Targeting hedgehog signaling in cancer: research and clinical developments

Jingwu Xie
Christopher M Bartels
Scott W Barton
Dongsheng Gu
Wells Center for Pediatric Research, Division of Hematology and Oncology, Department of Pediatrics, Indiana University Simon Cancer Center, Indiana University, Indianapolis, IN, USA

Abstract: Since its first description in Drosophila by Drs Nusslein-Volhard and Wieschaus in 1980, hedgehog (Hh) signaling has been implicated in regulation of cell differentiation, proliferation, tissue polarity, stem cell maintenance, and carcinogenesis. The first link of Hh signaling to cancer was established through studies of Gorlin syndrome in 1996 by two independent teams. Later, it was shown that Hh signaling may be involved in many types of cancer, including skin, leukemia, lung, brain, and gastrointestinal cancers. In early 2012, the US Food and Drug Administration approved the clinical use of Hh inhibitor Erivedge/vismodegib for treatment of locally advanced and metastatic basal cell carcinomas. With further investigation, it is possible to see more clinical applications of Hh signaling inhibitors. In this review, we will summarize major advances in the last 3 years in our understanding of Hh signaling activation in human cancer, and recent developments in preclinical and clinical studies using Hh signaling inhibitors.

Keywords: hedgehog, smoothened, PTCH1, cancer, signal transduction, clinical trials, animal model

Introduction
Remarkable progress has been made since the hedgehog (Hh) mutant phenotype was first described in fruit fly in 1980. Three vertebrate homologues of Hh and their receptors were identified in the 1990s. As an essential pathway during development, the Hh pathway is critical for maintaining tissue polarity and stem cell population. The first link between Hh signaling and cancer was shown in tumor-prone Gorlin syndrome in 1996. In early 2012, Hh signaling inhibitor GDC-0449 (Erivedge/vismodegib; Hoffmann-La Roche Ltd, Basel, Switzerland) was approved by the US Food and Drug Administration for treatment of locally advanced and metastatic basal cell carcinomas (BCCs).

The general signaling mechanisms of the Hh pathway are conserved from flies to humans. Mammalian Hh signaling molecules include ligands (sonic Hh, Indian Hh, and desert Hh), patched receptors (PTCH1, PTCH2), signal transducer smoothened (SMO), and transcription factors (Gli1, Gli2, Gli3) (see Figure 1). In the absence of ligands, SMO serves as the key signal transducer, whose function is inhibited by another transmembrane protein patched (PTCH1). Upon binding of an active Hh ligand, this inhibition is released, allowing SMO to signal downstream, eventually leading to activation of Gli transcription factors. Gli molecules can bind the specific consensus sequences located in the promoter region of the target genes to regulate target gene expression.
In the last 3 years, there has been significant progress regarding Hh signaling and its significance in cancer development and therapeutics. The total number of publications on Hh signaling in the last 3 years is close to 30% of all Hh-related publications, and progress has been made in the following areas: 1) better understanding of Hh signal transduction and the associated target genes, 2) more reliable mouse models linking Hh signaling to human malignancies, 3) better understanding of Hh signaling mechanisms during cancer development and metastasis, 4) an increasing number of clinical and preclinical studies on cancer treatment using Hh signaling inhibitors, and 5) emerging evidence of Hh signaling in supporting residual cancer cells and cancer stem cells.

