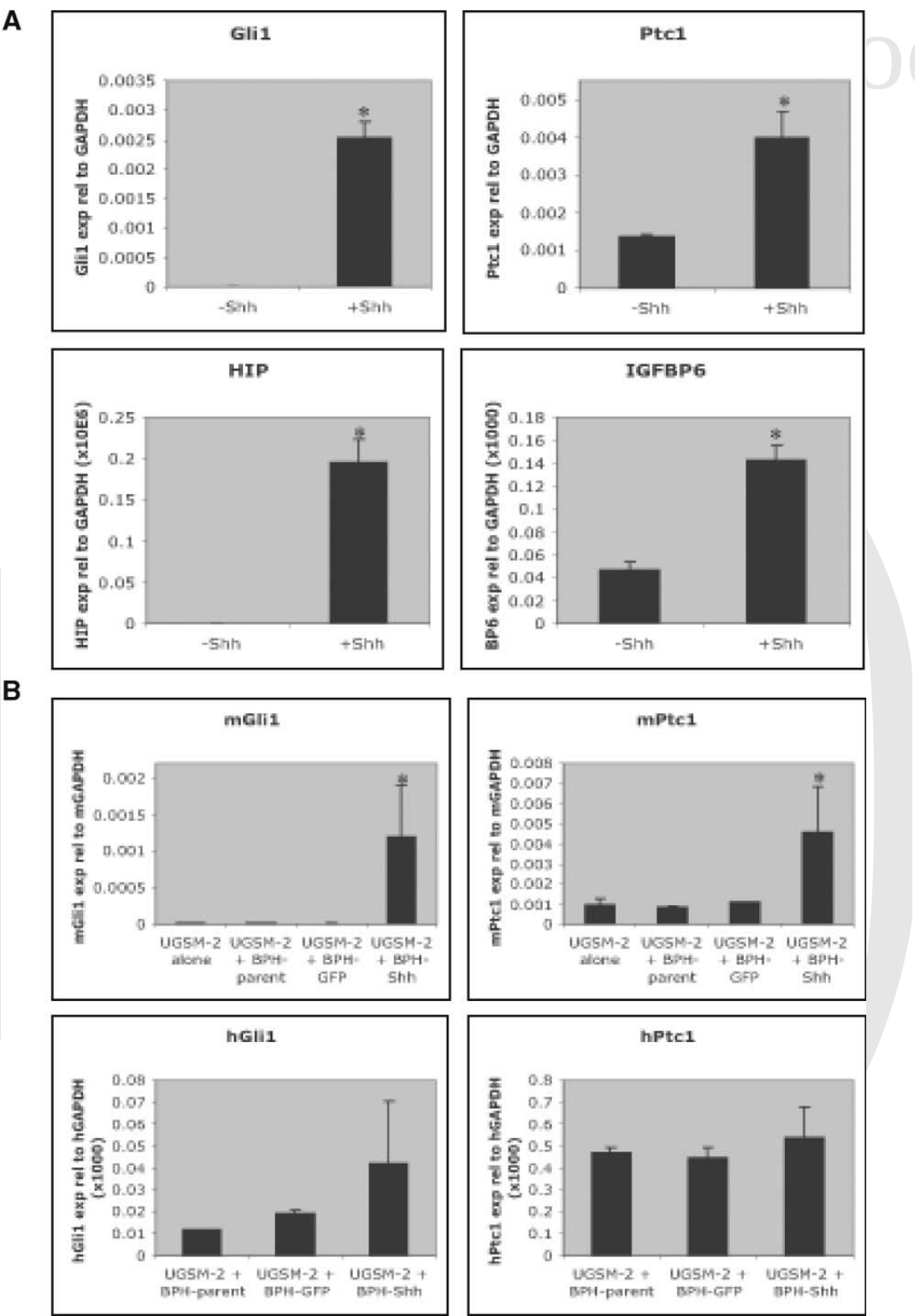


Fig. 6. Xxxxx.

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 forma-

tion [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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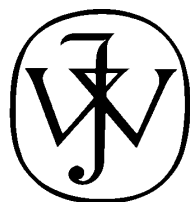
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AUTOCRINE HEDGEHOG SIGNALING IS NOT INVOLVED IN GROWTH OF HUMAN PROSTATE CANCER CELL LINES IN VITRO

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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

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ABSTRACT

Introduction: Several reports have highlighted the role of Hh pathway activation in prostate cancer growth, but the relative contributions of autocrine and paracrine signaling remain unclear. Divergent reports of the presence or absence of autocrine signaling in established human prostate cancer cell lines has not clarified the matter.

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 independent of any discernable effect on canonical Hh pathway activity.

INTRODUCTION

Hedgehog (Hh) signaling is required for normal prostate development¹⁻⁷ and appears to exert both autocrine and paracrine signaling activities. The Hh ligands Shh and Ihh are expressed in the urogenital sinus epithelium and tips of the developing ducts. This is mirrored by expression of the Hh target genes Ptc and Gli1 in the adjacent mesenchyme and associated with concentrated epithelial expression of Ptc and Gli1 at the tips of the buds and growing ducts^{6,8}.

Recent studies have shown that Hh signaling is active in human prostate cancer and accelerates tumor growth. However, the relative contribution of paracrine versus autocrine signaling to tumor growth and the role of ligand-dependent versus ligand-independent mechanisms of pathway activation in autocrine signaling remain unclear. Ligand-dependent pathway activation occurs when Hh ligand binds to its receptor Patched (Ptc), relieves inhibition of Smoothed (Smo) activity and activates Gli mediated target gene expression. Ligand-independent pathway activation results from mutations that abrogate regulatory mechanisms that prevent pathway activity in the absence of ligand. The studies reported here comprehensively examine ligand-dependent and ligand-independent pathway activity in the cell lines used most commonly to model Hh signaling in prostate cancer.

MATERIALS AND METHODS

Cell Lines. All the prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the medium recommended by ATCC. BPH1 cells were a generous gift from Dr. Simon Hayward (Vanderbilt University, Nashville, TN) and were grown in RPMI 1640 medium plus 5% fetal calf serum (FCS). Four cDNA samples from independent human prostate epithelial cultures were kindly provided by Dr. 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 a density of 1×10^5 cells/well. RNA was harvested after 3 days for the comparison of Hh pathway gene expression in different cell lines. For the assay of gene expression after SHH/cyclopamine treatment, serum concentration was reduced to 1% after 1 day attachment, and either 1nM octylated N-SHH (Curis, Inc., Cambridge, MA) or 5uM cyclopamine (Toronto Research chemicals, Ontario, Canada) was added to the medium and RNA was harvested after 48 hours treatment. Each experiment was repeated three times. UGSM-2 cells⁹ and MEFs were isolated in our laboratory.

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 set on top of UGSM-2 cells at the same density. 5uM cyclopamine or 1nM octylated N-SHH was added to the medium. RNA was harvested after 24 hours treatment.

