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Review

Sonic Hedgehog signaling in advanced prostate cancer

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Abstract. The Hedgehog family of growth factors activate a highly conserved signaling system for cell-cell communication that regulates cell proliferation and differentiation during development. Abnormal activation of the Hedgehog pathway has been demonstrated in a variety of human tumors, including those of the skin, brain, lung and digestive tract. Hedgehog pathway activity in these tumors is required for cancer cell proliferation and tumor growth. Recent studies have uncovered the role for Hedgehog signaling in advanced prostate cancer and demonstrated that autocrine signaling by tumor cells is required for proliferation, viability, and invasive behavior. The level of Hedgehog activity correlates with the severity of the tumor and is both necessary and sufficient for metastatic behavior. Blockade of Hedgehog signaling leads to tumor shrinkage and remission in preclinical tumor xenograft models. Thus, Hedgehog signaling represents a novel pathway in prostate cancer that offers opportunities for prognostic biomarker development, drug targeting and therapeutic response monitoring.

Key words. Hedgehog; prostate cancer; metastasis.

Introduction

Since its discovery about 30 years ago, the Hedgehog (Hh) signaling pathway has become widely recognized as one of the major mechanisms for cell-cell communication in developing organisms. While many of the core components and interactions between members of the Hh signaling pathway are conserved from flies to man, mammalian-specific components and mechanisms add to the complexity of the system. More recently, the role Hh signaling plays in medical syndromes such as birth defects and cancer has been the focus of much attention. In 2004, a series of articles defined the role of Hh signaling in the growth and metastasis of advanced prostate cancer [1–4]. Molecular mechanisms and pathway components discovered in biochemical, genetic and molecular studies of Hh signaling during development, particularly in stem cell biology, are now demonstrated to be behind characteristics of tumor cells such as proliferation and invasive behavior.

This review attempts to integrate these recent results as well as giving the reader a general context in which to place them. A comprehensive discussion of the detailed mechanisms of Hh signaling and the developmental systems in which it operates is not the goal of this review, and for more information along these lines we suggest that the reader explore the following recent review [5]. The breadth of research on Hh signaling leads to the dissemination of results in many types of journals and meetings ranging from development to cell signaling to cancer therapeutics. For example, a literature search of PubMed-recognized publications in 2004 listed over 250 articles in journals ranging from *Biophysical Journal* through *Development* to the *Journal of Nutrition* and *Investigative*

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Opthalmology and Visual Science. Thus the identification and integration of critical information is one of several obstacles to the rapid translation of basic studies in the Hh field to clinical treatment. This review attempts to critically integrate the biological data and outline trends and associations in the Hh field. In doing so we hope it both provides insight into the role of Hh in cancer biology, and directs scientists to the future questions and opportunities present in the Hh field in particular and developmental cancer biology in general.

Discovery of the Hh signaling pathway

Many of the core components of the Hh signaling pathway were originally identified in the 1970s and 1980s by the now classic genetic screens of Nusslein-Volhard and Wieschaus in the fruit fly *Drosophila* [6, 7]. These studies of the patterning of the *Drosophila* embryo produced the first mutations in *ptc* and *smo* as well as in *hh* itself. Analyses by many groups led to the general understanding of Hh as a secreted signaling factor that controlled elements of pattern formation in many tissues of the developing fly. The cloning of *hh* in 1992 [8–16] opened the door for the simultaneous identification of vertebrate homologs of *hh* in mouse, chick and zebrafish a year later [11–13], and since then the field has grown dramatically. Twenty-five years after the initial publication describing mutations in *hh*, successful genetic and molecular screens are still being carried out to identify additional members of the Hh signaling pathway [14–18]. While many of the genes discovered are conserved from flies to man, there are some aspects of Hh signaling that appear to be specific to mammalian systems.

A much simplified description of the Hh pathway includes the Hh ligands, their receptor and a few of the molecules that transduce the signal (see pathway in figure 1). In mammals there are three distinct Hh ligands with different patterns of expression: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). All three Hh ligands bind the transmembrane receptor Patched1 (Ptch1). Although a Patched2 protein exists, its function has not been clearly delineated. In the absence of Hh ligand, Ptch1 inhibits a second transmembrane protein Smoothened (Smo). Hh binding to Ptch1 relieves the repression of Smo and allows Smo to further transduce the Hh signal. This Hh signaling results in the activation of the Gli family of transcription factors: Gli1, Gli2 and Gli3. *Gli1* and *Ptch1* as well as the gene encoding the Hedgehog Interacting Protein (Hip) are Hh response genes, and their expression has been used as evidence for active Hh signaling. Expression of *Ptch1* or *Hip* starts a negative feedback loop that shuts down Hh signaling by sequestering Hh ligand. In the cytoplasm the Suppressor of Fused [Su(fu)] protein also inhibits transduction of the Hh signal. For a detailed description of the components of the Hh pathway and their function, we suggest that the reader consult a recent review focused on the mechanisms of Hh signal transduction [5].

Basic research has focused on questions such as the cellular and molecular mechanisms that establish localized concentrations of Hh activity, the role of Hh growth factors in a wide range of developmental decisions, the interactions between the Hh pathway and other signaling pathways and the relationship between Hh structure and function. These studies have pinpointed the involvement of heparan sulfate proteoglycans and enzymes that synthesize or modify heparan sulfate chains [19], enzymes that transfer lipid moieties [20], intracellular kinases [21, 22], Hh multimers [23, 24] and most recently lipoprotein particles [25] in the correct functioning of the Hh pathway during development.

Hhs and development

The original *hh* allele was identified due to its role in the segmentation of the *Drosophila* embryo and the patterning of the adult wing. In vertebrate models such as chick and mouse, the function of Shh in limb development and central nervous system patterning has been well established. In mammals three Hh growth factors have been identified: Shh, Ihh and Dhh [11]. All three Hh proteins work through the transmembrane protein Smo. While the role of Shh in mammalian development has been extensively examined [26], the role of Ihh in chondrogenesis [27] and the role of Dhh in Schwann cell biology have only recently been addressed [28, 29]. These invertebrate and vertebrate studies are the basis of the current model of the Hh family of growth factors as morphogens that form growth factor gradients, originating at the cellular source of Hh production. At a given position in the gradient the concentration of Hh proteins is translated by the activation of different Hh-dependent transcription factors into discrete cell types. The determination of different cell fates at reproducible distances from Hh expressing cells results in overall tissue patterning.