**Signal transduction of the Hh pathway**

All Hh proteins are secreted molecules, functioning at short range on nearby cells or at long range to distant cells during development.\(^{15-17}\) Hh protein precursors undergo post-translational modifications (autocleavage to release the N-terminal fragment [HhN], covalently binding to a cholesterol moiety at the C-terminal end, and palmitoylation by a palmitoyltransferase at the N-terminus of HhN).\(^{18-21}\) Molecules involved in Hh protein transport and distribution include the transmembrane transporter-like protein dispatched (Disp),\(^{22-24}\) metalloproteinases,\(^{25}\) the hepan sulfate proteoglycans Dally-like (Dlp) and Dally,\(^{26,27}\) or their regulators,\(^{28}\) as well as enzymes such as sulfatase and tout velu.\(^{29-31}\)
Figure 1 shows the mammalian Hh signaling pathway with major players in the diagram. Several molecules are engaged in reception of Hh ligands, with patched (PTC, one PTC in fly, and two PTCs in vertebrates: PTCH1 and PTCH2) as the major receptor. Studies from cultured cells indicate that PTC inhibits SMO at a substoichiometric concentration. Hh-interacting protein (HIP) can compete with PTC on Hh binding, resulting in negative regulation of Hh signaling. On the other hand, interference Hh or its vertebrate homologues cell adhesion molecule related/downregulated by oncogenes (CDO) and BOC (brother of CDO), GAS1, and glypican-3 (GPC3) serve as coreceptors of Hh. In contrast to the inhibitory effect of glypican-3, glypican-5 (GPC5) and other heparan sulfate proteoglycans are shown to stimulate Hh signaling by promoting binding of sonic Hh to PTCH1. The effect of GPC5 and interference Hh homologues requires another secreted extracellular molecule: Wnt inhibitory factor-1 (WIF1). It is still not entirely clear how binding of Hh proteins results in the pathway activation. It is proposed that PTC limits SMO signaling via transporting endogenous small molecules specifically targeted to SMO. Candidates of these small molecules include PI4P, lipoproteins, and provitamin D3. It is currently not very clear how these molecules regulate SMO signaling.

It is now known that glucocorticoid molecules can modulate SMO signaling through regulating its ciliary localization. Several recent reports support SMO to G protein coupling, but the physiological relevance of the G protein coupling of SMO in carcinogenesis has not been convincingly demonstrated. G0 can also regulate Gli proteins independent of SMO. It is quite clear that two important events occur during SMO signaling in mammalian cells. First, SMO protein undergoes conformational change to favor SMO signaling, although the regulatory mechanism underlying this conformational change is not clear. Second, ciliary translocation of mammalian SMO protein is critical for Hh signaling. Several reports now link neureilin 1/2 (Nrpl/2) to SMO signaling.

Several molecules are identified to be genetically downstream of SMO in Drosophila, including COS2, suppressor of fused (SuFu), and fused. A COS2 homologue, kinesin like-protein KIF7, functions in the Hh pathway but not directly associated with SMO suggesting that KIF7 does not contain all COS2 functions in vertebrates. In contrast, the phenotype of fused mice is very different from Shh null mice, indicating that fused is not critical for Hh signaling during early embryonic development in mice.

In addition to the Drosophila homologues, mammalian cells have several novel cytoplasmic regulators of Hh signaling, including Rab23 and tectonic. Rab23 and tectonic are all negative regulators downstream of SMO. We have shown that Rab23 is involved in Gli–SuFu interaction (see Figure 1). Unlike many Rab proteins, we found that Rab23 is localized both in the nucleus and in cytoplasm, suggesting that Rab23 may have other unrevealed functions apart from membrane trafficking.

The ultimate effect of Hh signaling is activation of downstream Gli transcription factors, which regulate target genes by directly binding a consensus binding site (5’-tgggtggtc-3’) in the promoter. The activity of Gli transcription factors can be regulated at several levels. First, nuclear–cytoplasmic shuttling of Gli molecules is tightly regulated. Protein kinase A can retain Gli1 protein in the cytoplasm via a protein kinase A site in the nuclear localization signal domain, whereas activated Ras signaling induces Gli nuclear localization. Second, ubiquitination, acetylation, and protein degradation of Gli molecules are regulated by several distinct mechanisms, including β-TRCP, and numb/itch-mediated Gli ubiquitination, sumoylation, and acetylation. In addition, Gli3 (Gli2 to a lesser extent) can be processed into transcriptional repressors, which may be mediated by the β-TRCP E3 ligase. SuFu not only prevents nuclear translocation of Gli molecules but also inhibits Gli1-mediated transcriptional activity. Other mechanisms to modify Gli functions include interaction with a negative regulator surose nonfermenting 5 (SNF5) and a positive regulator protein kinase C isoform tα.