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 Gli1-GFP, mGli2-GFP, Δ NmGli2-GFP, activated Smo*-GFP or GFP alone¹⁰ were kindly provided by Dr. Chen-Ming Fan (Carnegie Inst, Baltimore, MD). Cells were plated in a 24-well plate at density of 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/cells. Sonic hedgehog and cyclopamine were added at the same time. RNA was harvested and gene expression was determined as described below. Under these conditions, more than 90% of cells were infected according to GFP fluorescence 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 using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on BioRad iCycler (Hercules, CA) and using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard gene. Primer sequences used in this experiment are listed in Table I. Samples were run on 2% agarose gel.

Statistical analysis. An unpaired *t*-test was used to determine if there were statistically significant differences between treatment groups.

RESULTS

Hh pathway activity in prostate cancer cell lines. Comparison of Hh ligand expression in four prostate cancer cell lines (LNCaP, DU145, 22RV1, PC3) 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 benign prostate tissue as well as BPH1 immortalized normal prostate epithelial cells exhibited expression intermediate between LNCaP and PC3 (Figure 1c).

Ptc and Gli1 are primary targets of Hh transcriptional activation. Ptc expression is highest in LNCaP and 22 RV1, 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 well below the level of expression observed in the pooled normal prostate (Figure 2b).

Then we examined responsiveness of the tumor cell lines to exogenous Hh ligand. All cells were tested at 90% confluence and under a range of serum conditions. Treatment with 1nM Shh peptide elicited no detectable increase in the expression of either Ptc or Gli1 in any of the tumor cell lines tested (Figure 3).

Hh pathway responsiveness in prostate cancer cell lines. The tumor cell lines all express mRNA for the major components of the Hh signal transduction pathway (Figure 4a), however, the relative abundance of expression of each factor, determined by real-time RT-PCR, shows considerable variation (Figure 4b). It appears that the cells express all of the necessary machinery to respond to Shh, but do not respond. The lack of responsiveness to exogenous Shh ligand could result from 3 different mechanisms: a defect in the transmembrane receptor apparatus, a defect in the intracellular signal transduction mechanism or a specific block in the ability of Hh to induce Ptc and Gli1. To distinguish between these, we infected PC3 and 22RV1 cells with adenoviral vectors expressing either an activated form of hSmo (Smo*) or an activated form of mGli2 (Δ NmGli2). Smo* 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 data not shown, respectively). In contrast, infection with an adenovirus vector expressing Δ NmGli2 induced robust expression of both Ptc and Gli1 in 22RV1. Infection of PC3 with the Δ NmGli2 induced only a small and statistically insignificant increase in Ptc expression but produced a robust induction of Gli1 expression (Figure 5b). This difference is likely due to Gli1 being a more sensitive marker of induction because of its lower basal expression in the absence of ligand.

Effect of cyclopamine on Hh signaling. The plant steroidal alkaloid cyclopamine inhibits Hh signaling by an interaction with Smo. We examined the effect of 5 uM cyclopamine on Ptc and Gli1 expression in these tumor cell lines. Regardless of whether the assay was performed in 10%, 1% or 0.1% FCS, we observed no significant reduction in Ptc or Gli1 expression in any tumor cell line (Figure 6). Control studies performed with UGSM-2 cells showed complete blockade of Hh signaling with 5 uM cyclopamine (Figure 6 insert). We also observed no effect of cyclopamine on the tumor cell lines when the assay was performed in the presence of exogenous Shh peptide (data not shown).

Effect of cyclopamine on tumor cell proliferation. We examined the effect of cyclopamine on growth of 22RV1, PC3 and LNCaP cells in culture. Treatment with 5 uM 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 10uM 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).

It has been demonstrated that cyclopamine can inhibit growth of PC3 tumor xenografts. It has been assumed that this effect reflected the 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 cells. While cyclopamine did not affect expression of hPtc and hGli1 by the PC3 cells, it did efficiently inhibit mPtc and mGli1 expression by the UGSM-2 cells (Figure 8).

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 can accelerate tumor growth¹¹. Recently, we have shown that the paracrine growth effect in response to Shh signaling depends upon the nature of the tumor stroma (unpublished observations). This may explain why Hh signaling may occur equally in the normal adult prostate and in prostate cancer but elicit dramatically different growth effects. Several other studies examining the expression of Shh in localized and metastatic prostate cancer suggested variably increased Shh expression in localized tumors exerting a combination of autocrine and paracrine signaling activity, and dramatically increased pathway activation in metastatic disease¹¹⁻¹⁴. The possible contribution of autocrine signaling to tumor growth was examined by studying human tumor cell lines. LNCaP and PC3 cells were both found to express Shh, Ptc and Gli1^{12, 13}. Cyclopamine inhibited proliferation of LNCaP cells in culture^{13, 14} and was shown to inhibit Gli1 expression, suggesting that the effect was pathway specific¹³. Cyclopamine also inhibited proliferation of PC3 cells in culture¹²⁻¹⁴. Karhadkar et al¹² found that anti-Shh blocking antibody also inhibited PC3 proliferation in culture, but Sanchez et al¹³ found that PC3 proliferation was unaffected by either anti-Shh blocking antibody or exogenous Shh. The discrepancy in these results has not been resolved and highlights uncertainty in the relative contributions of ligand dependent and ligand independent pathway activation to PC3 proliferation. The effect of chemical blockade of Hh signaling on tumor growth was examined by effect of cyclopamine administration on xenograft tumor growth¹². 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 over-expressing Gli1 were resistant to the anti-tumor effect of cyclopamine. These exciting studies suggested that autocrine pathway activity promotes tumor cell proliferation and that treatment with Hh inhibitors in vivo could produce sustained regression of established tumors. However, these studies produced discrepant findings regarding the relative contribution of ligand-dependent versus ligand-independent pathway activation in these cell lines and did not consider the possibility that cyclopamine's action could involve an effect on paracrine signaling.

Our studies show that LNCaP, DU145, PC3 and 22RV1 all exhibit Hh ligand expression and expression of Gli1 and Ptc. The level of ligand expression varies, with the highest level of mRNA expression present in PC3 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. Similarly, the expression of Ptc and Gli1 in these cell lines is much lower than in the normal prostate.

Since the tumor cell lines express both Shh and Ihh, low level pathway activity could result from ligand-dependent autocrine pathway activation. However, our extensive studies of LNCaP, PC3 and 22RV1 found no evidence for a transcriptional response to Hh stimulation or cyclopamine inhibition. Our previous studies had shown that LNCaP was unresponsive to exogenous Shh¹¹, but the unresponsiveness of PC3 and 22RV1 was unexpected. 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 - arguing that the canonical Smo-mediated signal transduction pathway is non-functional. To ensure the validity of this negative result, we infected cells with an adenoviral vector expressing an activated form of Gli2 (Δ NmGli2) and demonstrated induction of Ptc and Gli in both cell lines. In these cells, the Hh pathway appears to be nonfunctional at the level of Smo, but the transcriptional response to Gli2 remains intact. These studies, which demonstrate that the Hh signal transduction mechanism is defective in both PC3 and 22RV1, are clearly consistent with a lack of responsiveness to Hh ligand.