The role of Hh growth factors during development is not limited to patterning. Various studies have now demonstrated the function of Hh signaling in the control of cell proliferation, especially for stem cells and stem-like progenitors. Studies of the vertebrate central nervous system have shown that Shh is required not only for patterning, but also for the proliferation of neuronal precursors in chick and mouse [30, 31]. Repressing Shh signaling with blocking antibodies decreases neural crest proliferation [30], while ectopic expression of *Shh* results in increased proliferation of spinal cord precursors [31]. Inhibition of Shh signaling also decreases proliferation of stem cells in the subventricular zone of the brain, while

added Shh increases proliferation of neurospheres derived from subventricular zone cultures [32]. In flies, mitotic activation of neural stem cells in the central nervous system is in direct proportion to the level of *hh* expression [33]. Manipulation of *hh* expression levels using inducible or temperature sensitive *hh* genes results in increased or decreased numbers of dividing neural stem cells, respectively. Constant exposure of neural stem cells to excess Hh results in premature cellular proliferation, suggesting that continually high levels of Hh signaling in stem cells results in aberrant cellular response [S. Datta., unpublished]. Hh also regulates the proliferation of other stem cells in both invertebrate and vertebrate systems, including epidermal stem cells and somatic stem cells of the gonad [34–36]. Thus, depending on timing and context, Hh growth factors are capable of regulating cell proliferation, especially of precursors or stem cells, and cell differentiation.

Shh and prostate development

The role of Shh in regulating induction, proliferation and differentiation of the developing mammalian prostate has been studied most extensively in mouse and rat models, and the interpretation of the resulting data has been the subject of some confusion. Despite the sometimes conflicting results, good evidence exists that Shh is required for ductal patterning in the developing prostate. The murine prostate develops from the simple tube of the urogenital sinus starting at about E17. At this stage the epithelium of the urogenital sinus begins to bud into the surrounding mesenchyme in response to testosterone. These epithelial buds grow and form the prostate ducts. The prostate ducts will elongate and branch extensively both prenatally and postnatally until the final elaborate branching structure of the mature prostate is achieved.

Hh function in the prostate seems to center around the establishment of ductal branching. *Shh* expression begins in the epithelial layers of the rodent urogenital sinus and concentrates at areas of epithelial budding [37]. Levels of *Shh* transcripts continue to be elevated as epithelial invaginations become ductal buds. During duct elongation, *Shh* transcripts localize at the growing distal tips [37–39]. The role of the other mammalian Hh proteins is less clear. *In situ* hybridization and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays do not detect expression of *Desert Hedgehog* during the time of prostate development [40, 41]. Very low levels of *Indian Hedgehog* were consistently observed using RT-PCR from E15 through P10 [41]. In comparison to the development of the prostate in rodents, human prostate development is also marked by a dramatic increase in *SHH* expression in the epithelial layers of the urogenital sinus, particularly in the areas of new prostatic budding [42]. *SHH* expression appears at approximately the same time as a surge in testosterone levels as monitored in the serum, suggesting that the two may be coordinated. *Shh* expression in the budding epithelium of the urogenital sinus correlates with a functional Hh pathway, as it results in upregulation of the Hh pathway proteins *Ptc1*, *Gli1*, *Hip* and *BMP4* in the adjacent mesenchyme [37–39]. Culture of urogenital epithelium with Shh does not induce *Ptc1* expression, nor does it upregulate *Gli1* expression, two indicators of Hh pathway activity [37]. In contrast, culture of urogenital mesenchyme with Shh does result in upregulation of both *Ptc1* and *Gli1*. Thus, the functional role of Hh appears to be as a paracrine factor made in the epithelium and activating downstream events in nearby mesenchymal cells.

Expression of *Shh* and activation of the Hh pathway appears to be required for ductal budding and branching. Acute or short-term inhibition of Shh signaling in rodent urogenital sinuses using Shh-blocking antibodies or the plant alkaloid cyclopamine, which targets the transmembrane protein Smo, resulted in inhibition of ductal morphogenesis either at the level of duct budding or branching [37, 38, 43]. The reason for developmental blockage at ductal budding in some assays and ductal branching in others is not clear. Since cyclopamine inhibits Smo function, it will also block signaling by Dhh or Ihh if they are active. In contrast, constitutive elimination of *Shh* expression by knock out of the *Shh* gene in transgenic mice resulted in defects in ductal budding [38]. Of note, the loss of Shh function could be rescued by increasing testosterone levels *in vivo* and in culture. These data suggest that the long-term removal of Shh signaling in *Shh* null male mice results in deficiencies in testosterone function that also affect prostate development. Thus, although there are still questions as to the role Shh plays in prostate development, it is clear that Shh produced by prostate epithelium signals to the prostatic mesenchyme. Shh signaling within the developing prostate is required for the complex pattern of ductal branching seen in the mature prostate. The role of Shh in ductal branching is particularly interesting given the observed role of Shh in branching morphogenesis in the mammary gland, lung and pancreas [44–46], three tissues in which aberrant Shh signaling has also been implicated in oncogenesis [47–50].

Reactivation of developmental pathways in cancer

The connection between development and oncogenesis has a long history rooted in both molecular and cell biology. Genes and processes generally considered to be important for oncogenesis have been shown to play important roles in development. For example, *ras*, now known to be of central importance in receptor tyrosine kinase

signaling – one of the major developmental signaling pathways – was originally isolated as an oncogene [51, 52]. The roles of genes required for cell cycle regulation and control of cell cycle phase in directing cell fate decisions are becoming more and more recognized. Finally, the role of apoptosis, a process subverted or eliminated in oncogenesis, is well accepted in normal development as a means of creating selective nerve synapses or removing extraneous cells during organ patterning.

