Targeting the Hedgehog pathway in cancer

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***Abstract*:** The Hedgehog (Hh) pathway is a major regulator of many fundamental processes in vertebrate embryonic development including stem cell maintenance, cell differentiation, tissue polarity and cell proliferation. Constitutive activation of the Hh pathway leading to tumorigenesis is seen in basal cell carcinomas and medulloblastoma. A variety of other human cancers, including brain, gastrointestinal, lung, breast and prostate cancers, also demonstrate inappropriate activation of this pathway. Paracrine Hh signaling from the tumor to the surrounding stroma was recently shown to promote tumorigenesis. This pathway has also been shown to regulate proliferation of cancer stem cells and to increase tumor invasiveness. Targeted inhibition of Hh signaling may be effective in the treatment and prevention of many types of human cancers. The discovery and synthesis of specific Hh pathway inhibitors have significant clinical implications in novel cancer therapeutics. Several synthetic Hh antagonists are now available, several of which are undergoing clinical evaluation. The orally available compound, GDC-0449, is the farthest along in clinical development. Initial clinical trials in basal cell carcinoma and treatment of select patients with medulloblastoma have shown good efficacy and safety. We review the molecular basis of Hh signaling, the current understanding of pathway activation in different types of human cancers and we discuss the clinical development of Hh pathway inhibitors in human cancer therapy.

***Keywords*:** basal cell carcinoma, cancer stem cells, GDC-0449, Hedgehog, medulloblastoma

Introduction

The Hedgehog (Hh) gene was initially discovered by Christiane Nusslein-Volhard and Eric F. Weischaus in 1980 in their screen for mutations that disrupt the *Drosophila* larval body plan [Nusslein-Volhard and Wieschaus, 1980]. The name Hedgehog originates from the short and 'spiked' phenotype of the cuticle of the Hh mutant *Drosophila* larvae, which resembled the spikes of a hedgehog [Varjosalo and Taipale, 2008; Ingham and McMahon, 2001]. The Hh family of proteins have since been recognized as key mediators of many fundamental processes in vertebrate embryonic development playing a crucial role in controlling cell fate, patterning, proliferation, survival and differentiation of many different regions. Hh signals have diverse functions in different contexts. They may act as morphogens in the dose-dependent induction of distinct cell fates within a target field, or may act as a mitogen in the regulation of cell proliferation controlling the form of developing organs [Ingham and McMahon, 2001]. The crucial developmental function of Hh signaling is illustrated by the dramatic consequences in human fetuses, with defects in the Hh signaling pathway resulting in fetuses with brain, facial and other midline defects such as holoprosencephaly (failure of forebrain development) or microencephaly, cyclopia, absent nose or cleft palate [Rubin and de Sauvage, 2006; Belloni *et al.* 1996; Roessler *et al.* 1996]. In adults, the Hh pathway remains active and is involved in regulation of tissue homeostasis, continuous renewal and repair of adult tissues, and stem cell maintenance [Hooper and Scott, 2005].

The Hh signaling pathway has also recently been recognized to be one of the most important signaling pathways and a therapeutic target in cancer. In adults, mutation or deregulation of this pathway plays a key role in both proliferation and differentiation leading to tumorigenesis or tumor growth acceleration in a wide variety of tissues. Basal cell carcinoma (BCC) and medulloblastoma are two well-recognized cancers with mutations in components of the Hh pathway [Tostar *et al.* 2006; Taylor *et al.* 2002; Dahmane *et al.* 1997]. Inappropriate activation of the Hh signaling pathway has been implicated

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in the development of several other types of cancer including lung, prostate, breast, and pancreas, as examples. In addition, some recent findings suggest that Hh might also promote tumorigenesis by signaling in a paracrine manner from the tumor to the surrounding stroma or in cancer stem cells (CSCs).

The first Hh pathway inhibitor to be identified was the naturally occurring plant alkaloid, cyclopamine. This was discovered as a teratogenic compound causing cyclopia and holoprosencephaly in lambs whose mothers had ingested corn lilies, a phenotype similar to Sonic Hedgehog (Shh) knockout mice [Bryden *et al.* 1971]. No untoward effect was seen in the adult sheep. The active chemical identified in the corn lily, cyclopamine, was subsequently shown to inhibit the Hh pathway by binding to and inactivating the Smoothened (SMO) transmembrane receptor protein [Chen *et al.* 2002; Cooper *et al.* 1998]. Cyclopamine is of low affinity, has poor oral bioavailability and suboptimal pharmacokinetics and thus more potent derivatives have been synthesized. Several synthetic, small-molecule SMO antagonists with higher potency than cyclopamine such as SANT1–SANT4, CUR-61414, HhAntag-691 and GDC-0449 are now available and have been tested in preclinical models against a variety of solid tumors [Rudin *et al.* 2009; Scales and de Sauvage, 2009; Von Hoff *et al.* 2009]. In this review, we provide a brief overview of Hh signaling, discuss the roles of this pathway in solid tumors, and summarize the clinical advances in using therapeutic agents targeting the Hh signaling cascade.

Hedgehog signal transduction

Hh proteins are secreted signaling proteins that were first discovered in *Drosophila* along with many other components of their signal transduction machinery [Nusslein-Volhard and Wieschaus, 1980]. The mechanism of Hh protein processing, secretion, and signaling appear to be more or less conserved in evolution between *Drosophila* and higher organisms, although some differences exist. Drosophila has only one Hh gene, whereas vertebrate Hh signal transduction involves three Hh homologues with different spatial and temporal distribution pattern: *Sonic Hedgehog* (Shh), *Indian Hedgehog* (Ihh) and *Desert Hedgehog* (Dhh) [Ingham and McMahon, 2001; McMahon, 2000]. The Hh proteins undergo multiple processing steps before signaling. The Hh protein is made as a precursor molecule, consisting of a C-terminal protease domain and an N-terminal signaling unit. The precursor Hh molecule is cleaved to release the active signaling domain called HhNp. Then, the C-terminal domain of the Hh polypeptide catalyzes an intramolecular cholesteroyl transfer resulting in a formation of a C-terminal cholesterol modified N-terminal Hh signaling domain. The cholesterol modification results in association of Hh with membranes, facilitating the final processing step in which a palmitoyl moiety is added to the N-terminus of Hh (acylation), generating the fully active HhN [Varjosalo and Taipale, 2007; Porter *et al.* 1996]. The gene Rasp encodes the enzyme, likely located at the endoplasmic reticulum, required for the Hh acylation and the production of active Hh [Micchelli *et al.* 2002]. Hh is then released from the secreting cell by a dedicated transmembrane transporter *Dispatched* (Disp) protein. In embryonic development, the cells that synthesize Hh ligands are distinct from the responsive cells. These responsive cells may either be adjacent to, or at a significant distance from, the Hh secreting cell [Varjosalo and Taipale, 2007].

