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Hedgehog signaling in myofibroblasts directly promotes prostate tumor cell growth†

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Abstract

Despite strong evidence for the involvement of the stroma in Hedgehog signaling, little is known about the identity of the stromal cells and the signaling mechanisms that mediate the growth promoting effect of Hh signaling. We developed an *in vitro* co-culture model using microchannel technology to examine the effect of paracrine Hh signaling on proliferation of prostate cancer cells. We show here that activation of Hh signaling in myofibroblasts is sufficient to accelerate tumor cell growth. This effect was independent of any direct effect of Hh ligand on tumor cells or other cellular components of the tumor stroma. Further, the trophic effect of Hh pathway activation in myofibroblasts does not require collaboration of other elements of the stroma or direct physical interaction with the cancer cells. By isolating the tropic effect of Hh pathway activation in prostate stroma, we have taken the first step toward identifying cell-specific mechanisms that mediate the effect of paracrine Hh signaling on tumor growth.

Introduction

Hedgehog (Hh) signaling is highly conserved among vertebrates due to its important role in embryonic development and tissue regeneration.¹ Recent reports have linked aberrant Hh signaling activation with the development and progression of several cancers including colon,² skin,^{3,4} pancreas,^{5,6} breast^{7,8} and prostate cancer.^{9–12} Hh signaling is important for ductal branching and morphogenesis in the developing prostate and reactivation and overactivity of Hh signaling have been identified in prostate cancer tissue samples, suggesting a role for Hh signaling in development and progression of prostate cancer.

Sonic Hedgehog (SHH) is the most abundantly expressed hedgehog ligand in the developing prostate. SHH and members of its signaling pathway have been found to be highly expressed in prostate tumor specimens.^{11–13} Karhadkar *et al.* reported increased hedgehog signaling in prostate tumor specimens and showed a positive correlation between Hh pathway activation and advanced disease. A critical step in Hh signaling is binding of SHH to the transmembrane receptor Patched1 (*Ptch1*), an action that relieves repression of another transmembrane protein, Smoothened (SMO), and triggers transcriptional activation of Hh target genes including *Gli1*, *Ptch1* and *Hip* (Fig. 1). Blocking Hh signaling with antagonists of SMO inhibits prostate tumor growth.^{12,14} While initial reports suggested a key role for

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autocrine activation of Hh signaling in prostate cancer cells,¹⁴ more recent studies have suggested that SHH induced tumor growth is mainly paracrine mediated. Fan *et al.* performed *in situ* hybridization studies of human prostate cancer showing expression of SHH in the tumor epithelium and transcriptional activation of *Gli1* in the adjacent stroma. Further studies in our lab have shown that overexpression of SHH in xenograft tumors made by LNCaP cells that overexpress SHH led to significantly increased tumor growth.¹¹ In this model, paracrine hedgehog signaling via activation of hedgehog target genes (*Gli1*, *Ptch1*) in the mesenchyme—not in the epithelium—led to accelerated tumor growth. Similarly, paracrine Hh signaling has been detected in a subset of human colorectal, skin, pancreatic and ovarian tumors supporting the role of the tumor stroma in hedgehog-mediated tumor growth.¹⁵

Defining the mechanisms by which the stroma mediates Hh signaling is important for the development of therapies that effectively target Hh-driven tumor growth. We have shown that activation of hedgehog signaling in the myofibroblast component *in vivo* is sufficient to induce tumor growth.¹⁶ Moreover, we correlated active Hh signaling and tumor growth promotion with the reactive status and developmental characteristics of the stroma in human prostate tissue samples. The mechanisms by which the Hh activated stroma promotes tumor growth *in vivo* are unknown. Whether active Hh signaling in the stroma directly induces a proliferative response in the epithelium or requires the presence of other mediator cells (e.g. endothelial cells, macrophages) is not known. Further, the factors responsible for Hh-mediated tumor growth have not been identified. The complexity of the tumor microenvironment and the lack of appropriate models have hindered the identification of the mechanisms by which activation of Hh signaling in the stroma promotes tumor growth. Attempts to Hh paracrine signaling in prostate tumor growth *in vitro* has presented multiple challenges. A viable model requires the presence of tumor cells that secrete Hh ligand and stromal cells that respond to the secreted ligand. The standard prostate cancer cell lines do not express high levels of SHH and human stromal cell lines have minimal response to Hh ligand. While Hh ligands can be added exogenously or overexpressed in tumor cells, obtaining an adequate response in human stromal cell lines has been problematic and the mechanisms that regulate responsiveness of stromal cell lines to Hh ligand remain uncertain. Mouse-derived primary stromal cells and immortalized stromal cell lines are generally responsive when cultured in serum-starved culture conditions.¹⁷ However, even attempts to use these in co-culture with human tumor cells have failed to replicate the trophic effect of Hh paracrine signaling on tumor cell proliferation. This raised the distinct possibility that the growth effect of paracrine Hh signaling is indirect and/or depends upon other elements of the tumor stroma.