Cyclopamine has been shown to inhibit Hh signaling by interfering with pathway activation by Smo. We observed no changes in the expression of Hh target genes Ptc and Gli1 in LNCaP, PC3 or 22RV1 treated with 5uM cyclopamine under a range of culture conditions. These observations are consistent with the aforementioned idea that Smo-mediated Hh signaling is defective in these cells. Although these observations stand in contrast to the studies of Karhadkar et al¹², they examined the effect of cyclopamine on expression of a Gli-reporter construct, rather than expression of endogenous Ptc and Gli1. It is therefore possible that they observed an effect of cyclopamine that does not accurately reflect the effect of cyclopamine on the expression of endogenous target genes. Similarly, we observed that treatment of cells in culture with 10 uM cyclopamine decreased cell number but without any discernable effect on Hh pathway activity. These findings strongly suggest that inhibition of cell proliferation in vitro by cyclopamine is not the result of canonical Smo-mediated Hh pathway inhibition.

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 is supported by our co-culture studies and suggests that the effect of cyclopamine on paracrine signaling in xenograft tumors may be an important, but unrecognized effect in the previously published studies.

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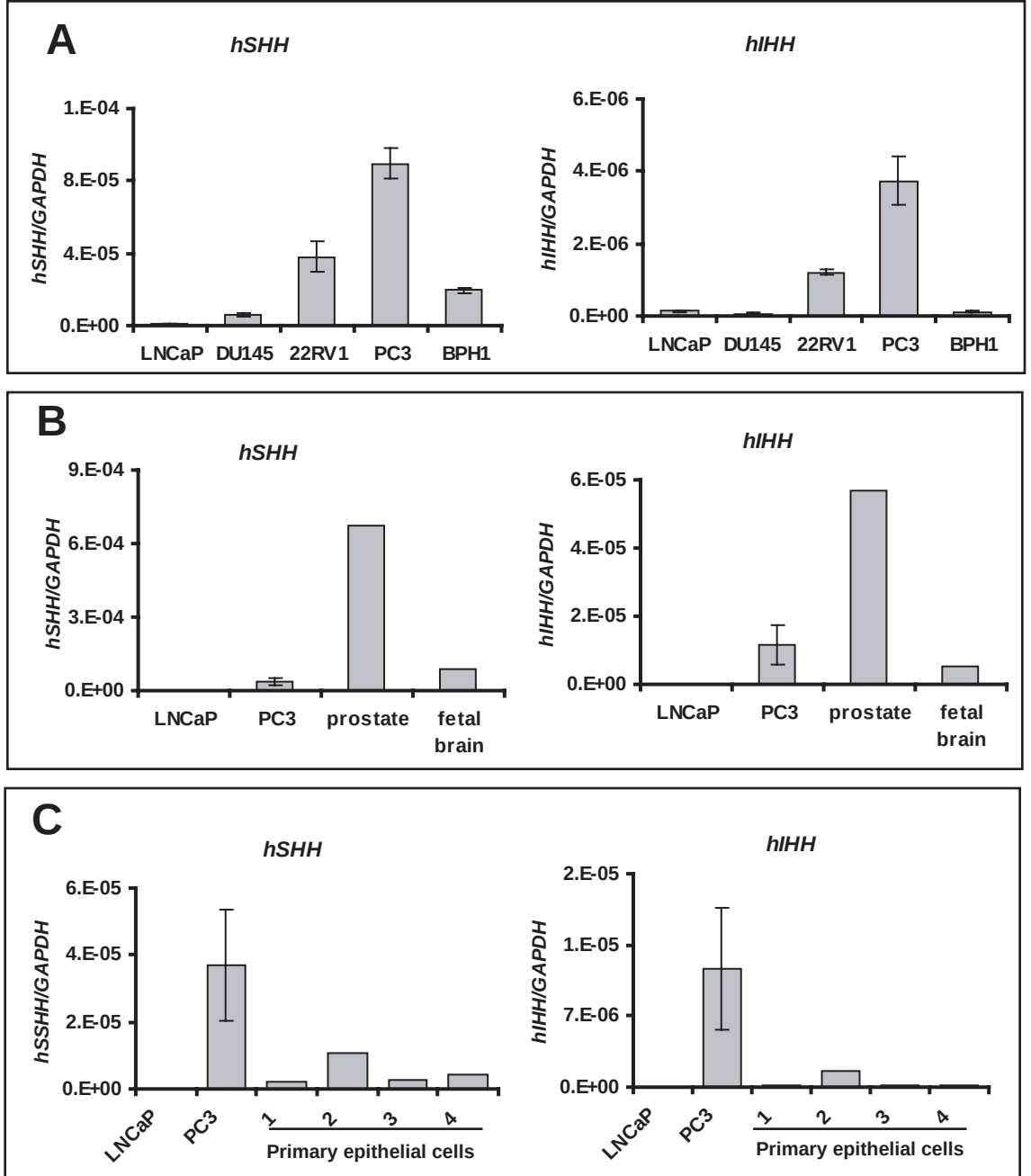


Figure 1 (A) *Shh* and *Ihh* expression in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the immortalized BPH-1 cell line. (B) Comparison of expression in LNCaP and PC3 with expression in the human fetal brain and a pooled sample of normal adult prostate RNA. (C) Comparison of expression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