In humans, the Hh signaling cascade is initiated in the target cell by the Hh ligand binding to the *Patched 1* protein (PTCH), a 12-span transmembrane protein (Figure 1). In the absence of a Hh ligand, PTCH catalytically inhibits the activity of the seven-transmembrane-span receptor-like protein, SMO, potentially by affecting its localization to the cell surface. It is also proposed that an endogenous intracellular small molecule that acts as an agonist for SMO is transported outside the cell by PTCH, preventing its binding to SMO. Binding of Hh to PTCH results in the loss of PTCH activity and the consequent activation of SMO, which transduces the Hh signal to the cytoplasm [Taipale *et al.* 2002]. The Hh signal is transmitted via an alteration of the balance between the activator and repressor forms of the Ci (cubitus interruptus)/GLI family of zinc-finger transcription factors. In *Drosophilia*, the Hh signal is transmitted via a protein complex which includes the atypical kinesin-like protein, Costal 2 (Cos2), Fused (Fu) and Suppressor of Fused (SuFu) and the transcription factor, Ci. In higher organisms, the Cos2 and Fu are not conserved, although SuFu still seems to play an important role in signal transduction. In mammals, the Hh signaling takes place in the nonmotile cilia to which the SMO and other downstream pathway components must need to

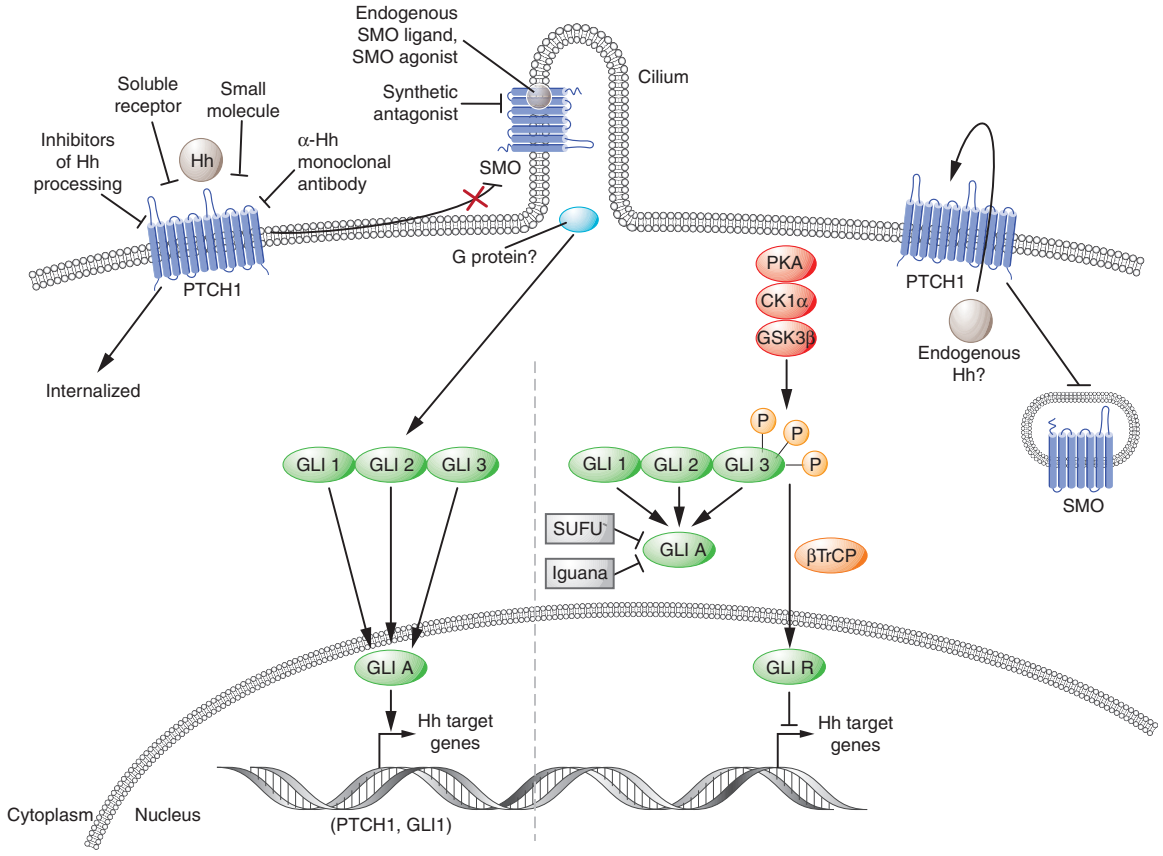

Figure 1. Hedgehog signaling pathway in vertebrates. The above model illustrates our current understanding of the vertebrate Hedgehog (Hh) pathway signaling. Hh signaling cascade is initiated in the target cell by the Hh ligand binding to the *Patched 1* protein (PTCH), a 12-span transmembrane protein located on the plasma membrane. *Smoothened* (SMO), a 7-transmembrane-span protein receptor, is located on the membrane of the intracellular endosome. In mammalians, the Hh signaling takes place in the nonmotile cilia to which the SMO and other downstream pathway components transit to in order to activate the GLI transcription factors [Rubin and de Sauvage, 2006; Corbit *et al.* 2005; Huangfu and Anderson, 2005; Huangfu *et al.* 2003]. An endogenous small molecule acting as a SMO agonist is transported outside the cell by PTCH, preventing its binding to SMO. In the absence of a Hh ligand, PTCH catalytically inhibits the activity of SMO by affecting its localization to the cell surface. Full-length GLI proteins are thus proteolytically processed to generate the repressor GLI^R, largely derived from GLI 3, which represses Hh target genes. Binding of Hh to PTCH, internalizes and destabilizes PTCH, so that it can no longer transport the endogenous SMO agonist molecules outwards. Intracellular accumulation of this agonist molecule activates SMO which translocates to the plasma membrane, apparently concentrating in the cilia. Relief of SMO inhibition promotes generation of the activator GLI^A, largely contributed by GLI 2 and the subsequent expression of the Hh target gene [Taipale *et al.* 2002]. CK1α, casein kinase 1α; GPCR, G-protein-coupled receptor; GSK3β, glycogen synthase kinase 3β; PKA, protein kinase A. Reprinted by permission from Macmillan Publishers Ltd: Rubin, L.L. and de Sauvage, F.J. (2006) Targeting the Hedgehog pathway in cancer. *Nat Rev Drug Discov* 5: 1026–1033.