In this study, we used a compartmentalized microculture platform to develop an *in vitro* model of paracrine Hh signaling and investigate the role of the stroma in Hedgehog-induced prostate tumor cell growth. The confined diffusion-dominant environment and close proximity among the tumor and stromal compartments favored paracrine interaction and enabled, for the first time, *in vitro* recapitulation of previous *in vivo* observations and demonstration that Hh activated mouse-derived myofibroblasts can directly promote tumor cell growth. Further, we have been able to induce myofibroblast differentiation of normal human prostate fibroblasts by treatment with TGF- β , capacitate stromal responsiveness to Hh ligand and recapitulate the growth promoting effect of paracrine Hh signaling in an all-human cell *in vitro* model. Our results establish a practical *in vitro* model of Hh signaling and provide valuable insights into the control mechanisms involved in Hh-mediated tumor growth.

Results

Tumor cell derived SHH enhances tumor cell growth through paracrine signaling

UGSM-2 is an Hh responsive, immortalized myofibroblast cell line derived from the embryonic day 16 mouse urogenital sinus.¹⁸ These cells are responsive to Hh ligand under conditions of growth arrest—confluence and low serum—that induce expression of primary cilium, a tubulin cell surface sensor essential for transduction of the Hh signal.^{17,19} Initial efforts to model paracrine Hh signaling *in vitro* by compartmentalized co-culture of UGSM-2 with LNCaP cells transfected with a human SHH over-expression vector (SHHv–LNCaP) or vector alone (v-LNCaP) were unsuccessful in achieving induction of *Gli1* and *Ptch1* expression in UGSM-2 cells co-cultured with SHHv–LNCaP cells and proliferation of SHHv–LNCaP was not increased compared to v-LNCaP (ESI[†], Fig. 1A–C). The absence of detectable Hh signaling in this experiment appears attributable to dilution of secreted SHH protein in the culture media (ESI[†], Fig. 1D).

To achieve compartmentalized co-culture under conditions that minimize dilution, we used a multi-compartment membrane-free microchamber platform that allows co-culture of different cell populations in separate small volume (2 μ L) compartments arranged in close proximity (500 μ m). This platform has been previously shown to enhance paracrine signaling by providing a diffusion dominant environment that increases sensitivity to secreted factors.²⁰ We first determined culture conditions appropriate to sustain viability and hedgehog responsiveness of UGSM-2 cells in the microchamber platform. A serum concentration of 3.5% was sufficient to maintain a viability of 70% and 90% in UGSM-2 and LNCaP cells respectively (ESI[†], Fig. 2A). Under these conditions, SHH-induced expression of *Gli1* and *Ptch1* in UGSM-2 cells was similar in wells and microchambers (ESI[†], Fig. 2B and C).

SHHv–LNCaP or v-LNCaP were co-cultured with a confluent monolayer of UGSM-2 cells in adjacent compartments (ESI[†], Fig. 3). The cells in each compartment were lysed individually and gene expression analysis was performed using human-specific and mouse-specific primers. As shown in Fig. 2, increased proliferation of SHHv–LNCaP when co-cultured with UGSM-2 (B) correlated with activation of *Gli1* and *Ptch1* expression in UGSM-2 cells (C and D). Chemical inhibition of paracrine signaling by addition of the SMO antagonist AZ75 to the culture medium abrogated induction of *Gli1* and *Ptch1* in UGSM-2 and eliminated the proliferative advantage of SHHv–LNCaP cells in co-culture. As previously reported²¹ we observed no evidence of autocrine hedgehog pathway activation with SHH over-expression (ESI[†], Fig. 4A–C). Physical contact among tumor and stromal cells was not observed during the experiments, indicating that soluble factor paracrine signaling is responsible for tumor cell growth.