It is also becoming more and more apparent that genes and pathways initially identified due to their importance in development are frequently upregulated in cancer cells. The gene encoding Wingless, a founding member of the Wnt family of growth factors, was originally uncovered in the same series of genetic screens that produced mutations in *hh* [6]. The mammalian Wnt, on the other hand, was originally identified as an oncogene in studies of mouse models of breast cancer using mouse mammary tumor virus. Recently, studies have suggested that Wnt signaling activates the proliferation of precursor cells to produce tumors [53, 54]. Other signaling molecules originally discovered in a developmental context are also being implicated in oncogenesis. These factors include Notch [55], the Bone Morphogenetic Proteins [56] and, of course, the Hhs (see below).

Another argument for the study of developmental processes in oncogenesis comes from the burgeoning evidence that some tumors contain cells with characteristics similar to stem cells. Stem cell-like cells have been isolated from acute myeloid leukemia, breast cancer, gliomas and most recently medulloblastoma [57]. Evidence that these cells are stem cell-like is found in their possession of stem cell characteristics: large proliferative capacity, apparent self-renewal, expression of stem cell markers and in some cases the ability to generate new tumors upon transplantation more efficiently than bulk tumor cells [57]. It is unclear as to whether these 'cancer stem cells' are endogenous stem cells that have become dysregulated or tumor cells that have acquired stem cell-like properties. Yet the prediction is that understanding the mechanisms of stem cell self-renewal and proliferation control will provide insights into oncogenesis. Perhaps not surprisingly, among the genes known to control stem cell proliferation are the Hh growth factors.

Hhs and Cancer

Activation of the Hh pathway has been implicated in a variety of cancers. For example, patients suffering from Gorlin's syndrome have increased susceptibility to basal cell carcinomas and medulloblastomas. The discovery that these patients with basal cell carcinoma carry mutations in *PTCH*, the Hh receptor, that result in hyperactivation of the Hh pathway was the first suggestion that activation of Hh signaling might be critical for carcinogenesis [58, 59]. Shortly thereafter studies demonstrating that overexpression of *Shh* produced basal cell carcinoma-like tumors in mouse and human skin were published [60, 61]. At the same time, transient expression of the Hh downstream activator *Gli1* was shown to induce skin tumors in tadpoles [62]. Further analysis revealed that activating or missense mutations in *SMOH*, a Hh co-receptor, are present in human basal cell carcinomas and medulloblastomas [63, 64]. Additional data now exist suggesting various roles for the Hh pathway in breast, esophageal, lung, pancreatic, and stomach tumors [49, 64–73]. Thus it appears that the Hh pathway is affected in multiple tumor types. In addition multiple pathway components are affected (fig. 1). An understanding of these changes in different tumor types demonstrates both the ubiquitous nature of the Hh pathway in tumorigenesis but also suggests key areas for study in specific tumor types.

Drug targeting has also led to insights into Hh activity in cancer. Initial studies demonstrated that the plant alkaloid cyclopamine affects pattern development, in particular midline facial features, leading to cyclopian dysmorphic changes. Further research demonstrated that the effects of cyclopamine occur at the level of cellular proliferation and that the drug blocks the Hh signaling pathway by binding to and inactivating Smo [74–77]. In cancer studies cyclopamine-treatment of human glioma and medulloblastoma cell lines resulted in inhibition of cell division [78]. Subsequent studies demonstrated that addition of cyclopamine to murine medulloblastoma cells inhibited their proliferation, downregulated expression of a neural stem cell marker and stimulated expression of differentiation-associated genes [79]. Addition of cyclopamine to human medulloblastoma cells also resulted in loss of cell viability, suggesting the drug's potential as a chemotheraputic. Finally, systemic administration of cyclopamine in a mouse model for medulloblastoma reduced tumor size and tumor cell number while increasing apoptosis [80].

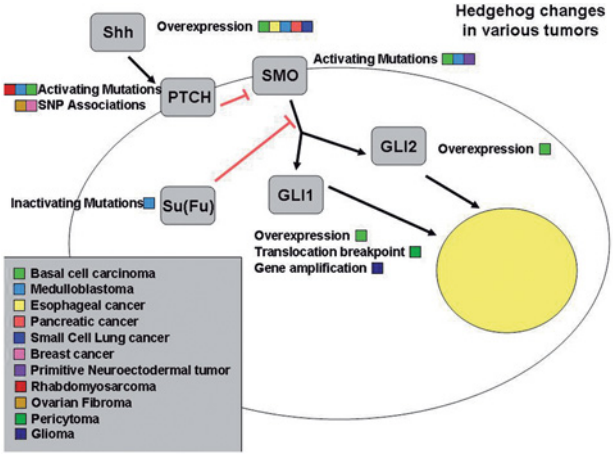

Figure 1. Tumor changes in the Hh pathway.

The observation that Shh signaling is required for lung development [46, 81–83] led to the possibility that hyperactivation of the pathway might lead to lung cancer. Similarly to prostate development Hh signaling in the lung is paracrine, with *Shh* expression during murine lung development occurring in the epithelial compartment, while *Ptc1* expression is found in the adjacent mesenchyme [73, 81]. However, upon lung injury and regeneration, both *Shh* and *Gli1* are expressed in the regenerating epithelium, suggesting a shift to autocrine signaling during tissue repair [73]. Analysis of samples from small cell lung cancer (SCLC), a highly aggressive form of lung cancer which demonstrates neuroendocrine properties, showed that half of the clinical samples expressed both *SHH* and *GLI1*, in comparison to only 10 percent of the more typical non-small cell lung cancers (NSCLCs). This tendency was confirmed in cell lines, where most SCLC cell lines expressed both *SHH* and *Gli1*, but none of the NSCLC cell lines expressed *Gli1*. Interestingly, NSCLC cells express *SHH*, much like normal developing lung epithelium, suggesting they are still capable of *SHH* production for paracrine signaling to adjacent cells. Thus, while NSCLC cells retain more developmentally patterned paracrine Hh signaling, SCLC cell lines appear to have developed a level of reliance on Hh signaling whether autocrine or paracrine. As observed before for medulloblastoma, cyclopamine treatment of SCLC cell lines expressing both *SHH* and *GLI1* inhibited their proliferation, downregulated stem cell markers and increased apoptosis. Subcutaneous injection of cyclopamine into animals carrying xenografts of SCLC cell lines but not NSCLC cell lines decreased tumor growth. Similar studies have been carried out to demonstrate the role of Shh signaling in stomach, pancreatic, esophageal, urothelial and breast tumors [47–49, 84]. So far, all the cancers that are Hh dependent arise from tissues in which Hh signaling also plays an important role during development. Whether this remains true will be an interesting question to address, and may shed light on the mechanism of initial Hh pathway activation.