Several feedback regulatory loops exist in this pathway to maintain a certain level of Hh signaling in a given cell. PTC, HIP, GAS1, neureilins, and Gli1 are components, as well as the target genes of this pathway. PTC and HIP provide negative feedback regulation, whereas Gli1 and Nrp1/2 form positive regulatory loops. On the other hand, GAS1 is downregulated by the Hh pathway but is a positive regulator for Hh signaling. Alterations of these loops would lead to abnormal signaling of this pathway, such as inactivation of PTCH1 in BCCs.

**Activation of the Hh pathway in human cancer**

The initial link between Hh signaling and human cancers was made from the discovery that mutations of human *PTCH1* are associated with a rare and hereditary form of BCC, basal cell nevus syndrome (BCNS) (also Gorlin syndrome). Gorlin syndrome is a rare autosomal genetic disease with two
distinct sets of phenotypes: an increased risk of developing cancers such as BCCs, medulloblastomas, rhabdomyosarcomas, and meningiomas, as well as developmental defects such as bifid ribs and ectopic calcification.\textsuperscript{104}

Almost all BCCs and about 30\% of medulloblastomas have activated Hh signaling via gene mutations in \textit{PTCH1}, SMO, or other Hh pathway molecules.\textsuperscript{105–109} In addition, cancers associated with Gorlin syndrome, including rhabdomyosarcoma\textsuperscript{10,111} and meningiomas,\textsuperscript{112–114} are reported to have gene mutations in the Hh signaling pathway or elevated Hh target gene expression. Activated Hh signaling has been detected in a variety of human cancer types, either in the tumor or in the stroma.\textsuperscript{110,115–117}

Genetically engineered mice with \textit{Ptch1} and \textit{Smo} genes have generated more convincing evidence for the critical role of Hh signaling in cancer. In addition to BCCs and medulloblastomas, rhabdomyosarcomas develop in mice with expression of oncogenic \textit{SmoM2} or knockout of \textit{Ptch1}.\textsuperscript{118–121} One surprising finding from tissue-specific \textit{Ptch1} knockout is the development of gastrointestinal stromal-like tumors (GIST),\textsuperscript{122} suggestive of a role of Hh signaling in GIST. Even in the situation of a small cell lung cancer (SCLC) mouse model, expression of oncogenic \textit{SmoM2} increases the tumor number, whereas \textit{Smo} knockout reduces the tumor number.\textsuperscript{123} Recent study of Barrett’s esophagus indicates that sonic Hh expression in the epithelium of the esophagus can lead to stromal expression of Hh signaling target genes, which is similar to the human situation.\textsuperscript{124,125} In contrast, tissue-specific expression of oncogenic Smo molecule \textit{SmoM2} has no effects on K-Ras-induced pancreatic cancer\textsuperscript{126} or on prostate cancer.\textsuperscript{127} The negative data, however, do not rule out the promoting effects of Hh signaling for tumor metastasis, a major factor for cancer mortality. Currently, there are only a limited number of mouse models for cancer metastasis. Even for the available mouse models for cancer metastasis, several variable factors make cancer metastasis models less robust, and these factors include mouse genetic backgrounds, low incidence, and long duration to observe metastasis in mice.