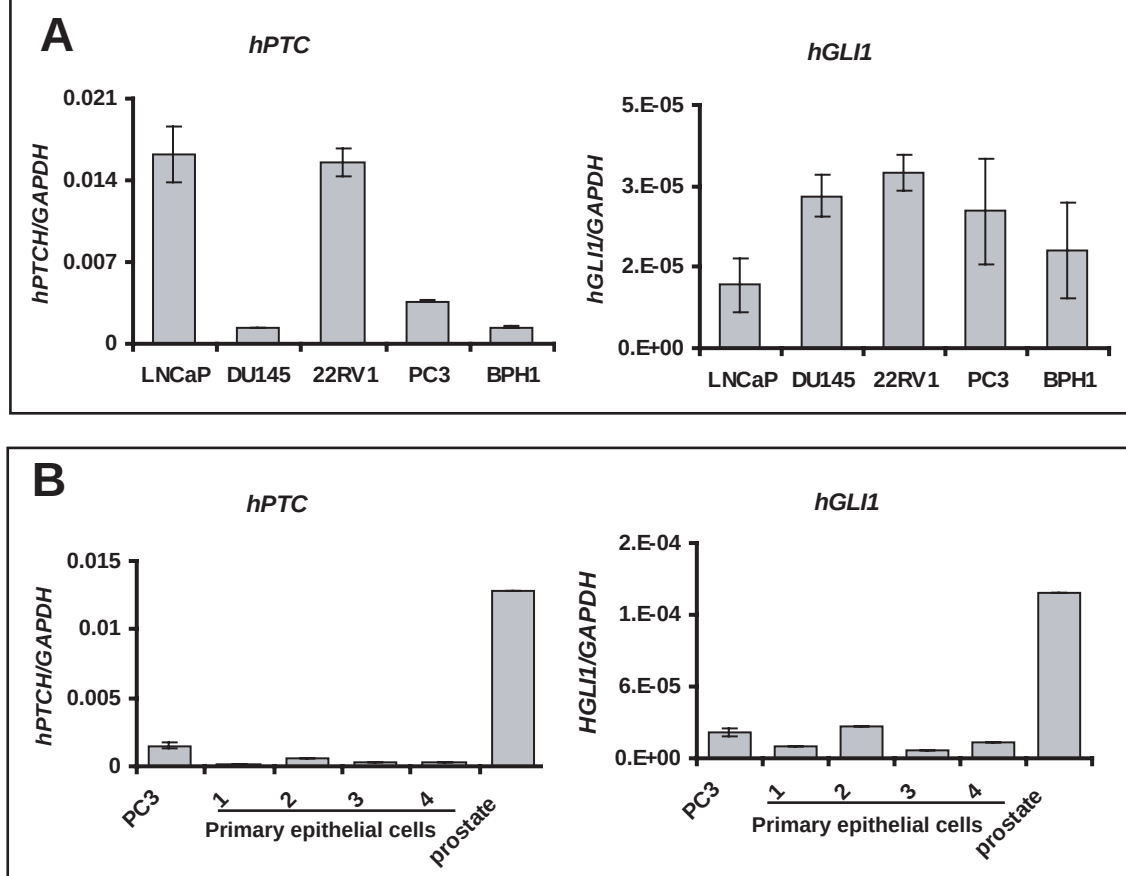


Figure 2 (A) Expression of the conserved Hh target genes *Ptc* and *Gli1* in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the immortalized BPH-1 cell line. (B) Comparison of *Ptc* and *Gli1* expression in PC3 and four primary benign prostate epithelial cell lines.

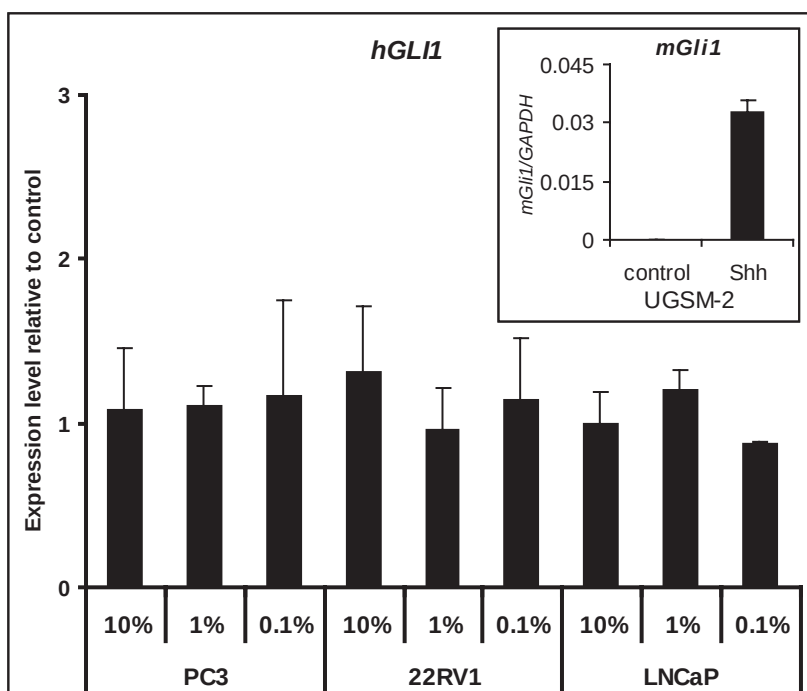
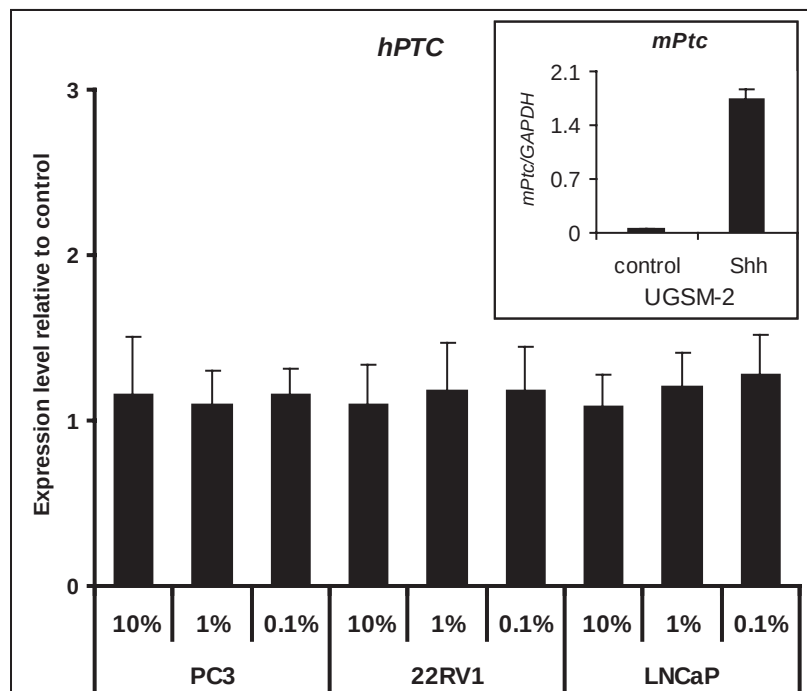


Figure 3 Lack of induction of Ptc1 and Gli1 expression in Shh treated PC3, 22RV1 and LNCaP cells. Shh treatment was in media supplemented with 10%, 1%, or 0.1% FCS for 48hr which is sufficient to significantly induce Ptc1 and Gli1 in UGSM-2 cells (0.1% FCS), $p < 0.005$ (insert).