Prostate cancer

There will be an estimated 230,000 new prostate cancer diagnoses in the United States in 2005. Risk factors associated with prostate cancer include both environmental and genetic components, with the interplay between genes and environmental exposures most likely determining an individual's risk for developing clinically significant prostate cancer. The strongest environmental associations include dietary fats, micronutrients such as zinc and/or selenium, and soy proteins [85]. Prostate cancer also is estimated to have a significant genetic component [86] and familial genetic mapping studies have identified a series of chromosomal regions that are associated with familial prostate cancer risk. These include regions on chromosomes 1p36, 1q24–25, 8p22, 17p and Xq27–28 that have been identified in individual cohort studies [87–96]. Additional chromosomal regions that may contain prostate cancer susceptibility genes have been recently published by the International Consortium for Prostate Cancer and include regions on chromosomes 5q12, 8p21, 15q11, 17q21 and 22q12 [97]. Thus it is clear that multiple genes in concert with environmental factors will play a role in a person's ultimate susceptibility to clinically significant prostate cancer.

The majority of patients diagnosed with prostate cancer will have tumor localized to the prostate gland and will be treated with surgical removal of the prostate (radical prostatectomy), radiation, or hormonal therapy. For these patients the 10-year disease free survival will be in the 70% range, with the remaining 30% demonstrating disease recurrence with elevated blood levels of the tumor marker prostate specific antigen (PSA). If the initial treatment fails to control the spread of tumor, or if the prostate tumor has spread throughout the body at the time of diagnosis, the options for treatment decrease significantly. In patients with demonstrable metastatic disease the survival drops to 10% at 10 years. Advanced prostate cancer tends to spread to regional lymph nodes and then to bone, with a significant source of patient pain and suffering associated with tumor as it grows in the bone marrow space. Modulation of the testosterone (androgen) signaling pathway for prostate cancer cell growth has been the mainstay of treatment for advanced prostate cancer, yet the subsequent development of androgen-independent tumor cells has been the hallmark of refractory tumor, occurring between 1 and 3 years after treatment initiation. Thus, the identification of signaling pathways that regulate the growth of advanced prostate cancer, in particular of androgen-independent tumor cells, is of utmost importance. These signaling pathways may represent both biomarkers for the development of advanced androgen refractory prostate cancer and targets for drug design that could be used, alone or in concert with hormonal treatments, to stop the growth of advanced prostate cancer.

Clinical significance of Shh expression in the prostate

The requirement of Shh for prostate development as well as the misregulation of Shh signaling in a variety of cancers suggested to us that expression of *Shh* and/or activation of the Hh pathway might be critical for the development of prostate cancer. Furthermore, it is interesting to note that multiple components of the Hh pathway are present within chromosomal regions associated with susceptibility to human prostate cancer (fig. 2).

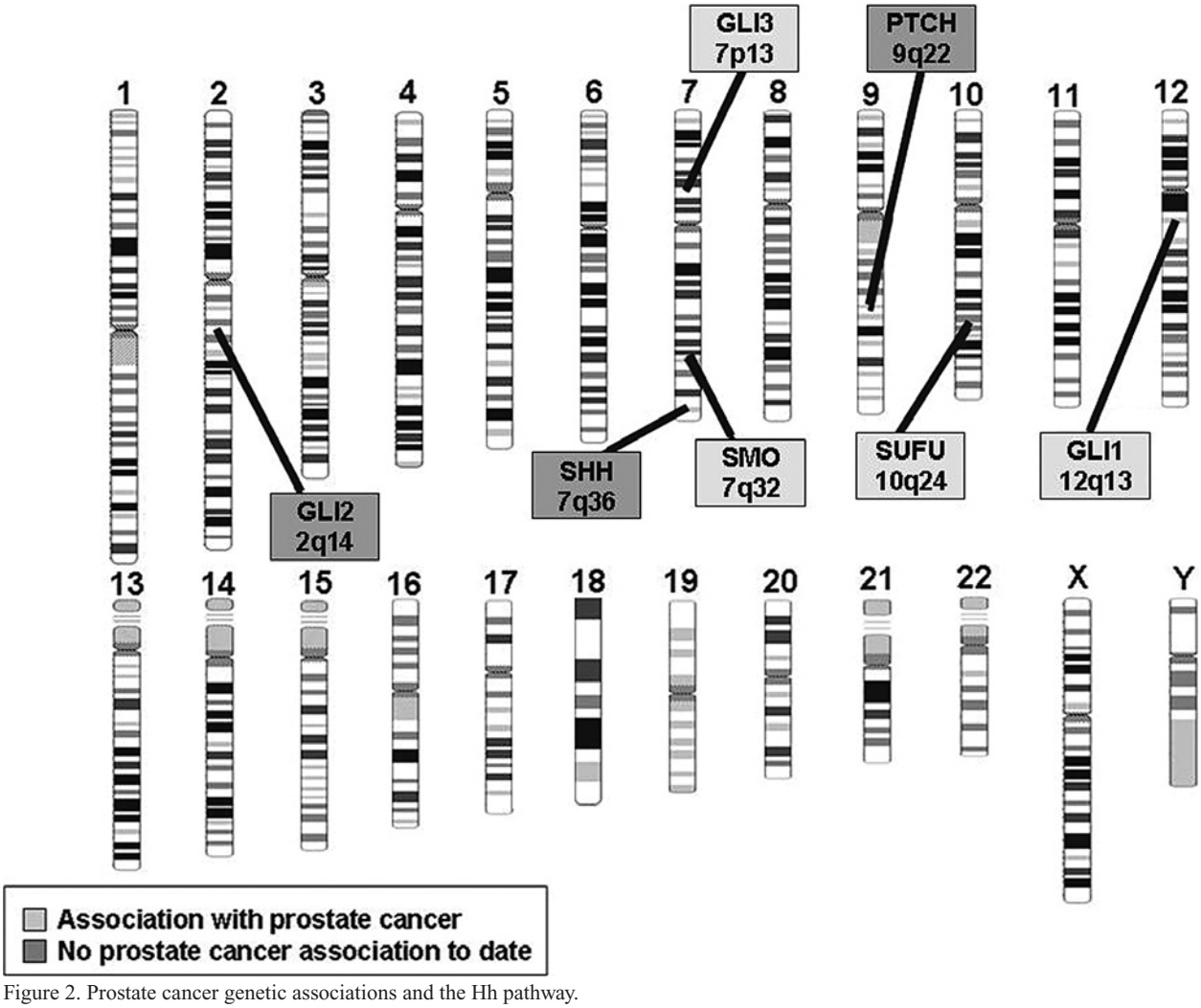

Figure 2. Prostate cancer genetic associations and the Hh pathway.