SHH peptide abundance was compared among v-LNCaP and SHHv–LNCaP cultured in transwells, 96 well plates and microchambers (ESI[†], Fig. 4D). The active form of secreted SHH ligand (19 kDa) was detected in conditioned media obtained from microchambers but not in SHHv–LNCaP conditioned media collected from transwells and 96 wells. This is best explained by the differences in cell-to-media volume ratio: at a constant surface cell density the relative volume per cell ratios are 1, 6 and 100 in microchambers, 96 wells and transwells, respectively.

The growth advantage conferred by tumor cell over-expression of SHH could be mimicked by addition of exogenous SHH peptide to the culture medium. Parental LNCaP were cultured alone or co-cultured with UGSM-2 in adjacent compartments $\pm$ 5 nM SHH.

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Exogenous SHH induced a significant increase in UGSM-2 expression of *Ptch1* and *Gli1* and increased proliferation of co-cultured LNCaP. Addition of AZ75 abrogated induction of *Ptch1* and *Gli1* expression and reduced LNCaP proliferation to baseline (Fig. 3). These studies show that SHH-ligand mediated activation of the Hh pathway in myofibroblast cells is sufficient to promote tumor cell proliferation.

Ligand independent Hh signaling promotes tumor cell growth

To test whether ligand-independent Hh pathway activation in myofibroblast stromal cells is sufficient to promote tumor cell proliferation, parental LNCaP cells were co-cultured with stromal cells in which the Hh pathway is rendered constitutively active by either inactivation of *Gli3* or over-expression of SMO. Stromal cells lacking *Gli3* (UGli3^{-/-}), a negative regulator of Hh target gene transcription, were made by isolation from the urogenital sinus of an embryonic day 16 *Gli3* mutant mouse.¹⁶ USMO stromal cells were made by transfecting UGSM-2 cells with an activated form of SMO.^{17,22} The basis for these studies was our previously reported observation that LNCaP xenograft tumors generated by co-injection of LNCaP and UGLi3^{-/-} exhibited basal activation of the Hh pathway in the stromal compartment and accelerated tumor growth.¹⁶ Co-culture of LNCaP with USMO resulted in a significant increase in proliferation as compared to co-culture with control cells (Fig. 4). Addition of AZ75 to the media reduced *Gli1* and *Ptch1* expression and abrogated the increase in tumor cell proliferation. Co-culture with UGLi3^{-/-} cells also significantly increased proliferation rates in LNCaP cells. Since *Gli3* acts downstream from SMO, the site of AZ75 inhibition, addition of AZ75 to the culture media neither inhibited the expression of *Gli1* and *Ptch1* nor abrogated the increase in tumor cell proliferation (Fig. 5). The growth-promoting effect of Hh pathway activation in stromal cells is not unique to LNCaP cells. Both the benign prostate hyperplasia cell line BPH-1 and the androgen independent prostate cancer cell lines PC-3-MM2 and C4-2B exhibited increased proliferation when co-cultured with UGLi3^{-/-}.

TGF- β enables Hh responsiveness and tumor promoting characteristics of prostate stromal cells

Three normal primary prostate fibroblast cell lines (NPF, N2-1 and N5-2) were tested for responsiveness to Hh ligand. These cells express vimentin and very low levels of alpha-smooth muscle actin (α -SMA) (ESI[†], Fig. 5) consistent with a fibroblast phenotype and exhibit primary cilia and *Ptch1* receptors (Fig. 6A and B). Cells were seeded in microchambers and treated with SHH for 48 h. Analysis of *Gli1* and *Ptch1* expression showed no significant changes in response to SHH ligand (Fig. 6C). Recent studies have implicated myofibroblasts as an important stromal locus of Hh signaling.^{13,15,16,23} TGF- β is a factor known to induce myofibroblast differentiation *in vitro* and in tissues.^{24,25} To determine whether a myofibroblast phenotype can mediate paracrine Hh signaling, we treated fibroblasts with TGF- β and examined their response to exogenous SHH ligand. After 4 days of exposure to TGF- β , normal prostate fibroblast cells expressed high levels of α -SMA and exhibited nuclear translocation of phosphorylated SMAD-3 (Fig. 7A and B), a factor known to be involved in TGF- β signaling. Cells exhibited abundant levels of α -SMA localized to stress fibers—a characteristic of the myofibroblast phenotype (ESI[†], Fig. 5). These changes persisted up to three days after removal of TGF- β from the culture medium. TGF- β treatment increased *Gli1* expression in NPF cells but did not capacitate Hh-induced *Gli1* expression in any of the three stromal cell lines (Fig. 7C). *Gli2* expression was significantly increased in N5-2 cells by TGF- β treatment and was further increased by co-treatment with SHH peptide (Fig. 7D). These changes may be attributable to non-canonical activation of the Hh pathway by TGF- β .²⁶ NPF cells pre-treated with TGF- β were cultured with LNCaP cells to examine their ability to promote tumor growth in response to Hh signaling. AZ75 was used to differentiate between SHH (SMO dependent) and TGF- β