transit to activate the Ci ortholog in mammals, the GLI transcription factors [Rubin and de Sauvage, 2006; Corbit *et al.* 2005; Huangfu and Anderson, 2005; Huangfu *et al.* 2003]. The GLI transcription factors exist as three separate zinc-finger proteins, GLI 1 and GLI 2 functioning as transcriptional activators and GLI 3 as a transcriptional repressor [Ruiz i Altaba, 1997]. The expression of GLI 1 is highly dependent upon active Hh signaling and is thus often used as a readout of pathway activation. In the absence of a Hh ligand, PTCH blocks SMO activity and full length GLI proteins are proteolytically processed to generate the repressor GLI^R, largely derived from GLI 3, which represses Hh target genes. Hh binding to PTCH relieves SMO inhibition, promotes generation of the activator GLI^A, largely contributed by GLI 2 and the subsequent expression of the Hh target genes. Ubiquitous mammalian Hh target genes include

GLI 1, PTCH1, Hh interacting protein (Hhip) and other cell-specific genes such as Cyclin D, Myc, Bmi1, Bcl-2, VEGF (vascular endothelial growth factor) and Snail depending upon the cell type [Scales and de Sauvage, 2009; Ferretti *et al.* 2005]. GLI activation is regulated at several different levels via phosphorylation by inhibitors such as SuFu, Ren, protein kinase A (PKA), glycogen synthase kinase 3β (GSK3β) and activators such as Dyrk1, Ras and Akt [Varjosalo and Taipale, 2007; Ferretti *et al.* 2005]. Hh and PTCH are subsequently internalized and degraded in the lysosomes.

Although the extent of Hh signaling is significantly lower in the adult compared with the embryo, it is still detectable at a few sites such as the central nervous system (CNS) neural stem cells [Palma *et al.* 2005; McMahon, 2000]. Hh also plays an important role in the maintenance and proliferation of continuously renewing tissues such as the gut epithelium [van den Brink *et al.* 2004] and is reactivated at sites of tissue damage and repair [Beachy *et al.* 2004; Mirsky *et al.* 1999; Parmantier *et al.* 1999].

Alteration of the Hedgehog pathway and cancer

In recent years, it has become increasingly clear that the aberrant activation of the Hh signaling pathway can lead to cancer. Three basic models have been proposed for Hh pathway activity in cancer (Figure 2A–C) [Scales and de Sauvage, 2009; Rubin and de Sauvage, 2006]. The first discovered were the type I cancers harboring Hh pathway-activating mutations which are Hh ligand independent, such as BCCs and medulloblastomas. Type II cancers are autocrine (or juxtacrine) ligand dependent, meaning that Hh is both produced and responded to by the same (or neighboring) tumor cells. Type III cancers, which are paracrine ligand dependent, have been described recently. In paracrine signaling, Hh produced by the tumor cells is received by the stroma, which feeds other signals back to the tumor to promote its growth or survival [Scales and de Sauvage, 2009; Rubin and de Sauvage, 2006].

Type I Hedgehog signaling: ligand independent, mutation driven

The first hint to the involvement of the Hh pathway in human cancer was appreciated when inactivating mutations in PTCH were identified in the rare condition Gorlin's syndrome [Hahn *et al.* 1996; Johnson *et al.* 1996]. Patients with Gorlin's syndrome develop numerous BCCs during their lifetime and are at an increased risk of other tumors including medulloblastoma, a tumor of the cerebellar progenitor cells, and rhabdomyosarcoma, a muscle tumor. This link was further strengthened when ligand-independent activation of the Hh pathway was observed in a majority of sporadically occurring BCCs [Dahmane *et al.* 1997]. Most of these tumors either had inactivating mutations in PTCH (85%) or activating mutations in SMO (10%) [Xie *et al.* 1998]. Furthermore, about one third of all medulloblastomas and occasional rhabdomyosarcomas were shown to have inappropriate Hh pathway activation, often due to PTCH mutations and sometimes due to SuFu mutations [Tostar *et al.* 2006; Taylor *et al.* 2002]. Dysregulated Hh signaling led to increased cell proliferation and tumor formation. These observations have been confirmed in various mouse models as well. Mice that are heterozygous for a PTCH mutation have a higher frequency of developing medulloblastoma, and susceptible to formation of UV-induced BCC, similar to patients with the Gorlin's syndrome [Aszterbaum *et al.* 1999]. Other mouse models with ectopic expression of various Hh signaling components have been shown to develop skin phenotypes with increased epidermal proliferation and BCC-like tumors as seen in Gorlin's syndrome [Rubin and de Sauvage, 2006; Svard *et al.* 2006]. The first clinical trials of Hh pathway inhibitor therapy included several patients with recurrent or metastatic BCC. Since these tumors are ligand independent, Hh pathway inhibitors must act at or below the level of SMO to be effective.