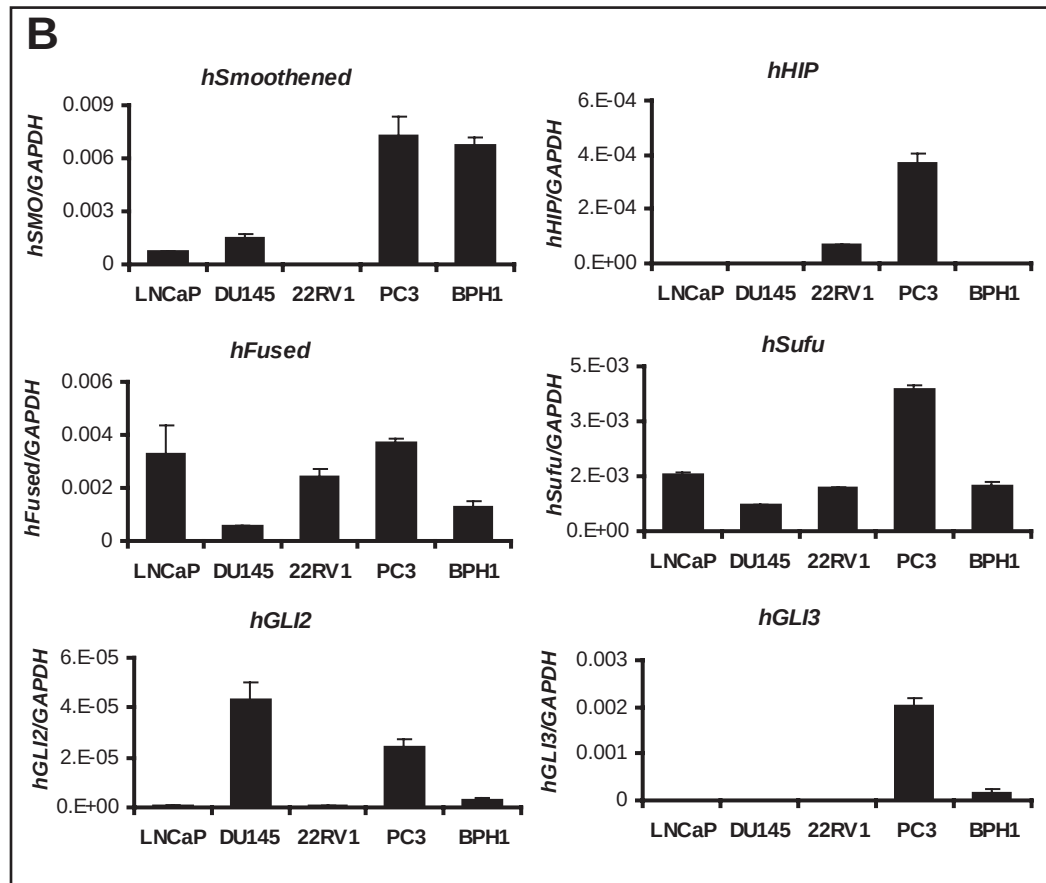
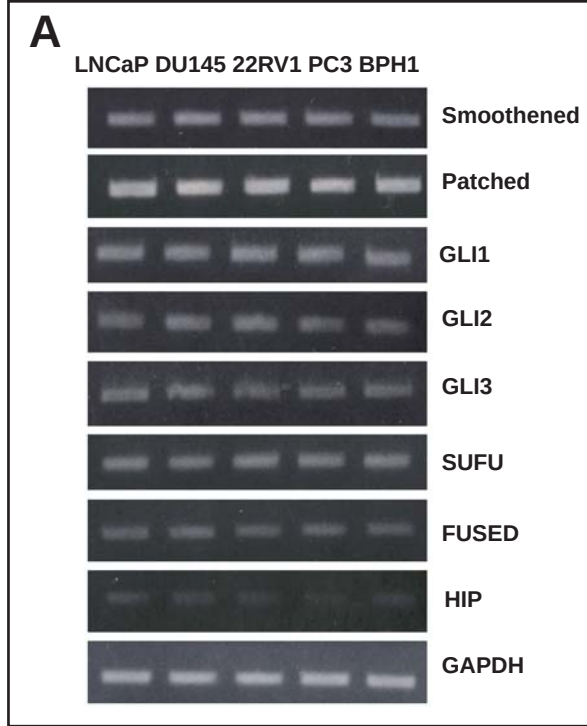


Figure 4 Expression of Hh pathway genes Smo, Ptc1, Gli1, Gli2, Gli3, SuFu, Fused and Hip in LNCaP, DU145, PC3, 22RV1 and BPH-1. (A) Resolution of RT-PCR products (40 cycles) on a 2% agarose gel using GAPDH as a loading control. (B) Quantitative real-time RT-PCR for the Hh pathway genes shows variations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

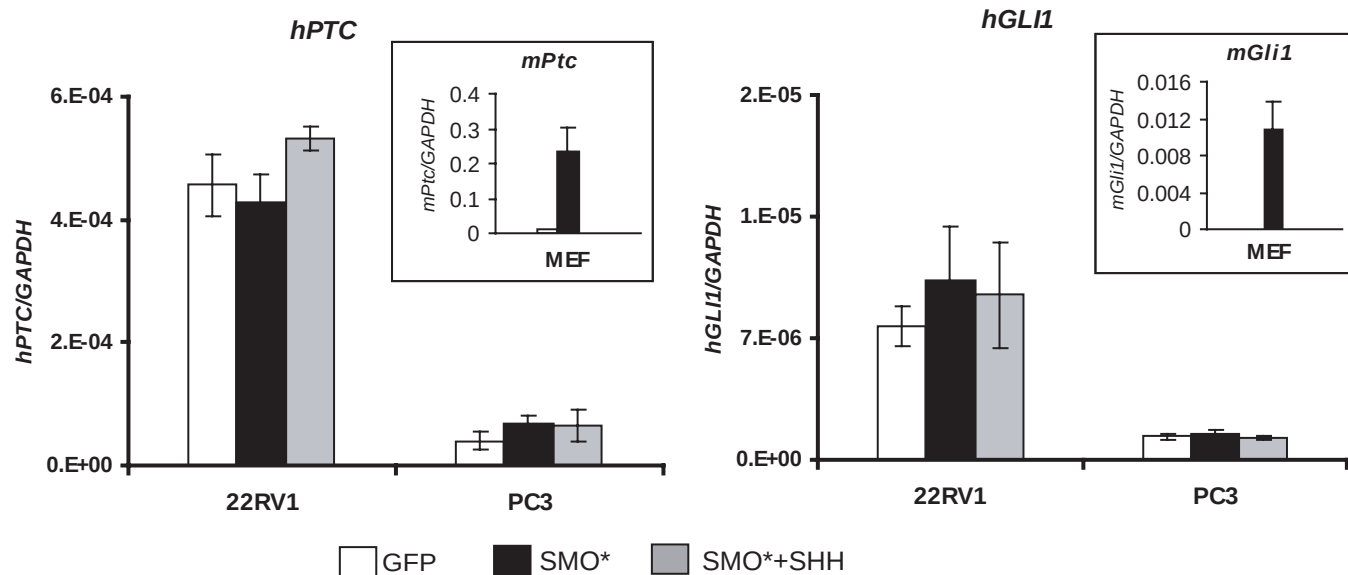
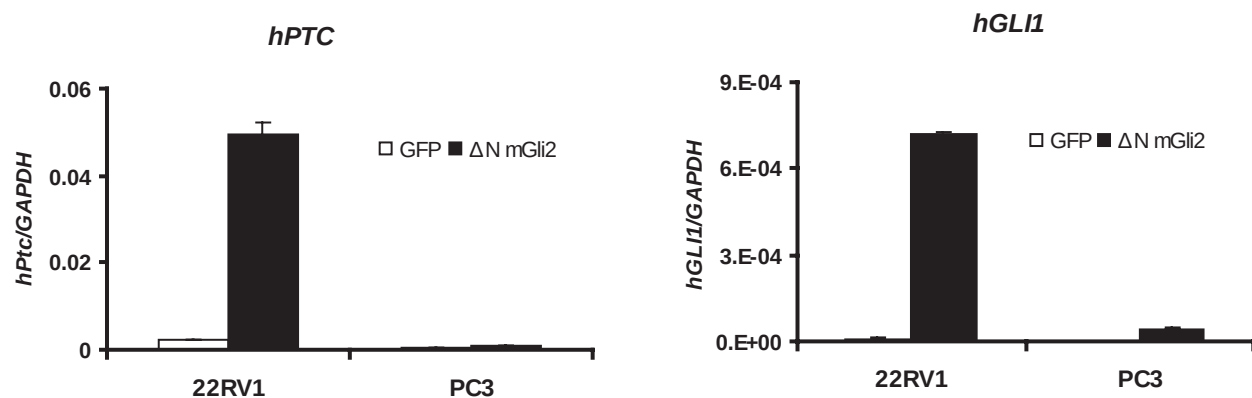
A**B**