(SMO independent) induced expression of *Gli1*. Tumor cell proliferation was dramatically increased by the addition of SHH ligand to co-cultures with NPF cells pre-treated with TGF- β (Fig. 8A). The significant increase in proliferation correlated with induction of *Gli1* ($p < 0.04$) but not *Ptch1* ($p < 0.07$). Nonetheless, we attribute the increase in proliferation to Hh pathway activation since increase in tumor cell proliferation and induction of Hh signaling by SHH were both abrogated by AZ75 (Fig. 8). Notably, AZ75 did not reverse the increased levels of *Gli1* or *Ptch1* expression in TGF- β pre-treated cells in the absence of SHH nor did it affect the rate of LNCaP proliferation in cultures performed in the absence of SHH.

Discussion

The studies reported here show that activation of Hh signaling in myofibroblasts is sufficient to accelerate tumor cell growth *in vitro*. Our previous studies of SHH overexpression in LNCaP xenografts excluded ligand-dependent Hh pathway activation in LNCaP tumor cells as a mechanism for growth promotion and firmly implicated actions of the tumor stroma (paracrine signaling) as the mechanism of interest. However, the relevant target cell(s) for SHH ligand remained uncertain. Hh signaling can affect multiple different cell types and processes including, for example, angiogenesis. We considered the possibility that Hh signaling would promote the development of new blood vessels that provide oxygen and nutrients facilitating tumor growth. Our more recently published studies showed that activation of Hh signaling in a myofibroblast cell line co-injected with LNCaP tumor cells accelerated tumor growth.¹⁶ This identified the myofibroblast as a key player in the paracrine response to Hh signaling that promotes tumor growth, however, these studies could not distinguish between a direct effect of myofibroblasts on tumor cell proliferation and an indirect effect mediated by or involving collaboration with other cell types to promote growth of tumor. Taken together with previous observations, our microchamber studies clearly show that stimulation of tumor cell proliferation requires neither a direct effect of Hh ligand on the tumor cell or other components of the tumor stroma nor an interaction of myofibroblasts in which the Hh signaling pathway is activated with other cell types.

The tumor stroma has been shown to play an important role in tumor growth and progression,²⁷ and reactive stroma has been implicated as a critical feature of tumorigenesis. Several studies show that reactive stroma is present in prostate intraepithelial neoplasia (PIN) and human prostate cancers but absent in normal tissues.²⁸ Xenograft studies show that reactive stroma regulates the development and progression of tumors indicating that reactive stroma in human prostate cancer is likely to function as a mediator of tumorigenesis.²⁹ We have shown both *in vitro* and *in vivo* that paracrine Hh signaling stimulates LNCaP and PC3 tumor cell proliferation. But further, we have shown that Hh signaling stimulates proliferation of the immortalized prostate epithelial cell line BPH-1. BPH-1 is a non-tumorigenic cell line that becomes transformed and forms tumors when combined with cancer associated fibroblasts.³⁰ Our finding that paracrine Hh signaling is able to promote proliferation of this cell suggests that paracrine Hh signaling could promote cell proliferation in pre-neoplastic lesions and supports the notion that reactive stroma plays a critical role in early stages of tumorigenesis.

A reactive tumor microenvironment is attributed to the presence of inflammation. Cancer-associated inflammation is characterized by the release of inflammatory cytokines and growth factors that induce phenotypic changes in stroma cells and stimulate tissue remodeling. The inflammation-induced changes in stromal are thought to promote formation of new blood vessels, stimulate cell proliferation and enable cell migration. These processes, canonical features of a healing wound, in the context of malignant change can facilitate tumor growth, invasion and metastasis. Tumors and most pre-neoplastic lesions are rich in