Type II Hedgehog signaling: autocrine, ligand dependent

Constitutive activation of the Hh pathway has been detected in a broad variety of tumors including lung, stomach, esophagus, pancreas, prostate, breast, liver and brain [Clement *et al.* 2007; Sicklick *et al.* 2006; Karhadkar *et al.* 2004; Kubo *et al.* 2004; Berman *et al.* 2003; Thayer *et al.* 2003; Watkins *et al.* 2003b]. Most of these tumors are dissimilar to BCC or medulloblastomas in that they do not harbor any somatic mutations in the Hh signaling pathway. Rather, they demonstrate an autocrine, ligand-dependent, abnormal Hh pathway activation. Most of these tumors have an elevated expression of the Hh ligand (Shh or Ihh) and/or ectopic PTCH and GLI expression within the epithelial compartment. Ectopic Hh

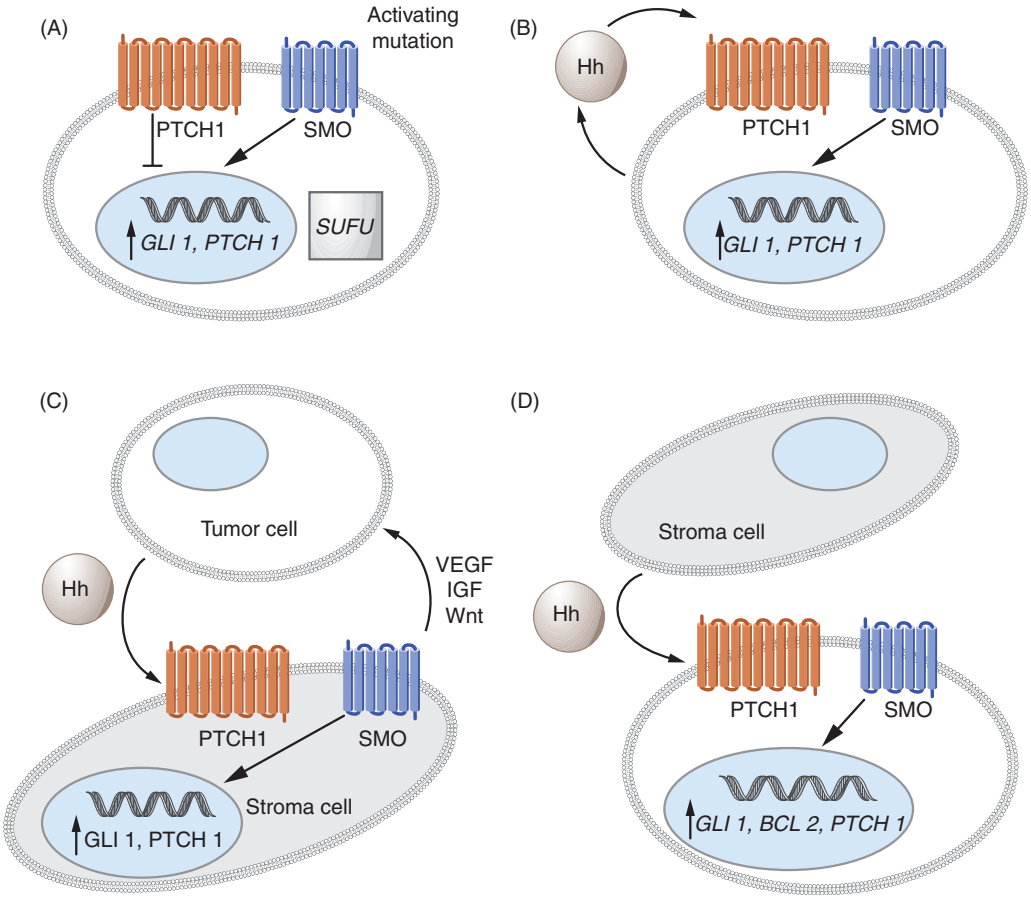

Figure 2. Different models of Hedgehog pathway signaling. (A) Type I ligand-independent cancers harbor inactivating mutations in *Patched 1* protein (PTCH) or activating mutations in Smoothened (SMO) leading to constitutive activation of the Hedgehog (Hh) pathway even in the absence of the Hh ligand. (B) Type II ligand-dependent autocrine cancers both produce and respond to the Hh ligand leading to support tumor growth and survival. (C) Type III ligand-dependent paracrine cancers secrete the Hh ligand which is received by the stromal cells leading to pathway activation in the stroma. The stroma in turn feeds back various signals such as IGF, Wnt, VEGF to the tumor tissue leading to its growth or survival. (D) Type IIIb reverse paracrine tumors receive Hh secreted from the stroma leading to pathway activation in the tumor cells and upregulation of survival signals. (E) Cancer stem cells (CSCs): Hh signaling occurs only in the self-renewing CSCs, from the Hh ligand produced either by the CSCs or by the stroma. CSC will give rise to more Hh pathway dependent CSCs or possibly may differentiate into Hh-pathway negative tumor cells comprising the bulk of the tumor. Reprinted from: Scales, S.J. and de Sauvage, F.J. (2009) Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends Pharmacol Sci* 30: 303–312, with permission from Elsevier.

ligand production occurring in all tumor cells or in a small number of tumor stem cells, acts upon itself or the neighboring tumor cells to support tumor growth and survival. This autocrine tumor growth can be effectively suppressed by various pathway inhibitors such as Hh neutralizing antibodies or SMO antagonists.

Type III Hedgehog signaling: paracrine, ligand dependent

A recent report by Yauch and colleagues highlighted that tumor Hh signaling may occur via paracrine mechanisms and emphasized the importance of Hh signaling in promoting the tumor microenvironment [Jiang and Hui, 2008; Yauch *et al.* 2008]. Paracrine Hh signaling is critical during development and for the maintenance of various epithelial structures such as the small intestine [Theunissen and de Sauvage, 2009; Varjosalo and Taipale, 2008; Ingham and McMahon, 2001]. Hh ligand secreted by the epithelium is received by the mesenchymal stroma and directly affects and stimulates proliferation in the mesenchyme. Upon Hh target gene activation, the mesenchyme produces additional molecules that feed back to the epithelium.