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 $\sim 90\%$.

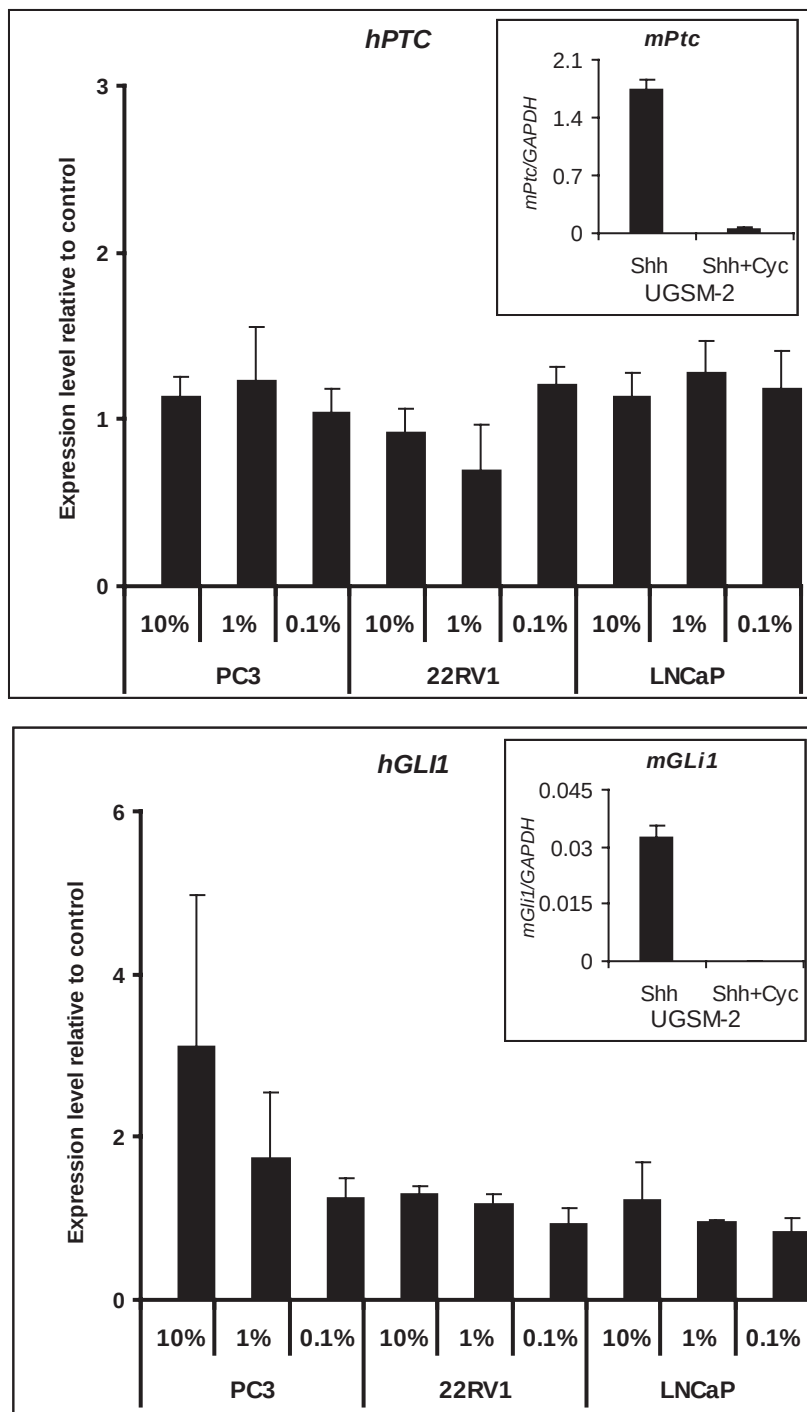


Figure 6 Cyclopamine treatment of PC3, 22RV1 and LNCaP cells in media supplemented with 10, 1, 0.1% FCS did not alter expression of the Hh target genes Ptc or Gli1. Target gene expression was induced by Shh and inhibited by 5uM cyclopamine in UGSM-2 cells $p < 0.005$, (insert).