Bioinformatic analysis of data from genetic studies of familial prostate cancer showed that regions implicated in prostate cancer susceptibility contained the genes coding for Hh pathway components, including GLI1, GLI3, SMOH and SU(FU) [3, 33, 90, 98–101]. Thus, while currently only speculative, the possibility exists that mutations or changes at multiple levels of the Hh pathway may lead to an increased risk of prostate cancer. This is coupled with data that indicate that the Hh pathway is present and expressed in human prostate tumors. Analysis of SHH protein immunoreactivity in both normal prostate tissues and tumors demonstrated an upregulation of Shh in prostate tumors, a finding that correlated with the more aggressive tumors, with the Shh-positive tumors having a higher Gleason grade [2–4]. A meta-analysis of the combined data on SHH and its downstream signaling molecules [2–4] reveals that while SHH is present in both benign and malignant prostate epithelial cells, the downstream targets PTCH and GLI1 are preferentially activated in metastatic prostate tumors (table 1). Of particular interest, analysis of the data presented in the initial three reports of SHH signaling in prostate cancer reveals that all the advanced metastatic prostate cancers studied to date have activated the SHH signaling pathway.

Although the statistical significance of the correlation between detection of the SHH signaling pathway and the presence of metastatic prostate cancer is exciting, it is important to remember that the presence of SHH protein alone does not truly indicate the activation of the pathway and does not correlate with tumor grade. Due to the complexity of the Hh pathway as demonstrated in other tumor types (fig. 1), myriad mechanisms exist that could induce activation of the pathway even in the absence of ligand. These would include the upregulation or activating mutations in the Hh receptor PTCH or its co-receptor SMO, or the downstream signaling Gli proteins. There is now a growing knowledge that similar mechanisms are in place regarding the Hh pathway and prostate cancer (fig. 3).

Table 1. Meta-analysis of tissue data for expression of Shh, Patched and Gli-1.

RT-PCR	localized tumor	metastatic tumor	
Shh			
High	16	15	
Low	2	0	p=0.4886
Patched			
Y	8	15	
N	10	0	p=0.0005
Gli-1			
Y	8	15	
N	10	0	p=0.0005
IHC	Shh High/Present	Shh Low/Absent	
localized tumor	32	77	
metastatic tumor	4	0	p=0.0091
Gleason 6 or less	11	38	
Gleason 7 or higher	25	76	p=0.9069

RT-PCR data (upper panel) from Figure 3 of Karhadkar et al. and Table 1 of Sanchez et al. [3]. Immunohistochemistry (IHC) data (lower panel) from additional data from Sheng et al. [4], and Table 2 of Sanchez et al. [3]. After a review of Figure 2 in Sheng et al., the IHC data was combined into low/absent expression and high/present expression. All statistics performed using the Fisher's exact test.

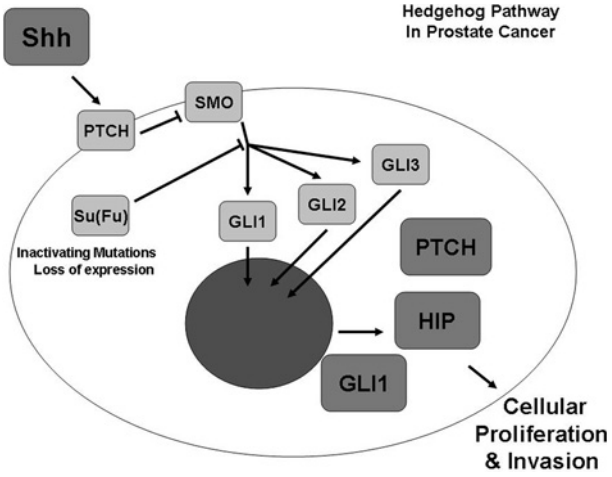

Figure 3. Prostate cancer changes and the Hh pathway. Dark grey molecules indicate overexpression.

For example, 7 out of 59 tumors assayed by Sheng et al. [4] were negative for SHH protein but positive for PTCH1 and HIP proteins, two indicators of Hh signaling activity. Continual activation of the Hh pathway in 4 of these tumors could be due to the lack of SU(FU) protein, an inhibitor of Hh signaling. The mechanism(s) resulting in pathway activation in the remaining 3 tumors are not known. Nine of 59 tumors were positive for SHH, PTCH1 and HIP proteins, but lacked SU(FU). Since loss of SU(FU) in this context would further increase the activity of the pathway, it may be significant that 8 of the 9 tumors were highly aggressive Gleason score 9 or 10 cancers. In 2 of these tumors, the surrounding normal tissue was positive for SU(FU), suggesting a tumor-specific loss of SU(FU) function. Cloning of the gene from the tumor tissue revealed mutations that result in premature stop codons, presumably inactivating the protein. The matched benign tissue carried a normal copy of the gene, indicating somatic loss of functional SU(FU). The loss of SU(FU) expression in aggressive tumors with active Hh signaling along with the function of SU(FU) as an inhibitor of the Hh pathway places *SU(FU)* as a tumor suppressor gene for prostate cancer and correlates well with its placement in a genomic region implicated in prostate cancer susceptibility [102]. In contrast, the presence of SHH is not always indicative of downstream Hh pathway activity. RT-PCR analysis of 12 localized tumors by Karhadkar et al. [2] showed detectable *SHH* transcripts in all of them, but only 3 of the 12 demonstrated Hh pathway activity as monitored by expression of *PTCH* and *GLI*. In our hands [3] [A. Hernandez, S. Datta and M. Datta, unpublished], 8 tumor samples showed detectable *SHH* message by qRT-PCR, and all had measurable levels of *PTCH1* and *GLI1* messenger RNA (mRNA). However, comparison of matched tumor and benign tissue showed that only five of the eight patients showed increases in *SHH*, *PTCH1* and *GLI1* in tumor versus normal samples. Thus, not all tumors regulate SHH at the RNA level. Yet, 1 of the 8 patients showed a large increase in *GLI1* expression in tumor tissue with no significant change in the level of *SHH* transcripts, indicating that activation of Hh occurred downstream of *SHH* expression. Thus, lack of SHH immunoreactivity in a tumor biopsy does not necessarily indicate that the Hh pathway is not active in the tumor. Conversely, some tumors that express *SHH* do not exhibit pathway activity.