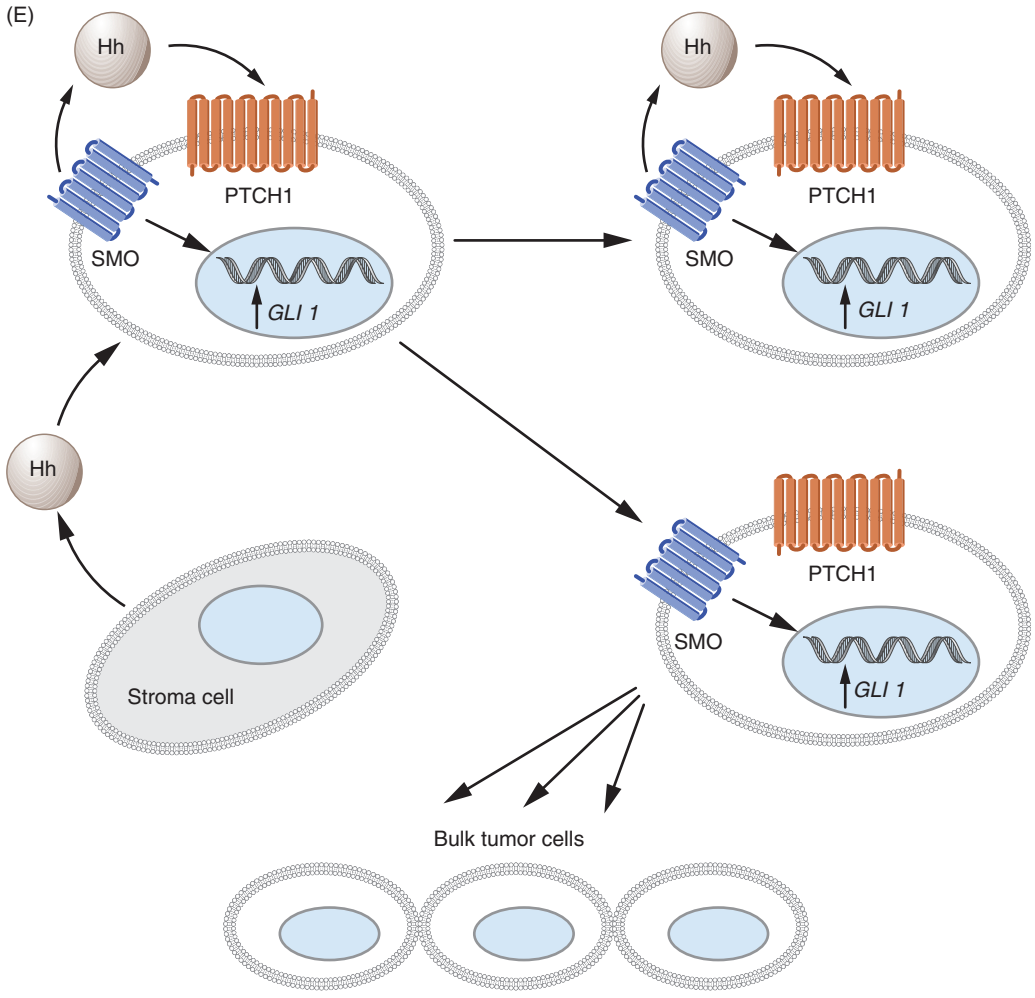

Figure 2. Continued.

Fan and colleagues first showed that at least one model of prostate cancer signals to the stroma through paracrine mechanisms, with an elevated expression of PTCH and GLI in the murine stroma in response to Hh production by human xenografts [Fan *et al.* 2004]. These results were extended recently by three reports which showed that the Hh ligand expressing cancers were refractory to the ligand, whereas the surrounding stroma was ligand responsive [Nolan-Stevaux *et al.* 2009; Theunissen and de Sauvage, 2009; Tian *et al.* 2009; Yauch *et al.* 2008]. Yauch and colleagues observed that the tumor-derived Hh from several naturally Hh overexpressing xenografts stimulated expression of GLI 1/GLI 2 and PTCH in the infiltrating stroma but not in the tumor itself. Treatment with both a Hh-blocking antibody 5E1 and a small-molecule SMO inhibitor downregulated these murine stromal genes and slowed tumor growth, implying that the stromal cells send growth or survival signals back to the tumor [Theunissen and de Sauvage, 2009; Yauch *et al.* 2008]. In addition, Nolan-Stevaux and colleagues recently showed that the genetic deletion of SMO from pancreatic cells did not substantially alter PTCH and GLI expression in the neoplastic ductal cells and more importantly did not affect the development or progression of *Kras* driven pancreatic adenocarcinoma [Nolan-Stevaux *et al.* 2009]. Conversely, Tian and colleagues showed that the epithelial expression of mutationally activated SMO, which triggers constitutive, ligand-independent activation of the Hh pathway, was not able to induce neoplastic transformation in murine pancreatic epithelium, nor affect tumor development and progression of *Kras* driven pancreatic ductal adenocarcinoma models [Theunissen and de Sauvage, 2009; Tian *et al.* 2009].

These studies support the paracrine model of Hh signaling in which tumor cells activate Hh

signaling in the surrounding stroma, resulting in the expression of soluble factors and extracellular matrix components that act upon the tumor epithelium to ultimately promote tumor growth [Theunissen and de Sauvage, 2009]. The exact mechanism of stromal feedback to the tumor remains to be determined but could involve components of the molecular signaling pathways involving insulin-like growth factor (IGF) and Wnt pathways, as IGF and Wnt signaling molecules in the tumor stroma were modulated similar to GLI and other Hh target genes in xenograft tumor models treated with Hh pathway inhibitors [Scales and de Sauvage, 2009; Yauch *et al.* 2008]. Inhibition of this paracrine signaling in epithelial tumors may be of therapeutic value as specific inhibition of Hh signaling in the stroma did result in growth inhibition of tumor xenografts, although the most effective way of treating these tumors would possibly be to use a combination of a Hh pathway inhibitor to target the stroma and other drugs to target the tumor cells.

Reverse paracrine signaling

Very recently, a 'reverse paracrine' signaling model has also been recognized in which Hh is secreted from the stroma and is received by the tumor cells (Figure 2D) [Theunissen and de Sauvage, 2009]. So far, this has only been observed in hematological malignancies such as multiple myeloma, lymphoma and leukemia, in which the Hh secreted from the stroma seems to be essential for the survival of the cancerous B cells via upregulation of the antiapoptotic factor Bcl2 [Scales and de Sauvage, 2009; Hegde *et al.* 2008; Dierks *et al.* 2007]. Stromal Hh was also found in high-grade, platelet-derived growth factor (PDGF)-induced gliomas in endothelial cells [Becher *et al.* 2008]. In the reverse paracrine signaling model, stromal Hh is thought to provide the appropriate microenvironment for potentiating tumor growth and would thus be a suitable therapeutic target as well.