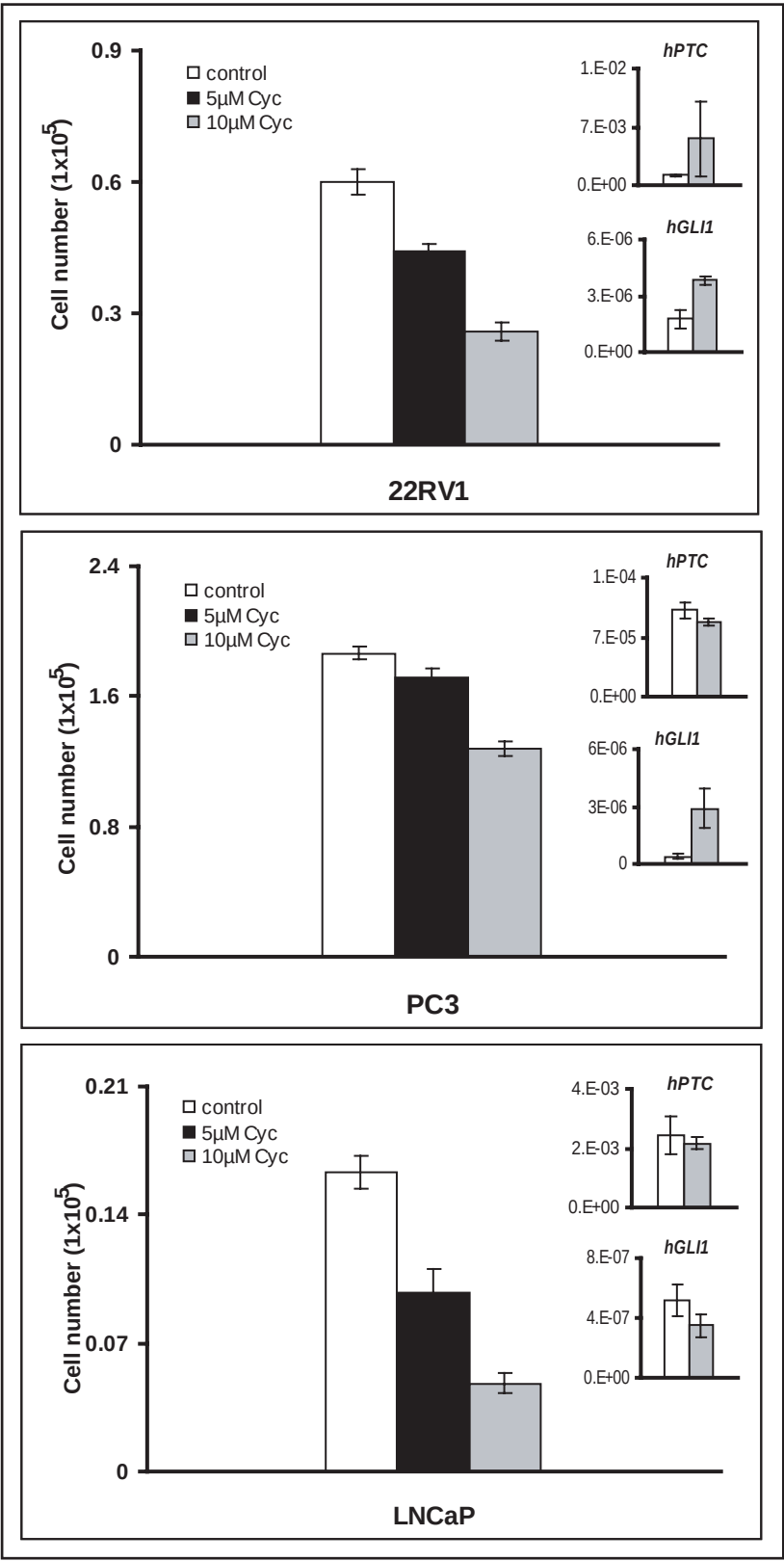


Figure 7 Proliferation of 22RV1, PC3, and LNCaP cells over 4 days was inhibited by cyclopamine in a dose dependent fashion ($p < 0.05$ at 10uM cyclopamine). In these cultures expression of the Hh target genes Ptc and Gli1 were not altered by 10uM cyclopamine suggesting that the reduction in proliferation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