The recent analyses of tumor samples have produced conflicting results on the importance of SMOH in prostate cancer. All 15 metastatic tumors from the published series showed active Hh signaling as assayed by expression of *PTCH1* and *GLI* as well as *SHH* by RT-PCR [2]. Of the 15 samples, 9 also expressed elevated levels of *IHH* message compared with localized tumors. Four of the metastatic samples showed very low levels of *SMOH* expression, and 11 of 12 localized tumors had no detectable *SMOH* message. Many of the latter also appeared to have no Hh activation as monitored by *GLI* and *PTCH* expression, suggesting that tumors that fail to utilize the Hh pathway may do so because they lack SMO function. The role of SMO in the prostate epithelium was demonstrated when

Karhadkar et al. examined Hh signaling components in the normal prostate epithelial cell line PE. No *PTCH*, *GLI1* or *SMOH* expression was seen. However, when the PE line was transfected to express *SMOH,* endogenous Hh signaling activity and the ability to respond to exogenous Shh ligand was restored. These data suggested that expression of *SMOH* might be the limiting factor for Hh pathway activation in normal prostate cells and at least a subset of tumors. Thus, changes in SMO expression or function may be a feature of advanced disease.

Autocrine versus paracrine Hh signaling in prostate cancer

Conflicting results on the role of autocrine and paracrine Shh signaling in prostate cancer cell growth have been obtained in different studies. These could be explained by differences in Hh signaling during development and carcinogenesis. Studies of Hh pathway components during prostate development demonstrate epithelial-stromal signaling, although low levels of Hh response gene expression were also observed in epithelial tissues. Signaling from the epithelium to the surrounding stroma was also observed by Fan et al. [1] in sections of prostate tumor used for *in situ* hybridization. When cells from the human prostate cancer epithelial cell line LNCaP were transfected to express *SHH*, they did not activate expression of human *PTCH1* or *GLI1*, indicating a lack of autocrine SHH signaling. Nor did incubation with 5 μM cyclopamine affect LNCaP proliferation. When LNCaP cells expressing *SHH* were used in murine xenograft studies, they induced murine *PTCH1* and *GLI1* in the stroma of the murine hosts, but not human *PTCH1* and *GLI1* in the transplanted epithelial cells. These findings suggest that *in vivo* prostate cancer cells are capable of paracrine but not autocrine SHH signaling. However, SHH overexpressing LNCaP cells produced tumor xenografts with significantly higher tumor growth rates than SHH null LNCaP xenografts. The increased size of the tumors was due to increased tumor cell mass rather than increased stromal cell mass, as judged by the relative percentage of epithelial versus stromal tissue in the tumors and the lack of mitotic figures in the tumor stromal tissue. Thus, the results suggest that while the LNCAP prostate epithelial cells can regulate SHH in a paracrine, but not an autocrine, manner, there appears to be an inherent advantage for Hh in tumor growth.

In contrast, expression of *SHH*, *PTCH1* and *GLI1* by LNCaP cells was observed by Karhadkar et al. [2] and addition of cyclopamine to LNCaP or primary prostate cancer cultures produced a dramatic drop in cell proliferation as well as a decrease in the expression of GLI1 in the LNCaP cells [3]. Our *in situ* hybridization studies [3] showed expression of *SHH*, *PTCH1* and *GLI1* in the epithelium of prostate tumor tissue, suggesting autocrine signaling. RT-PCR studies demonstrated that LNCaP cells as well as six primary human prostate cancer cell cultures express *SHH*, *PTCH1* and *GLI1*, further suggesting autocrine signaling. Growth of primary prostate cancer cells with a blocking antibody to SHH caused decreased cell proliferation in 75% of the cell cultures. Thus, despite the cell line experimental variation, the results from primary prostate tumors suggest autocrine signaling.

The discrepancy in the results between Fan et al. and Sanchez et al. can at least partially be explained by the differences in the characteristics of the LNCaP cell lines used. It is clear that the LNCaP line utilized by Fan et al. is not responsive to cyclopamine and does not have an activated Hh pathway. However, the LNCaP lines used by Karhadkar et al. and Sanchez et al. do have an active Hh pathway as demonstrated by expression of *PTCH1* and *GLI1*. Furthermore, our primary prostate cancer culture studies clearly establish cyclopamine sensitivity of both cell division and Hh pathway activity in prostate epithelial cells [3]. It is becoming increasingly apparent that diverse cell lines with the same designation in fact behave differently with regard to SHH signaling. These differences point to a real need for investigators to have access to primary prostate tumor specimens such that cell line results can be confirmed in primary cultures. It is less clear how to reconcile conflicting *in situ* hybridization data purporting to show autocrine [3] versus paracrine [1] SHH signaling in prostate tumors. However, we note that the number of *GLI1* expressing cells detected in the prostate tumor stroma by Fan et al. is very low, whereas a large number of cells in the tumor epithelium express *GLI1* as shown in Sanchez et al. [3]. The difference in hybridization pattern suggests that the sensitivity of the latter hybridizations may be higher, and therefore be detecting epithelial *GLI1* expression that was missed in Fan et al. Such differences could be explained by differences in tissue harvesting and fixation protocols used for the samples studied [Bushman and Datta, personal communication].