Hedgehog signaling in cancer stem cells

Most renewing tissues are maintained by small populations of stem cells that have the ability to both generate additional stem cells and give rise to all mature cell types of the tissue. Hh signaling is an important regulator of stem cell activity, stimulating self-renewal and proliferation of stem cells in various tissues (Figure 2E) [Taipale and Beachy, 2001; Zhang and Kalderon, 2001]. It is believed that tumor growth and propagation might be dependent on a small population of CSCs that are similar to normal tissue stem cells and are regulated by the same signaling molecules as the normal stem cells [Reya *et al.* 2001]. Growing evidence suggests that the abnormal formation and expansion of cancer is due to deregulation of the multiple signaling pathways in the stem cells including the Hh, Wnt, Notch and BMP pathways [Rubin and de Sauvage, 2006]. Hh signaling has been shown to regulate the self-renewal of CSCs in breast, glioma and multiple myeloma, and more convincingly in the maintenance of chronic myelogenous leukemia (CML) stem cells [Theunissen and de Sauvage, 2009; Dierks *et al.* 2008; Clement *et al.* 2007; Peacock *et al.* 2007; Liu *et al.* 2006]. Dierks and colleagues observed that CML stem cells (Bcr-Abl driven $Lin^{-}/Sca1^{+}/c\text{-}Kit^{+}$) with SMO knockout had a reduced ability to form tumors in irradiated mice whereas SMOM2 expression enhanced it [Dierks *et al.* 2008; Peacock *et al.* 2007]. Furthermore, SMO antagonists such as cyclopamine and Hh blocking antibody 5E1 both inhibited growth of the CML CSCs *in vitro* and *in vivo* and enhanced time to relapse after the end of treatment. A recent report showing that Hh signaling is essential for maintenance of CSCs in CML lends further support for this concept. The loss of SMO in the mouse hematopoietic system resulted in decreased induction of CML by the Bcr-Abl oncoprotein and induced Numb, causing depletion of CML stem cells. Cyclopamine treatment inhibited the growth of imatinib-resistant mouse and human CML indicating that Hh signaling may be an important target to avoid induction of imatinib-resistant CML [Zhao *et al.* 2009].

Tumors contain only a minority of CSCs, which can give rise to more CSCs as well as nontumorigenic cancer cells [Al-Hajj and Clarke, 2004; Beachy *et al.* 2004]. CSCs are typically resistant to conventional chemotherapy and radiation as they are slow growing and are thought to be the cause of cancer relapse after tumor debulking by conventional therapy. The fact that active Hh signaling has been identified in several types of CSCs makes Hh inhibition a promising therapeutic target to deplete the tumor-forming CSCs, ideally in combination with other tumor debulking agents or radiation to remove the differentiated bulk of the tumor [Scales and de Sauvage, 2009]. Another recent finding that Hh positively regulates the expression of drug transport pumps in stem cells, enabling them to resist

uptake of cytotoxic drugs [Sims-Mourtada *et al.* 2007], makes the strategy of using Hh inhibitors to target the CSCs more rational.

Hh signaling has also been shown to promote tumor metastasis by being actively involved in the *epithelial–mesenchymal transition* (EMT). EMT involves transforming polarized epithelial cells into motile mesenchymal cells facilitating invasive growth and ultimately causing metastasis. Hh exerts its effects on EMT via the upregulation of transcription factor SNAIL and downregulation of E-cadherin [Rubin and de Sauvage, 2006; Karhadkar *et al.* 2004]. This observation was first made by Karhadkar and colleagues in prostate cancer cell lines where they showed that the rarely metastasizing clone AT2.1 could be induced to metastasize by overexpression of GLI 1, and that the capacity of another cell line AT6.3 to metastasize to the lung was abrogated by cyclopamine [Karhadkar *et al.* 2004]. Similar observations in pancreatic cancer cell lines were made by Feldman and colleagues, who showed that ectopic expression of GLI led to increased invasiveness, whereas inhibition of the Hh pathway led to downregulation of Snail expression and reduction in invasive properties [Feldmann *et al.* 2007].

Targeting Hedgehog pathway signaling in solid tumors

Aberrant Hh signaling can be activated in a variety of cancers through various mechanisms, as discussed earlier. Understanding the specific mechanism of Hh activation in a particular tumor might help in selecting the most appropriate agent and strategy for optimizing the therapeutic benefit to be obtained by Hh pathway inhibition. Tumors such as BCC or medulloblastoma, which have a constitutive, mutation-driven activation of the Hh pathway, may be best treated with single-agent Hh inhibitors acting downstream of the activating mutation. Tumors with predominant autocrine or paracrine Hh signaling and CSCs might be more effectively treated with a combination of Hh antagonists and cytotoxic drugs targeting tumor cells [Scales and de Sauvage, 2009].

The first Hh pathway inhibitor to be identified, cyclopamine, inhibited the Hh pathway by binding to, and inactivating, SMO [Chen *et al.* 2002; Cooper *et al.* 1998]. However, cyclopamine has low affinity, poor oral bioavailability and suboptimal pharmacokinetics, and more potent derivatives have been synthesized. Several synthetic, small-molecule SMO antagonists with higher potency than cyclopamine such as SANT1–SANT4, CUR-61414, HhAntag-691, GDC-0449, MK4101, IPI-926 and BMS-833923 as examples, are now available and have been tested in preclinical models [Scales and de Sauvage, 2009]. Hh-blocking antibodies, which act upstream of SMO by preventing the binding of Hh to PTCH like 5E1, are also available and have demonstrated good preclinical activity [Scales and de Sauvage, 2009]. Multiple other drugs targeting different points of the Hh pathway, such as the natural Hh inhibitor Hhip mimetic, SUFU mimetics and GLI activity/transcription blocking agents (Gant 61 and Gant 58) are in various phases of development, as well [Lauth *et al.* 2007; Lauth and Toftgard, 2007]. Recently, a small molecule that binds the extracellular Shh protein, robotnikin, was isolated from small-molecule microarray-based screens [Stanton *et al.* 2009]. Targeting Shh ligands may be an interesting approach since the tumor-derived Shh ligands directly activate signaling in stromal cells. So far, only the SMO antagonists have been tested in the humans, and of these the CUR-61414 and GDC-0449 compounds, IPI-926, and BMS-833923 (XL139) are in the most advanced phase of clinical evaluation.