reactive stroma³¹⁻³³ and mouse xenograft studies have shown that reactive stroma was essential to stimulate incidence and growth rate of tumors formed by LNCaP cell.³⁴ TGF- β is a growth factor that exerts a range of activities affecting angiogenesis, matrix remodeling, cell growth and apoptosis.^{35,36} TGF- β is overexpressed at sites of wound repair and in many carcinomas, and is a primary inducer of myofibroblast differentiation—the key phenotypic hallmark of reactive stroma.^{24,28} Myofibroblasts are abundant cells in tumor tissues and the principal cellular component of reactive stroma in many epithelial cancers^{37,38} including prostate cancer.^{39,40} The studies did here reinforce the accumulating evidence that myofibroblasts are key mediators of the trophic effect of paracrine Hh signaling on tumor cell proliferation. Specifically, we used microchannel co-culture to demonstrate that normal human prostate fibroblasts treated with TGF- β exhibited the capacity to mediate paracrine Hh signaling and stimulate tumor cell proliferation. It is noteworthy that induction of myofibroblast differentiation of normal primary prostate fibroblasts by treatment with TGF- β was not sufficient to capacitate cells to respond to Hh ligand *in vitro* but required the presence of tumor cells in co-culture. This finding resonates with our previous study of Hh signaling in human prostate tissue specimens which implicated a dynamic interplay between myofibroblasts in the tumor stroma and tumor cells in recapitulating the pattern Hh regulated target gene expression characteristic of the developing prostate.¹⁶ In that study, we found that a fingerprint of 9 *bona fide* Hh target genes identified in the mouse fetal prostate, where Hh signaling promotes epithelial proliferation⁴¹ were re-expressed in the stroma of the LNCaP xenograft. Further, we found that Hh regulation of these target genes was strikingly evident specifically in tumors exhibiting a reactive stroma. Remarkably, Hh regulation of these target genes was absent from benign prostate tissue—despite a robust level of Hh signaling activity and the presence of a reactive stroma. We interpret these independent observations as strong suggestive evidence that cross-talk between epithelial and stromal cells which is an important determinant of a stromal response to Hh signaling that promotes epithelial (tumor) cell proliferation.

Materials and methods

Microfabrication

Fabrication and characterization of microchambers have been described in a previous publication.^{42,43} The array of microchambers is made out of polydimethylsiloxane (PDMS) using well established soft lithography methods (ESI).[†] Briefly, PDMS and crosslinker are mixed manually at a 10: 1 ratio, degassed under vacuum, poured over an SU-8 master mold and cured for 6 h at 60 °C. The PDMS microchamber array is then placed in a Soxhlet extractor overnight in ethanol to remove un-crosslinked oligomers.⁴⁴ PDMS microchambers are sterilized (autoclave) and mounted on top of a flat-bottom tissue-culture treated plate (Nunc, Rochester, NY) for enhanced cell attachment. Chambers have a volume of 2 μ L each and are separated by diffusion ports that are 500 μ m wide and 15 μ m tall. The diffusion dominant environment (laminar flow) and the fluid resistance imparted by the short height of the diffusion ports allows even cell distribution and prevents cells from getting mixed during seeding.

Cell culture

LNCaP, a human prostatic adenocarcinoma cell line, was obtained from ATCC (Manassas, VA). BPH-1 cells and normal prostate primary fibroblasts (NPF, N2-1 and N5-2) were obtained from Simon Hayward (Vanderbilt University, Nashville, TN). Generation of GFP (v-LNCaP) and SHH overexpressor GFP LNCaP (SHHv-LNCaP) cells was described before.¹¹ Indistinct behavior was verified for transfected cells as compared to the parental LNCaP cell line (ATCC). Isolation of the mouse prostate myofibroblasts, UGSM-2 and UGli3^{−/−}, was described in previous publications.^{16,18} All cells were mycoplasma free

(MycoAlert Assay, Lonza, Rockland, ME) and maintained in RPMI 1640 cell culture media (Cellgro, Mannansas, VA) supplemented with 2 mM L-glutamine, 1% Penicillin/Streptomycin, 4.5 g L⁻¹ of glucose, 10 mM HEPES, 1.5 g L⁻¹ sodium bicarbonate and 1 mM sodium pyruvate (complete media) with 10% or 3.0% (N5-2, N2-1 and NPF) of FBS and incubated at 37 °C with 5% CO₂.