\section*{Hh signaling in tumor initiation, promotion, and metastases}

Hh signaling plays different roles in different types of cancer.\textsuperscript{110} Based on the published data, we attempt to divide the functions of Hh signaling during cancer development into three types: the tumor driver, the tumor promoter, or the regulator for residual cancer cells after therapy. For example, activated Hh signaling can drive development of BCCs, medulloblastomas, rhabdomyosarcoma, GIST, and Barrett’s esophagus,\textsuperscript{118,119,122,124,128,129} and Hh signaling in these lesions serves as the tumor driver, at least in the mouse models. For SCLC, Hh signaling can promote cancer development but is not sufficient to drive tumor formation, and thus serves as a tumor promoter.\textsuperscript{123} In pancreatic cancer, inhibition of Hh signaling does not affect tumor formation but can promote tumor metastasis.\textsuperscript{130–137} For other cancer types, Hh signaling may regulate the number of cancer stem cells or the tumor microenvironment, such as leukemia and liver cancer.\textsuperscript{138,139} As more in vivo data are available, we predict more revelation of the tumor promoting role of Hh signaling. Tumor recurrence after therapy is a major issue in clinical care of cancer patients, such as chemotherapy or radiotherapy resistance, and will be discussed in “Hh signaling, cancer stem cell, and residual cancer cells.” For some cancer types, Hh signaling may not have any roles to play.

Activation of Hh signaling does not work in isolation but rather crosstalks with other signaling pathways during cancer development and metastasis. Earlier studies indicated that \textit{Ptch1\textsuperscript{1+/–}} mice with \textit{P53} knock out all developed medulloblastomas, whereas <30\% of \textit{Ptch1\textsuperscript{1+/–}} mice (with wild-type \textit{P53}) had this type of tumor.\textsuperscript{110} We have shown that skin-specific knockout of \textit{Stat3} or its upstream activator \textit{Il11ra} significantly reduced Hh signaling-mediated BCC formation.\textsuperscript{141} Increasing data have indicated close collaboration between Hh signaling and growth factor signaling pathways. Our earlier work indicated that platelet-derived growth factor \textit{α} (PDGFR\textit{α}) is regulated by Hh signaling and is responsible for cell proliferation in BCCs.\textsuperscript{142} Now more links are reported between Hh and other pathways, including epidermal growth factor, insulin growth factor, transforming growth factor β (TGF\textit{β}), mTOR/S6K1, RACK1, notch, and protein kinase C.\textsuperscript{140,143–151} Although some of these molecules are involved in regulation of tumor microenvironment, such as TGF\textit{β}, others are known to regulate cancer stem cells, such as PDGFR\textit{α} and notch. We will have more discussion on cancer stem cells in “Hh signaling, cancer stem cell, and residual cancer cells.”

Increasing evidence indicates that Hh signaling plays an important role during tumor metastasis in several types of cancer, such as pancreatic and breast cancers.\textsuperscript{135,136} Studies from many groups indicate activation of Hh signaling in the stromal as well as tumor compartments in metastatic pancreatic cancer.\textsuperscript{130,133–137,153} In fact, Hh signaling inhibitors are effective in suppressing tumor metastases of pancreatic cancer.\textsuperscript{135} Hh signaling also regulates bone homeostasis as well as bone metastasis in breast cancer independent of
the Hh ligands. During tumor metastasis, Hh signaling activation is observed both in the tumor compartment and in the stroma. The molecules mediating Hh’s metastatic functions remain largely untested, but there are reports to indicate the following molecules: snail, TGFβ, Wnt, HGF, and muc5 AC. Further studies will be needed to understand the molecular basis by which Hh signaling mediates cancer metastases.

### Hh signaling inhibitors: preclinical and clinical studies

More than 200 compounds have been disclosed to have inhibitory effects on Hh signaling. Of these, eight have been used for clinical trials (see Table 1 for the list). There are three major targeting sites for Hh signaling inhibitors identified so far: Hh molecules (Shh neutralizing antibodies, small molecule Robotnikinin), SMO protein (cyclopamine and its derivatives IPI-926 and CyeT, and synthetic compounds GDC-0449, XL-139/BMS833923, LDE-225, PF04449913, and LY2940680), and Gli inhibitors (HPI-1, HPI-2, GANT-56, and GANT-61). The major advances include successful clinical trials using GDC-0449 and US Food and Drug Administration approval of GDC-0449 for treatment of locally advanced and metastatic BCCs. However, combination of Hh signaling inhibitors with gemcitabine or Hh signaling inhibitors alone did not show any improvements in the outcomes of pancreatic cancer patients. We summarize these data below.