Basal cell carcinoma

BCC is the most common skin cancer in the United States, with an annual incidence of approximately 1,000,000 new cases. BCC was the first group of cancers in which the tumorigenic potential of deregulated Hh signaling was identified. This was based on the identification that patients with Gorlin's syndrome had a marked susceptibility to develop BCCs [Hahn *et al.* 1996; Johnson *et al.* 1996]. Using family-based linkage studies of kindred with Gorlin's syndrome, the causative mutation was mapped to the *Patched 1* gene (PTCH1) on chromosome 9 [Gailani *et al.* 1992]. It is believed that upregulation of Hh signaling is the sole and pivotal abnormality in all BCCs [Epstein, 2008; Hutchin *et al.* 2005]. Approximately 90% of the sporadic BCCs have an identifiable mutation in at least one allele of PTCH1 (loss-of-function mutation) and about 10% have activating mutations in SMO (gain-of-function mutation) [Epstein, 2008; Xie *et al.* 1998; Gailani *et al.* 1996]. These mutations cause constitutive Hh pathway signaling that mediate unrestrained

proliferation of basal cells of the skin, which has been confirmed in various mouse models of BCC, as well [Grachtchouk *et al.* 2000; Aszterbaum *et al.* 1999; Xie *et al.* 1998]. With such strong evidence of dysregulated Hh 'oncogene addiction' in BCC, blocking the Hh pathway would theoretically be a useful therapeutic approach for patients with metastatic BCC not controllable by other local therapies.

The first discovered steroidal alkaloid cyclopamine was used as a topical application by one group to induce regression in four BCCs [Tabs and Avci, 2004]. Several other synthetic cyclopamine derivatives have subsequently been developed as Hh pathway inhibitors, with better pharmacological and inhibitory properties than cyclopamine. Cur-61414, one of the earlier synthetic SMO inhibitors, prevented the formation of BCC-like 'basaloid nests' in Shh-treated *ex vivo* skin punches from $PTCH^{+/-}$ mice and also eliminated preformed BCC-like lesions [Scales and de Sauvage, 2009; Athar *et al.* 2004]. Interestingly, Cur-61414 selectively induced apoptosis and decreased proliferation in the BCC-like lesions, without any deleterious effects on normal surrounding skin [Scales and de Sauvage, 2009; Athar *et al.* 2004]. Cur-61414 was safe and well tolerated in other preclinical models, as well, and was thus formulated as a topical agent [Scales and de Sauvage, 2009; Flagella, 2006]. It was the first class of Hh antagonists to enter phase I clinical trials for use in sporadic BCC patients. However, it did not produce any clinical changes or reduction in Hh target gene GLI1 transcription when applied topically to BCC lesions, possibly because the formulation did not adequately penetrate the human skin [Fretzin *et al.* 2006].

GDC-0449, a second Curis-Genentech novel SMO inhibitor, was discovered by high-throughput screening of a library of small-molecule compounds and subsequent optimization through medicinal chemistry. GDC-0449 is a selective Hh pathway inhibitor with greater potency and more favorable pharmaceutical properties than cyclopamine, with good antitumor activity seen in preclinical models [Rudin *et al.* 2009; Von Hoff *et al.* 2009; Yauch *et al.* 2008]. The results of the phase I study of GDC-0449 demonstrating antitumor activity in patients with BCC and medulloblastoma were published recently [Rudin *et al.* 2009; Von Hoff *et al.* 2009]. Thirty-three patients with metastatic or locally advanced BCC received oral GDC-0449 at one of three doses, 150, 270 or 540 mg daily for as long as the patients had clinical benefit. Of the 33 patients, 18 had an objective response to GDC-0449, with 2 complete responses and 16 partial responses. Eleven other patients had stable disease with 4 patients having progressive disease. GDC-0449 has an unusual pharmacokinetic profile with high, sustained micromolar plasma concentrations and long terminal half-life. The median time to steady state was 14 days (range, 7–22 days). A consistent steady-state total plasma level of GDC-0449 was maintained throughout the treatment period of the study, with no apparent decline at the time of disease progression. Pharmacodynamic downmodulation in the Hh pathway was shown by a decrease in GLI1 expression as compared with pretreatment biopsy-sample analysis. The extent of GLI1 downmodulation did not correlate with pharmacokinetic levels of GDC-0449 in individual patients. Grade 3 adverse events related to the study drug included fatigue, hyponatremia, muscle spasm and atrial fibrillation. Other milder side effects included hair loss or thinning, altered taste sensation, nausea and vomiting, dyspepsia and weight loss. Interestingly, some of these toxicities might be attributable to the on-target effects of Hh in taste bud papillae formation and hair growth [Scales and de Sauvage, 2009]. High levels of GLI1 mRNA expression were observed in the tumors from responding patients, consistent with constitutive activation of the Hh pathway. Based on these promising results, GDC-0449 has now entered phase II trials in advanced BCC.

Medulloblastoma

Medulloblastoma, an aggressive childhood tumor of cerebellar origin, is another malignancy with a well-recognized dependency on aberrant Hh signaling. The first indication that alteration in the Hh signaling pathway contributes to medulloblastoma was the discovery that patients with Gorlin's syndrome, who have germline mutations in the PTCH-1 gene, have an increased incidence of medulloblastoma [Goodrich and Scott, 1998; Kimonis *et al.* 1997]. Although rare, the outcome of medulloblastomas is invariably poor. Primary management consists of surgical resection followed by radiation and chemotherapy, with serious treatment-related morbidity from these modalities. Patients with recurrent disease after primary therapy have a median survival of less than 6 months [Zeltzer *et al.* 1999].

Hh signaling has a critical role in the developing cerebellum. Shh released by the migrating Purkinje cells delays neuronal differentiation and induces proliferation of granular neuron precursors in the external germinal layer of the cerebellum [Berman *et al.* 2002; Wechsler-Reya and Scott, 2001; Wallace, 1999]. Although critical during embryogenesis, the Hh pathway is downregulated after early postnatal development in most tissues, including brain, and the constitutive activation of this pathway seems to give rise to medulloblastomas [Romer *et al.* 2004]. More than 30% of human medulloblastomas demonstrate high levels of GLI1 expression consistent with abnormal activation of the Hh pathway [Lee *et al.* 2003]. Hh pathway antagonists thus have potential therapeutic value in the treatment of medulloblastomas and have been tested successfully in preclinical models and most recently in the clinic as well.