Cell proliferation and differentiation

Cells were cultured in complete media supplemented with 3.5% FBS (microchambers) or 0.5% FBS (transwells). Tumor epithelial and stromal cells were seeded at a cell density of 350 cells mm⁻² and 750–800 cells mm⁻² respectively. In primary cell cultures, about 1000–1500 stromal cells were seeded in per side chamber. In transwells (Corning), LNCaP and UGSM-2 cells were seeded in the top (36 mm²) and bottom (192 mm²) compartments respectively. Transwell polyester membranes had a pore size of 0.4 µm which prevented cell migration. Culture media was changed at 24 h after seeding LNCaP. In microchambers, LNCaP and UGSM-2 cells were seeded in adjacent compartments. UGSM-2 cells were seeded a day before LNCaP cells to verify confluence. Cells were patterned in each micro-compartment *via* passive pumping.^{45,46} Briefly, a 5 µL droplet of cell culture medium was placed at the output port and a 2 µL droplet of cell suspension was delivered to the input port of each chamber. All micro-compartment were 250 µm height and held a volume of about 2 µL. The side chambers were connected to the center chamber through diffusion ports (15–18 µm high), thus allowing secreted soluble factors to diffuse freely between the chambers.²⁰ Epithelial cells were co-cultured with themselves (to keep a constant amount of volume per cell) in mono-culture conditions. Cell culture media was fully replaced (2 µL) after the first 24 h (day 1) and half replaced every two days in each compartment to prevent nutrient depletion and avoid washing away all diffused secreted factors respectively.

A concentration of 5 nM octylated-SHH (Curis, Inc., Cambridge, MA) and 1 µM AZ75 (Aztra Zeneca, Waltham, MA) were added 24 h after cell seeding and replenished during media changes. To induce a myofibroblast phenotype, 10 ng ml⁻¹ TGF-β (Preprotech, Rocky Hill, NJ) was incubated with primary fibroblasts for 4 days (replenish every 48 h). TGF-β was washed from culture prior to SHH and AZ75 treatments. Epithelial cell growth was monitored during the last 48 h of culture (day 5) using Click-iT 647 assay according to the manufacturer's recommendations (Molecular Probes, Eugene, OR).

Gene expression

To prevent RNA cross-contamination among human cells seeded in adjacent chambers, lysis buffer was added and collected one compartment at a time. RNA was isolated directly from cell lysates using Dynabeads mRNA DIRECT Micro Kit (Invitrogen, Carlsbad, CA). Total mRNA (20–30 ng) was reverse transcribed to generate cDNA in a volume of 30 µL using SuperScript III Reverse Transcriptase (Invitrogen). Primer sequences for mouse and human *GAPDH*, *α-SMA*, *Gli1*, *Gli2* and *Ptch1* have been described before.^{11,17} Relative mRNA quantity was determined by real-time RT-PCR using SYBR green (Molecular Probes, Carlsbad, CA), and iCycler instrumentation and software (BioRad, Hercules, CA). Data for each gene were normalized to the expression of *GAPDH*.

Cell live/dead assay

Cells were washed once with two volumes of 1× PBS prior to adding any reagent. A working solution of 2 µM calcein AM and 4 µM EthD-1 (Live/Dead viability/cytotoxicity kit for mammalian cells, Molecular Probes) was added directly to the cells and incubated for 15 min at room temperature prior to imaging. Two images were produced for each chamber, one representing the live stain (calcein/GFP) and the other representing the dead stain (ethidium bromide). A calibration curve that correlates intensity and cell number was

generated to estimate total cell per image. Viability was quantified by subtracting dead cell counts from total GFP-positive (v-LNCaP or SHHv-LNCaP) or calcein-positive (UGSM-2) cell counts divided by the total cell number.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were then permeabilized in 0.5% triton X-100 diluted in 1× PBS for 10 min. The following primary antibodies were used: mouse anti-acetylated tubulin (1: 400 dilution; T6793, Sigma), mouse anti- α -SMA (1: 400 dilution; A2547, Sigma-Aldrich), goat anti-human vimentin (1: 100 dilution; AB1620, Chemicon), goat anti-PTCH (1: 20; sc-6149, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-phospho-SMAD3 to the region Ser^{423/425} (1: 100 dilution; 9520, Cell Signaling, Boston, MA). Primary antibodies were detected with Alexa Fluor 488 anti-goat (1: 200 dilution; A11029, Molecular probes), Cy5 anti-mouse (1: 100; ab-6719, Abcam), Alexa Fluor 488 anti-rabbit (1: 200 dilution; A11034, Invitrogen) and Alexa Fluor 488 anti-mouse (1: 200; A11029, Invitrogen). A solution containing 3% BSA + 0.1% Tween 20 in 1× PBS was used for blocking (overnight, 4 °C) and antibody dilutions (1 h, 24 °C). Cells were incubated with 0.22 $\mu\text{g ml}^{-1}$ of Hoechst diluted in 1× PBS for 15 min prior to imaging.