It is curious that in prostate cancer tumors, SHH signaling appears to occur as an autocrine loop within cancerous epithelially derived cells, while during normal prostate development SHH signaling is a paracrine event between the epithelium and the adjacent stroma (fig. 4). This shift parallels the changes observed for SHH signaling in small cell lung cancer SCLC, described above [73]. As observed in the prostate, SHH signaling is paracrine during branching morphogenesis in lung development, but appears autocrine in SCLC lines. The change from paracrine to autocrine signaling is also observed during repair of lung injury, leading Beachy and colleagues to propose that abnormal SHH signaling initiated during the healing process may be a first step in carcinogenesis [103].

Shh action in prostate cancer metastasis

Analysis of tissue samples have clearly demonstrated the correlation between SHH pathway activation and the presence of prostate cancer. To determine whether SHH might play a role in the proliferation and invasiveness of prostate cancer cells, the activity of the SHH pathway in a number of prostate cancer cell lines of varying metastatic potential was investigated. Examination of five different human prostate cancer cell lines (LNCaP, PC3, DU145, TSU and 22RV1) by ourselves and others revealed high levels of *GLI1* and *PTCH1* expression in all lines, indicating activity of the Hh pathway [2–4]. *IHH* expression was also detected in each cell line assayed. Analysis of six rodent cell lines confirmed that those with high metastatic characteristics also showed a high level of activity of the Hh pathway [2]. An additional feature of metastatic prostate cancer is expression of stem cell markers such as the intermediate filament protein Nestin [104]. Expression of *NESTIN* in human prostate cancer cell lines PC3, 22RV1 and DU145 was inhibited by addition of cyclopamine, while ectopic expression of *GLI1* in a normal prostate cell line upregulated *NESTIN* expression [2], suggesting that this stem cell-like character is also under the control of Hh signaling.

Mirroring the differences observed in LNCaP isolates described previously, further differences were observed in the expression and function of Hh signaling in supposedly identical DU145 prostate cancer cell lines. Our RT-PCR assays of DU145 cells failed to show expression of *SHH*, although *SHH* transcripts were detected in LNCaP and PC3 cell lines [3]. In contrast, RT-PCR assays by Karhadkar et al. [2] demonstrated the presence of *SHH* message in all three cell lines. Although apparently conflicting, cyclopamine studies of DU145 in each laboratory back up their respective results. In our hands, cyclopamine did not inhibit DU145 cell proliferation, even when supplied at 10 µM [3]. In contrast, for Karhadkar's DU145 cell line treatment of that line with only 3 µM cyclopamine inhibited both cellular proliferation and the expression of Hh target genes [2]. It appears, then, that the characteristics of these two DU145 strains have changed during the course of cell culture and emphasizes the need for the backup of cell line data with studies in primary cultures and tumors.

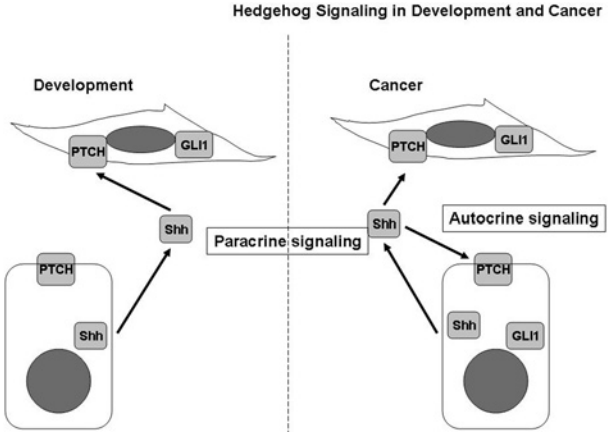

Figure 4. Autocrine and paracrine signaling changes in Hh and tumorigenesis.

Requirement for SHH signaling *in vitro* and *in vivo*

Expression studies in prostate tissue samples and prostate cancer cell lines made a strong case for the importance of SHH signaling in prostate cancer. The key question then became how activity of the pathway affects cellular behaviors associated with tumorigenesis and metastasis. As alluded to in the previous section, several studies demonstrated a requirement for SHH signaling for the proliferation of prostate cancer cell lines or primary cultures [2–4]. Application of cyclopamine or knockdown of *GLI1* expression by *GLI1* small interfering RNA (siRNA) produced significant inhibition of proliferation in all three studies. In addition, both Sheng et al. and Karhadkar et al. noted decreased cell viability and increased apoptosis. Ectopic expression of *GLI1* protected cancer cells from cyclopamine-induced apoptosis and decreased viability, demonstrating that the effects of cyclopamine derive from inhibition of the Hh signaling pathway [2]. The proliferation studies accurately mimicked the *in vivo* situation, as shown by data indicating that xenograft tumors derived from PC3 or 22RV1 cells grew more slowly and even regressed upon cyclopamine treatment. Remarkably, animals treated with the highest dose of cyclopamine, which caused complete tumor regression, remained in remission even 70–148 days post-treatment. There are some inconsistencies in the three groups' results, possibly due to laboratory-specific differences in the characteristics of prostate cancer cell lines and experimental methodologies. A thorough evaluation of the relevant phenotypes of the established cell lines used in a given laboratory may well identify the sources that explain the variation in the experimental data. These differences highlight the need for studies on primary cell cultures derived from human tumor samples. Such studies would provide an added level of significance to human prostate cancer.

This observation raises the tantalizing possibility that the inhibition of Hh pathway activity may have caused the apoptosis of all the cancer cells, as opposed to selecting for a more resilient cell type such as is thought to occur upon anti-androgen therapy. As expected, xenograft tumors derived from 22RV1 cells transfected to express *GLI1* were insensitive to cyclopamine treatment, and even appeared to grow more rapidly than control cells without cyclopamine treatment.

Shh pathway activity was also linked to metastatic behavior [2]. Incubation with cyclopamine or the Shh-blocking antibody decreased the invasiveness of the 22RV1 cell line in a matrigel assay, but had no effect on the invasiveness of *GLI1*-transfected cells, suggesting that invasive behavior was a function of Hh pathway activation. Furthermore, the low-invasion rat cell line AT2.1 could be transformed into a high-invasion cell line simply by overexpression of *GLI1*. Most excitingly, this translated into differences in metastatic behavior *in vivo*. The AT2.1 cell line has low levels of Shh signaling activity compared with AT6.3 as monitored by levels of *Ptc* expression. Xenografts of AT2.1 failed to metastasize; however, transfection of AT2.1 for *GLI1* expression resulted in a cell line that yielded numerous visceral metastases. Limited cyclopamine treatment of tumors derived from the high Shh activity line AT6.3, on the other hand, resulted in decreased numbers of metastatic tumors.