Cyclopamine was shown to decrease the rate of growth of mouse medulloblastoma cells both in culture and in mouse allograft models [Berman *et al.* 2002; Dahmane *et al.* 2001]. Interestingly, cyclopamine inhibited the *in vitro* growth of all human medulloblastoma cell lines, although only about one third would be expected to harbor Hh pathway mutations, suggesting Hh antagonists could be broadly effective in treating all medulloblastomas [Scales and de Sauvage, 2009; Berman *et al.* 2002]. Romer and colleagues used another small-molecule SMO-binding Hh antagonist, Hh-Antag to treat endogenous medulloblastomas in PTCH1$^{+/-}$p53$^{-/-}$ mice models, where tumors develop with 100% incidence [Romer *et al.* 2004]. Hh-Antag completely eliminated the medulloblastomas by blocking tumor cell proliferation and stimulating apoptosis, without adversely affecting the surrounding cerebellum [Romer *et al.* 2004]. Rudin and colleagues recently reported a patient with metastatic medulloblastoma, refractory to multiple therapies responding to the novel Hh pathway inhibitor, GDC-0449 [Rudin *et al.* 2009]. Treatment resulted in rapid regression of the tumor burden and reduction of symptoms, although resistance to drug developed rapidly. Molecular analyses of the patient's tumor specimens obtained before treatment showed increased expression of Hh target genes including GLI1, PTCH1, PTCH2 and secreted frizzled-related protein 1 (SFRP1), suggesting activation of the Hh pathway. Genomic analysis of the PTCH1 locus in tumor cells showed loss of heterozygosity and somatic mutation with no such alterations seen in the normal skin tissue biopsies [Rudin *et al.* 2009]. There is currently an ongoing phase II trial evaluating the efficacy and safety of GDC-0449 in the treatment of adults with recurrent or refractory medulloblastoma (see www.clinicaltrials.gov). The use of Hh pathway inhibitors in the treatment of medulloblastomas may offer a more effective therapeutic option and may avoid some of the serious adverse effects of current treatments. Since the Hh pathway also regulates various developmental pathways, it is unclear what the adverse effects of Hh pathway blockade may be in prepubescent children.

Other solid tumors

Multiple other solid tumors that do not harbor any somatic mutations in the Hh signaling pathway, such as BCC or medulloblastoma, also demonstrate a ligand-dependent activation of the Hh pathway. Constitutive activation of the Hh pathway has been detected in a broad variety of tumors including lung, stomach, esophagus, pancreas, prostate, breast, liver and brain [Clement *et al.* 2007; Sicklick *et al.* 2006; Karhadkar *et al.* 2004; Kubo *et al.* 2004; Berman *et al.* 2003; Thayer *et al.* 2003; Watkins *et al.* 2003b]. Although preclinical xenograft and animal models of many of these Hh overexpressing tumors show tumor growth inhibition on treatment with cyclopamine [Karhadkar *et al.* 2004; Berman *et al.* 2003; Thayer *et al.* 2003; Watkins *et al.* 2003a; Watkins *et al.* 2003b], the potential usefulness of Hh pathway inhibitors have yet to be tested in a clinical setting.

In addition to the above effect of Shh signaling in cancer and stromal cells, inhibition of the Shh pathway seems to augment the formation of desmoplasia in pancreas cancer [Olive *et al.* 2009]. The expression of Shh was found to cause desmoplasia formation in pancreatic cancer [Bailey *et al.* 2008]. IPI-926, a synthetic, small-molecule SMO antagonist, combined with gemcitabine was shown to improve the gemcitabine delivery to this pancreatic tumor model by depleting tumor-associated stromal tissue.

There are multiple Hh pathway inhibitors in development, including SANT1–SANT4, CUR-61414, HhAntag-691, GDC-0449, MK4101, IPI-926, BMS-833923 and itraconazole [Kim, 2009; Scales and de Sauvage, 2009]. The orally available SMO inhibitor GDC-0449 is

the farthest along in development and is the major Hh antagonist actively being tested for use in ligand-dependent cancers. Two trials utilized GDC-0449 as maintenance therapy, one in patients with ovarian cancer in a second or third complete remission and the other for first-line therapy for metastatic colorectal cancer in combination with concurrent chemotherapy and bevacizumab (see www.clinicaltrials.gov and Scales and de Sauvage, 2009). Two other trials evaluating the use of GDC-0449 for the treatment of extensive-stage small cell lung cancer in combination with chemotherapy and unresectable pancreatic cancer in combination with erlotinib have recently been opened and are actively recruiting patients (see www.clinicaltrials.gov).

Conclusions

The last decade has seen extraordinary progress in understanding the roles and mechanism of action of Hh proteins in development and cancer. Targeting the Hh signaling pathway provides a new and exciting therapeutic option for a broad variety of cancers. Novel associations with dysregulated Hh signaling and the formation of cancer continue to emerge. Although all mechanisms of the Hh signaling pathway are not completely understood, it is clear that aberrant Hh signaling causes tumor growth and proliferation, increases tumor aggressiveness and raises the frequency of metastasis. Inhibition of the Hh pathway is thus a promising new approach for the treatment of select advanced malignancies. These include cancers such as BCC and medulloblastoma, which have mutations leading to constitutive activation of the Hh pathway, as well as other tumors which are Hh ligand dependent for tumor growth either by autocrine or paracrine mechanisms. Initial clinical trials of the oral SMO antagonist GDC-0449 show good efficacy and safety in BCC and medulloblastoma [Rudin *et al.* 2009; Von Hoff *et al.* 2009]. Although Hh pathway inhibitors seem to be safe in adults, their safety in children, especially for the treatment of medulloblastoma, is yet to be ascertained. The use of Hh antagonists in the treatment of ligand-dependent cancers is also to be determined, with multiple ongoing clinical trials in other solid tumors (see www.clinicaltrials.gov). Hh signaling also seems to be important for regulating stem cells in various tissues and Hh pathway inhibition might represent another method to target these relatively resistant and slow-growing CSCs. Optimally this approach would warrant the combination of systemic Hh pathway inhibition with other cytotoxic inhibitors of tumor growth. To maximally exploit the Hh pathway for therapeutic purposes, a better understanding of the precise Hh signaling mechanisms in various tumors is required.

It has been exciting to follow the advances of Hh pathway inhibitors in the ongoing preclinical and clinical trials including the recently reported use in advanced and metastatic BCC. These preliminary studies have set the stage for using these inhibitors in other cancers. Hh pathway inhibitors truly represent an important new class of therapeutic agents, which are bound to have far-reaching implications in oncology.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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