Western blot

v-LNCaP and SHHv-LNCaP were seeded at a cell density of 350 cells mm^{-2} in a total culture volume of 700 (transwells), 50 (96 wells) and 4 μL (microchambers). Cells were cultured in media containing 0.5% (96 wells, transwells, microchambers) and 3.5% serum (microchambers). These serum concentrations were previously determined for co-culture experiments based on the response of UGSM-2 to SHH ligand. After 48 h, conditioned media was collected to detect secreted SHH in culture. Proteins present in conditioned media (15 μL) were separated on a 4–12% Bis-Tris gel (NP0321, NuPAGE Novex, Invitrogen) and then transferred to a nitrocellulose membrane (162-0095, Biorad, Boston, MA). Octylated-SHH was used as positive control. The membranes were blocked (overnight, 4 °C) with LI-COR blocking buffer (LI-COR, Inc., Lincoln, NE) and probed with rabbit anti-SHH (1: 1000; #2207, Cell signaling) and anti-rabbit, coupled to an infrared fluorescence marker with emission wavelength of 800 nm (1: 10000; IR800, Rockland Immunochemicals, Gilbertsville, Pa) for 1 h at room temperature (24 °C). Blots were imaged with an Odyssey Infrared Imaging System (LI-COR, Inc.).

Imaging and statistical analysis

Fluorescent images were taken using an inverted Nikon Eclipse Ti with NIS Elements 3.1 software and Digital Sight DS-2Mv camera. Images were taken using a 4× (NA: 0.10) and 10× (NA: 0.3) magnifications. For cell proliferation analysis, images were obtained as follows: one image per microchamber (entire chamber) and two images per well (96 well or transwell insert). Primary cilium was imaged with a 4× objective (NA: 0.13) using confocal microscopy (BD pathway fluorescent microscope), SPOT RT Color camera and SPOT Basic software. Fixed samples were kept in PBS 1× and imaged at room temperature (25 °C). Fluorescent nuclear counts and GFP intensities were determined using ImageJ v1.38 (NIH). % EdU (+) cells was obtained by dividing total EdU (+) cells to total cell number (Hoescht-nuclear stain) multiplied by 100. Two-sample t-tests were used to compare gene expression levels among culture conditions. Cell proliferation was assessed for significant differences by Wilcoxon Mann–Whitney test. Significant differences have a p -value <0.05.

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Insight, innovation, integration

We apply micro-scale co-culture systems to examine the role of the stroma in Hedgehog signaling-mediated tumor cell growth. The use of micro-scale culture systems allowed, for the first time, the recapitulation of paracrine Hedgehog signaling *in vitro* which had been previously observed in xenograft mouse models. Our findings provide new biological insights showing that activation of Hedgehog signaling in the stromal compartment is sufficient to accelerate tumor cell growth. The compartmentalized co-culture system enabled the conclusion that this effect does not require direct physical interaction or collaboration of other elements of the stroma with cancer cells. By isolating the tropic effect of Hedgehog pathway activation in prostate stroma, we have taken the first step toward identifying cell-specific mechanisms that mediate the effect of paracrine Hedgehog signaling on tumor growth.

Fig. 1.

Ligand-dependent activation of the Hh pathway. In the absence of ligand, *Ptch1* inhibits SMO activity. This inhibition leads to transcriptional repression mediated by *Gli3* (GLI3-R). In the presence of ligand, SHH binds to *Ptch1*, this relieves the repression of *Ptch1* on SMO. Activation of SMO removes transcriptional repression imparted by GLI3-R allowing transcription of Hh target genes (e.g. *Ptch1* and *Gli1*) by the transcriptional activators GLI2-A and *Gli1*.

Fig. 2.

Secreted SHH induces tumor cell proliferation through paracrine signaling. Representative image of cells co-cultured in adjacent compartments of the microchamber platform (A). Proliferation of LNCaP cells in response to paracrine Hh signaling was examined (B). Cells were seeded in adjacent compartments and incubated $\pm 1 \mu\text{M}$ AZ75 for 5 days. Proliferation of SHHv-LNCaP was significantly up-regulated in co-culture with UGSM-2 cells. AZ75 significantly reduced the number of proliferating SHHv-LNCaP co-cultured with UGSM-2. Expression of *Gli1* and *Ptch1* in the mouse myofibroblast cell line UGSM-2 was evaluated by qRT-PCR (C and D). *Ptch1* and *Gli1* expression levels in UGSM-2 co-culture with LNCaP were calculated relative to the expression of *GAPDH*. Graph shows that co-culture with SHHv-LNCaP significantly up-regulated the expression of *Gli1* (C) and *Ptch1* (D) in UGSM-2. Columns show mean of three independent experiments. Error bars represent ± 1 SD.