Table 1 shows the list of Hh signaling inhibitors in clinical trials, with all eight small molecules targeting SMO. Clinical trials with GDC-0449 in BCCs are the most successful. The successful Phase II clinical trials were preceded with a remarkable Phase I clinical trial in patients with metastatic BCCs. This drug is well tolerated by patients. Two independent groups used GDC-0449 to treat BCNS patients and sporadic BCCs, respectively, via oral administration. Although the overall outcomes were very encouraging, the responses of two groups of patients were quite different. Although BCNS patients had virtually a 100% response rate, sporadic BCCs had only a 33% response rate. Previous studies in mouse models indicate that tumors acquire somatic mutations in Smo or other signaling pathways following GDC-0449 administration, which may explain why not all sporadic BCCs responded well. A more rational way to treat sporadic BCCs is topical application. Two groups (one from Novartis AG and one from Hoffmann-La Roche Ltd/Genentech) indeed tested that possibility with BCNS patients and obtained impressive responses. Mechanisms to Smo antagonist resistance include mutations in the target SMO gene or alterations in the PI3K pathway. Several ways have been explored to mitigate drug resistance to SMO antagonists, such as itraconazole and arsenic trioxide, polymeric nanoparticle-encapsulated Hh signaling inhibitors, or vitamin D3. Hopefully, some of these combined treatments will provide benefits to BCC patients.

Studies in animal models demonstrated significant inhibition of Hh signaling inhibitors on medulloblastoma development. For example, oral administration of IPI-926 or PF-5274857 can reduce tumor development, leading to a longer lifespan in mouse medulloblastoma models. However, an early clinical trial on a medulloblastoma patient using GDC-0449 yielded only a transient therapeutic effect, due to an SMO mutation occurring soon after treatment. The outcome data of current medulloblastoma clinical trials are not available, but there is still a high expectation.

There is evidence to support that rhabdomyosarcoma is very responsive to Hh signaling inhibitors. First, gene expression analyses revealed elevated Hh target gene expression in embryonic rhabdomyosarcomas. Second, preliminary studies used forskolin or SMO inhibitor to shrink tumors in mouse models. In addition, evidence for Hh signaling in meningiomas and SCLC is quite clear, and

### Table 1 A list of hedgehog signaling inhibitors in clinical trials (from http://clinicaltrials.gov)*

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Other names</th>
<th>Phase</th>
<th>Tumor types</th>
<th>FDA approval</th>
<th>Company</th>
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<td>GDC-0449</td>
<td>Vismodegib/erivedge</td>
<td>I/II/III</td>
<td>BCCs and solid tumors</td>
<td>BCCs</td>
<td>Hoffman-La Roche Ltd</td>
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<td>BMS833923</td>
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<tr>
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<td></td>
<td>I/II</td>
<td>SCLC/advanced cancer</td>
<td></td>
<td>Eli Lilly and Company</td>
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Notes: All small molecules target smoothened molecule. GDC-0449 has been approved by the FDA to treat locally advanced and metastatic BCCs. There are no ongoing clinical trials for LEQS06, TAK-441, and BMS833923.

Abbreviations: BCC, basal cell carcinoma; FDA, US Food and Drug Administration; SCLC, small cell lung cancer.
Hh signaling inhibitors should be effective in these tumor types as well.

**Hh signaling, cancer stem cell, and residual cancer cells**

Increasing evidence indicates that Hh signaling is critical for cancer stem cell maintenance and function.\(^{138,175,176}\) For example, leukemia stem cell maintenance and expansion are dependent on Hh signaling.\(^{138,175}\) The effect of Hh signaling on a normal hematopoietic stem cell population, however, is still quite controversial, with some showing effects but others with no effects.\(^{138,177-180}\) Based on cancer stem cell theory, it is anticipated that Hh signaling activation exerts resistance to cancer chemotherapy and radiotherapy.\(^{181}\) Several studies have indeed shown that Hh signaling activation is associated with chemotherapy or radiotherapy resistance.\(^{182-185}\) Hh signaling inhibitor IPI-926 enhances delivery of the chemotherapeutical drug gemcitabine in a mouse model of pancreatic cancer. Relevance to the cancer stem cell theory is the link between Hh signaling activation and cancer relapse from drug resistance.