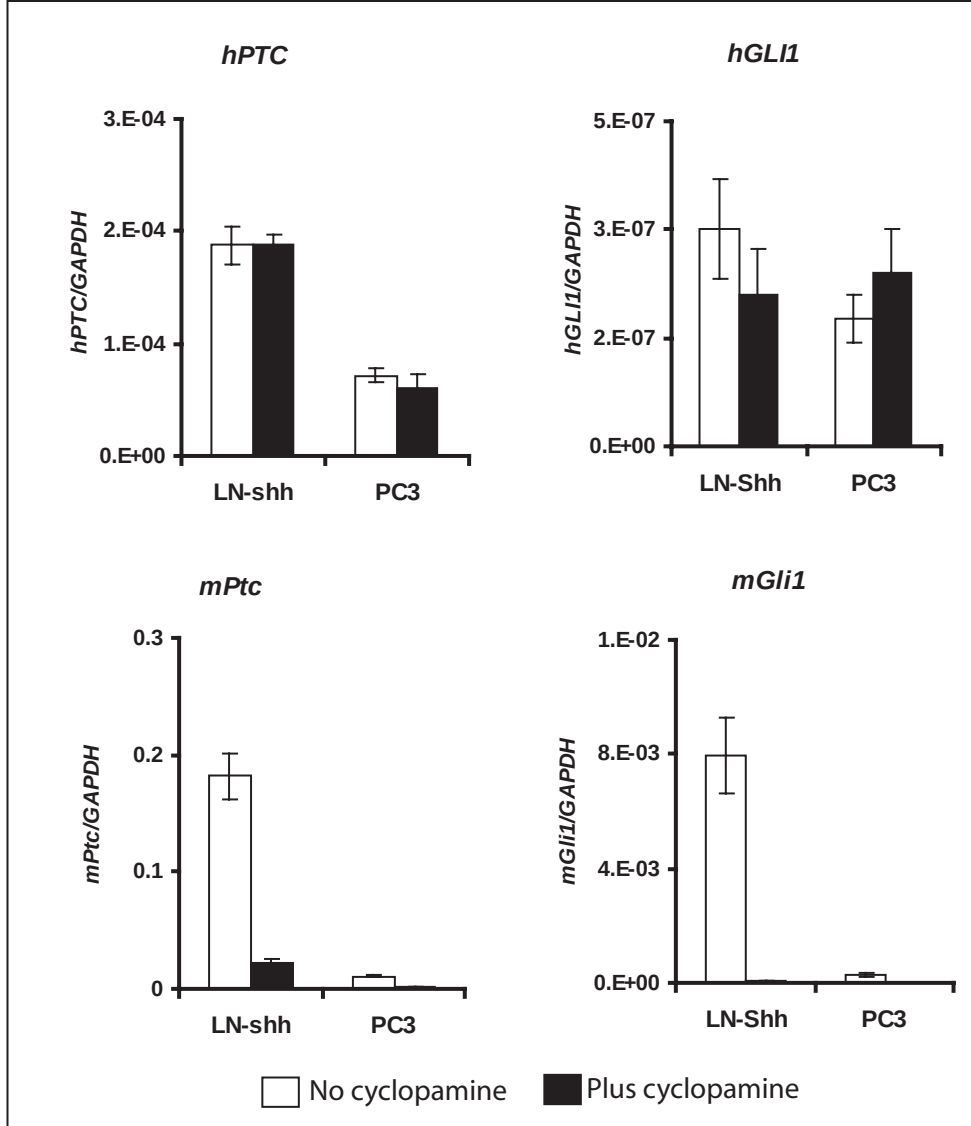


Figure 8 Effect of 10uM cyclopamine on autocrine and paracrine pathway activity in co-cultures of either LNCaP cells over-expressing Shh (LN-Shh) or PC3 cells co-cultured with mouse UGSM-2 cells. There is no effect on expression of *hPtc* and *hGli1* (upper panel). However inhibition of paracrine signaling 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. 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 reference controls. 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. 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. 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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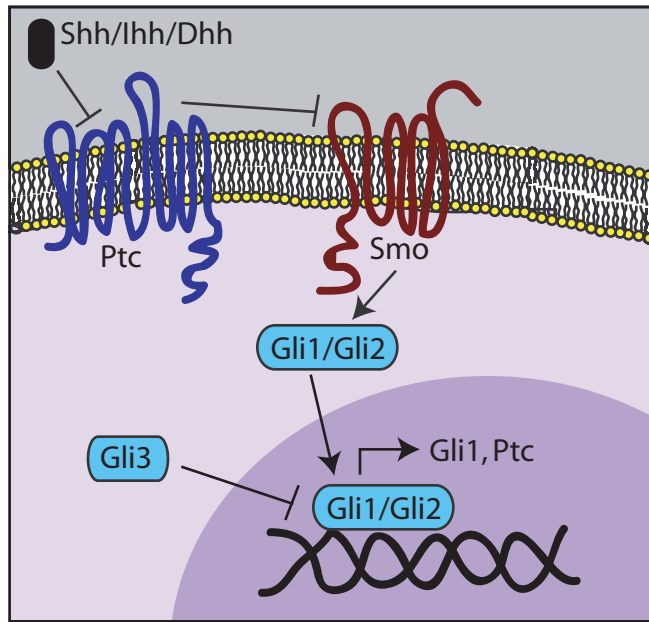
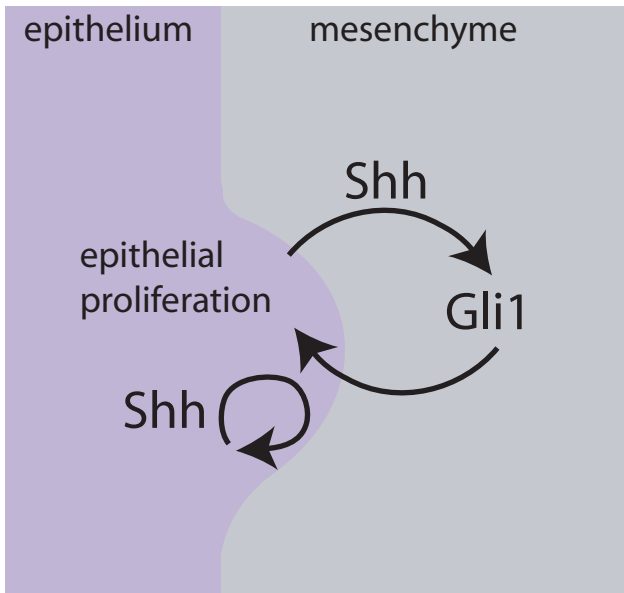


Figure 1. The mammalian Hh signaling pathway. Hedgehog ligands Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) bind to the transmembrane receptor Patched (Ptc) and relieve constitutive repression of Smoothened (Smo). Smo activation curtails transcriptional repression by Gli3 and 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

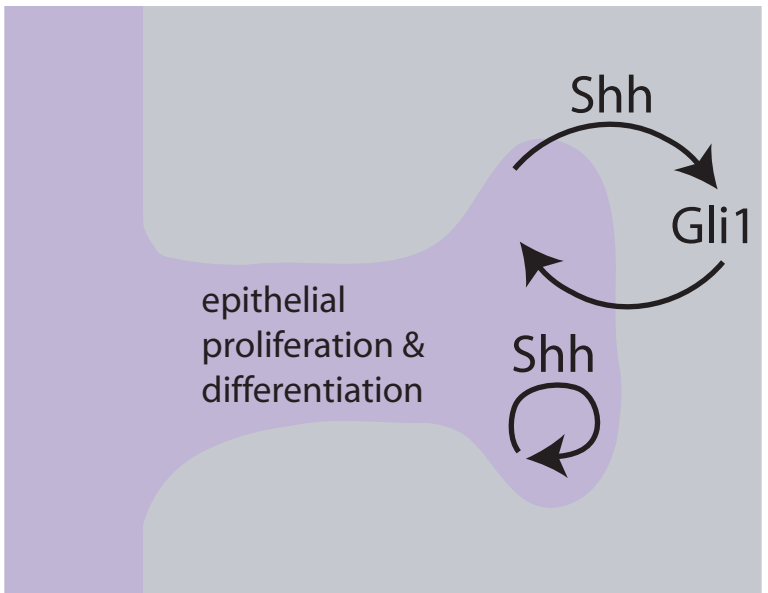
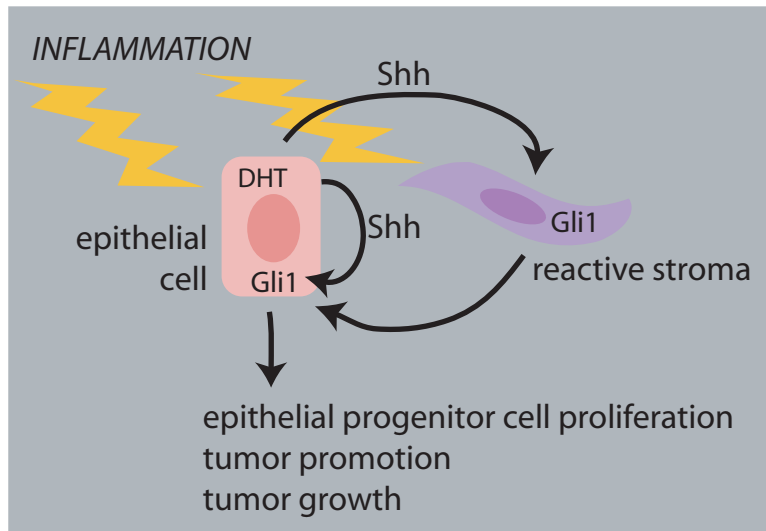


Figure 2. Postulated actions of Hh in prostate development. (Left) Androgen-dependent ductal budding is associated with focal expression of Shh in the epithelium of nascent buds. Shh acts on adjacent mesenchyme to activate expression of Hh target genes and increases epithelial proliferation (paracrine 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

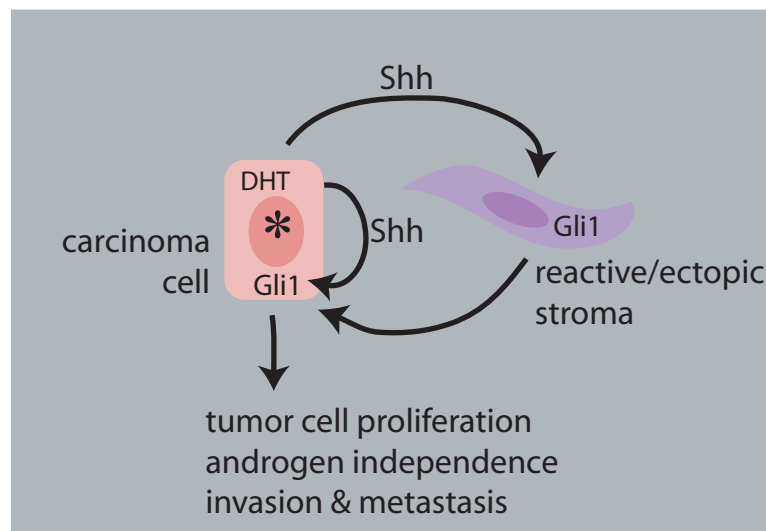


Figure 3. Postulated actions of Hh in prostate cancer. (Top) Injury and inflammation induce ligand-dependent autocrine stem cell activation and proliferation while paracrine signaling elicits growth stimulating responses from the reactive stroma. This creates an environment that promotes tumor formation 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.