Fig. 3.

Exogenous SHH promotes tumor cell growth through paracrine signaling. LNCaP cells were cultured alone (control) or co-cultured with UGSM-2 cells in microchambers. Cells were incubated ± 5 nM SHH ± 1 μ M AZ75 for 5 days. Expression levels of *Gli1* (B) and *Ptch1* (C) in UGSM-2 co-cultured with LNCaP was evaluated relative to GAPDH. Columns are mean of triplicates. Error bars represent ± 1 SD. Data are representative of three independent experiments.

Fig. 4.

Overexpression of SMO in the stroma promotes tumor cell growth. LNCaP cells were co-culture with UGSM-2 or USMO cells 1 μ M AZ75 for 5 days. Expression levels of *Gli1* (B) and *Ptch1* (C) in $\pm$ UGSM-2 + SHH monocultures and UGSM-2 and USMO co-cultured with LNCaP were evaluated relative to *GAPDH*. Columns are mean of three independent experiments. Error bars represent ± 1 SD.

Fig. 5.

UGli3^{-/-} accelerates proliferation rates of prostate cells. Cells were culture in microchambers $\pm 1 \mu\text{M}$ AZ75 for 5 days. Graphs (A–D) show the average total EdU (+) epithelial cell in mono-culture (control) and co-culture (UGSM-2 or UGli3^{-/-}) conditions. RT-PCR analysis of *Gli1* (E) and *Ptch1* (F) expression in UGSM-2 and UGli3^{-/-}. *Ptch1* and *Gli1* expression levels in UGSM-2 + SHH monocultures and UGSM-2 and UGli3^{-/-} co-cultured with LNCaP were calculated relative to *GAPDH*. Columns are mean of three independent experiments. Error bars represent ± 1 SD.

Fig. 6.

Expression of cilia and Ptch receptors in normal human prostate fibroblasts. Primary fibroblasts express cilia (A). Cells were plated at sub-confluence (80% confluence) in microchambers at for 48 h. UGSM-2 cells were plated at confluence and used as a positive control for cilia expression. White arrow heads indicated ciliary localization. Nuclei are stained with Hoechst (blue), cilia and microtubules are stained with anti-acetylated tubulin (green) and proliferating cells are stained with Alexa fluorophore 647 (red). Ptch receptors are expressed in primary fibroblasts (B). White arrows indicated clumps of Ptch receptors in the cell membrane. Nuclei are stained with Hoechst (blue) and patched receptors are stained with anti-Ptch (green). (C) Fibroblasts were incubated ± 5 nM SHH for 48 h. Expression levels of *Gli1* and *Ptch1* were compared relative to *GAPDH*.

Fig. 7.

TGF- β -treated myofibroblast-like cells show high levels of *Gli1*. Cells were treated with TGF- β for 4 days. Expression of phosphorylated SMAD-3 (A) and mRNA levels of α -SMA (B) were evaluated to confirm myofibroblast phenotype. Following TGF- β treatment, cells were incubated $\pm$ 5 nM SHH for 48 h. Expression levels of *Gli1* (C) and *Gli2* (D) were compared among primary fibroblasts and TGF- β -treated cells. Myofibroblast-like cells are indicated by TGF- β or TGF- β + SHH. Columns are mean of three independent experiments. Error bars represent $\pm$ 1 SD.

Fig. 8.

Myofibroblast-like cells promote tumor cell growth in response to SHH. LNCaP cells were cultured with normal primary fibroblasts (NPF) or myofibroblast-like cells (myo-NPF) for 72 h in microchambers. Cells were incubated ± 5 nM SHH $1 \mu\text{M}$ AZ75. Proliferation of LNCaP cells was determined by the number $\pm$ of EdU positive cells per total cells (Click-IT). RNA was isolated from the stromal compartment (co-culture adjacent to LNCaP) and expression of *Gli1* and *Ptch1* was determined relative to *GAPDH*. Data represent mean of three independent experiments. Error bars represent ± 1 SD.