Based on the published data, we propose that Hh signaling may help maintain the stemness of cancer stem cells, which are generally insensitive to chemotherapy and radiotherapy. There is evidence to indicate that Hh signaling regulates expression of cancer stem cell-related markers, such as aldehyde dehydrogenase, Bmi1, snail, Wnt2, PDGFRα, jagged-1, CD44, and c-MET.\(^{135,155,194-188}\) The level of Hh expression is often higher in the cancer stem cell population in several cancer types.\(^{189-193}\) Thus, we have reasons to believe that inhibition of Hh signaling may be effective in reducing the number of cancer stem cells, which may play an important role in chemotherapy and radiotherapy resistance.

Chemotherapy and radiotherapy play an important role in the clinical care of cancer patients, but resistance to these treatments remains a major obstacle in cancer patient care. Recent studies revealed a few examples for the role of Hh signaling in chemotherapy and radiotherapy resistance. Resistance to docetaxel is a major clinical challenge for prostate cancer patients. A recent study revealed an important role of Hh signaling on docetaxel resistance in prostate cancer.\(^{194}\) Combination of notch and Hh signaling inhibitors was able to reverse docetaxel resistance both in cultured cells and in xenografts. Activation of Hh signaling via PI3 K is also reported in tamoxifen-resistant breast cancer.\(^{195}\) and a combination of Hh signaling inhibitor GDC-0449 with tamoxifen significantly reduced cell colony formation and tumor development in xenografts. In addition, activated Hh signaling is shown to be responsible for drug resistance in ovarian cancer, cervical cancer, and myeloid leukemic cells.\(^{196-198}\) A recent study also suggests that Hh signaling may be associated with antiepidermal growth factor receptor therapy (targeted therapy) resistance observed in head and neck cancer.\(^{144}\) The exact mechanisms by which Hh signaling activation confers drug resistance are not entirely clear, but it is reported that Hh signaling can regulate expression of several drug resistance-related genes such as ABCG2 and MDR.\(^{198,199}\) The cancer stem cell theory can also explain some of the mechanisms.

Overcoming recurrence to radiotherapy is also very challenging, but recent studies suggest that inhibiting Hh signaling may help mitigate radiotherapy resistance in pancreatic and head/neck cancer. For pancreatic cancer, we found that a combination of Hh signaling inhibitor BMS833932 (see Table 1 for details) and radiation could significantly reduce the number of lymph node metastasis.\(^{135}\) Similarly, high expression of Gli1 is reported to be associated with lymph node metastases and tumor progression after radiotherapy in squamous cell carcinomas of the head/neck.\(^{200}\)

**Summary and future perspectives**

In summary, the link of Hh signaling activation to a variety of human cancer implies the relevance of studying Hh signaling to human health. Rapid advancement in the discovery of novel Hh signaling inhibitors has provided many opportunities for developing novel cancer therapeutic strategies. It is not surprising to learn that several major challenges still exist to prevent the use of Hh signaling inhibitors in clinics. These challenges include a lack of basic understanding of the molecular mechanisms by which Hh signaling mediates carcinogenesis; no clear criteria to identify the right tumors for therapeutic application; only a few reliable, physiologically relevant, and reproducible mouse models for cancer metastases to test and optimize drug dosages in order to minimize side effects; and a lack of clear strategies to mitigate drug resistance. Over the last 3 years, research in this area has greatly improved, as indicated in this review. It is anticipated that additional novel therapeutic strategies will be developed for cancer clinical trials using Hh signaling inhibitors in the next few years.

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Disclosure
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