The direct link between Hh pathway activity and *in vivo* prostate tumor growth and metastasis is incredibly exciting. Even more so are the studies showing that inhibition of the pathway not only slows xenograft tumor growth *in vivo*, but apparently can result in complete regression and remission of tumor presentation.

Future directions

The establishment of Hh signaling as a major player in the pathogenesis of advanced prostate cancer opens the door to a wide array of basic and translational questions and issues to be resolved. For example, clinical studies might ask whether some measure of Hh signaling activity could provide a more accurate diagnosis of prostate cancer than the PSA test. Development of such an assay would not only identify patients with advanced prostate cancer, but identify those for whom there would be an obvious treatment – inhibition of the Hh pathway. Given the correlation of the level of Hh activity and tumor aggressiveness, evaluation of biopsy material for *GLI1* or *PTCH1* expression might separate those patients who need intervention quickly from those for whom monitoring is the best approach. For patients with metastatic disease, the treatment options are limited. In these patients circulating cancer cells can be detected in their bloodstream [105]. One could envision an assay system where circulating cancer cells are isolated from a patient and interrogated for Hh pathway activity and drug response (fig. 5). Such an assay, as demonstrated here with quantum dot nanoparticles, would allow for the rapid detection of treatment-sensitive cancer cells and the monitoring of treatment efficacy and tumor resistance.

Since the Hh pathway was implicated in cancers such as basal cell carcinoma almost 10 years ago, development of pharmaceutical approaches to block Hh signaling is already ongoing. Indeed, a collaboration between Curis and Genentech has resulted in the development of a series of small molecule antagonists of Hh signaling based on cyclopamine function. One of these candidate pharmaceuticals is expected to enter Phase 1 clinical trials this year. However, a note of caution should be considered – given the ubiquity of Hh signaling in development and stem cell maintenance, systemic inhibition of Hh signaling could be detrimental, especially in young pa-

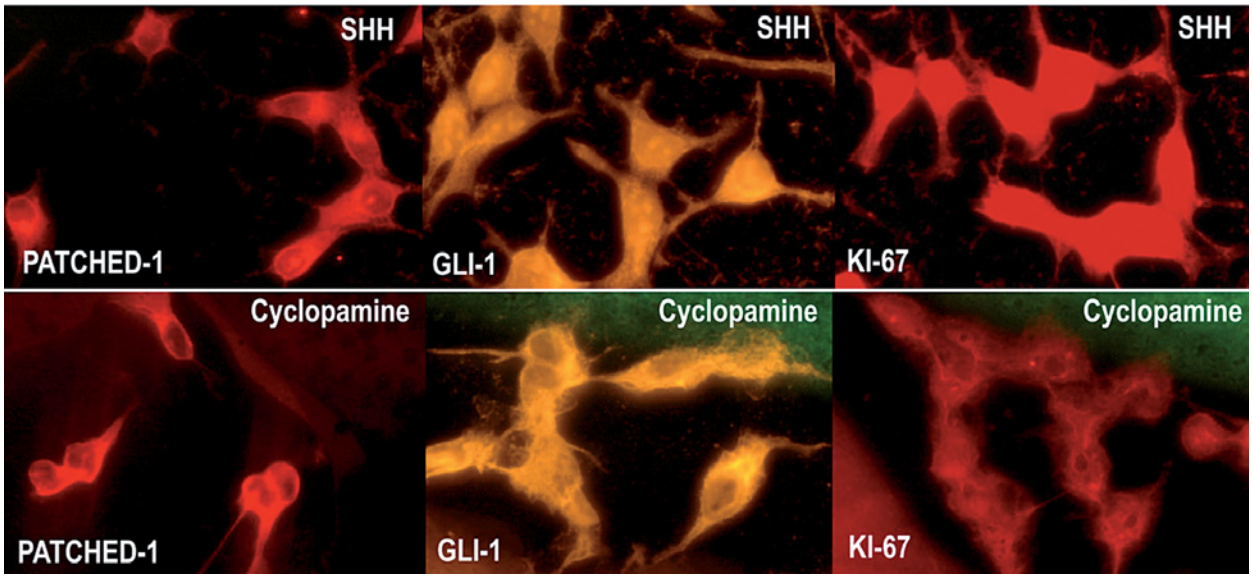

Figure 5. Quantum dot interrogation of prostate cancer cells treated with Shh (activation) or cyclopamine (inhibition). PTCH, Patched1 receptor, cytoplasmic and cell surface, GLI1, Gli-1 cytoplasmic and nuclear (when activated), Ki67, proliferation marker Ki-67 cytoplasmic and nuclear (when activated).

tients. While the average age of a prostate cancer patient would seem to obviate this concern, even mature adults rely on stem cell proliferation to replenish battered blood cells, maintain the integrity of their skin and the functioning of the large and small intestine. Therefore, one of the clinical challenges ahead will be to either design targeted delivery mechanisms that result in inhibition only in prostate cancer cells or to devise treatment regimens that eliminate all prostate cancer cells while having a minimal impact on normal stem cell populations and their function.

From a basic to translational point of view, identifying new members of the Hh pathway will undoubtedly produce new drug targets. Understanding their function may lead to a greater insight into the risk factors for prostate cancer and the development of means to counteract that risk. For example, the recent identification of lipoprotein particles as mediators of Hh movement [25] may suggest a mechanism for the long-standing recognition of cholesterol levels as a risk factor for prostate cancer. The question of how, when and where Hh pathway activation occurs is both intriguing from a basic science point of view and crucial from a translational viewpoint; understanding this process could result in therapies or preventatives that would dramatically cut the number of cases that progress towards advanced stages. It has already been postulated that chronic injuries that require Hh signaling for repair might increase an individual's chances of turning on Hh signaling in an abnormal fashion [102]. Finally, the knowledge that the Hh pathway is also of critical importance in so many other devastating forms of cancer lends impetus to the search; what we discover in prostate cancer will be applicable to so many other diseases and so many other patients.

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