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Review

Clinical implications of hedgehog signaling pathway inhibitors

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Abstract

Hedgehog was first described in *Drosophila melanogaster* by the Nobel laureates Eric Wieschaus and Christiane Nüsslein-Volhard. The hedgehog (Hh) pathway is a major regulator of cell differentiation, proliferation, tissue polarity, stem cell maintenance, and carcinogenesis. The first link of Hh signaling to cancer was established through studies of a rare familial disease, Gorlin syndrome, in 1996. Follow-up studies revealed activation of this pathway in basal cell carcinoma, medulloblastoma and, leukemia as well as in gastrointestinal, lung, ovarian, breast, and prostate cancer. Targeted inhibition of Hh signaling is now believed to be effective in the treatment and prevention of human cancer. The discovery and synthesis of specific inhibitors for this pathway are even more exciting. In this review, we summarize major advances in the understanding of Hh signaling pathway activation in human cancer, mouse models for studying Hh-mediated carcinogenesis, the roles of Hh signaling in tumor development and metastasis, antagonists for Hh signaling and their clinical implications.

Key words Hedgehog, Smoothened, PTCH1, cancer, signal transduction, clinical trials, animal model

Major advances in understanding the hedgehog (Hh) pathway have been made in the last 30 years. The Hh gene was identified in 1980 through genetic analyses of *Drosophila* fruit fly segmentation[1]. In early 1990's, three vertebrate homologues of the Hh gene were identified[2-6]. As an essential signaling pathway in embryonic development, the Hh pathway is critical for maintaining tissue polarity and stem cell population. In 1996, inactivation of this pathway was linked to the hereditary developmental disorder holoprosencephaly, whereas hyperactivation of this pathway was linked to human cancer [7-11]. More recently, an inhibitor of Hh signaling was successfully used in clinical trials of human cancer, further indicating the feasibility of Hh signaling inhibitors for cancer therapeutics. Figure 1 lists the major milestones of research on Hh signaling as related to cancer.

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The general signaling mechanisms of the Hh pathway are conserved from flies to humans [12]. In the absence of Hh ligand, Smoothened (SMO), the seven transmembrane domain containing protein, serves as the key signal transducer, whose function is inhibited by another transmembrane protein Patched (PTC). An active Hh ligand (Shh, Ihh, Dhh, or the fly Hh homologue) binds to its receptor PTC and relieves this inhibition, allowing SMO to signal downstream, leading to the activation of Gli transcription factors. As a transcription factor, Gli protein associates with specific

1980	Discovery of Hh in fly
1987	Discovery of GLI in glioma
1993	Cloning of vertebrate Hhs
1996	Linking Hh to developmental defects
1996	Linking Hh to cancer- Ptch1 as first TSG
1998	Linking Hh to cancer- SMO as first oncogene
1998	Linking cylopamine to Hh signaling
2002	Synthesis of Hh signaling inhibitors
2003-	Linking Hh signaling to more common cancers
2009-	First successful clinical trial in human cancer

Figure 1. Major milestones in the studies of hedgehog signaling as related to human diseases, particularly cancer. For all references, please see the text for details.

consensus sequences located in the promoter region of target genes, regulating target gene expression [13,14]. Figure 2 shows a simplified diagram of the Hh signaling pathway.

Signal Transduction of the Hedgehog Pathway

Hh proteins [one Hh in *Drosophila* and three Hhs in mammals—Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh)] are secreted during development, functioning at short range to nearby cells and at long range to distant cells [15-17]. After translation, the Hh protein precursor undergoes auto-processing to release its N-terminal fragment (HhN), which is then covalently bound to a cholesterol moiety at the C-terminal end. Palmitoylation mediated by the Skinny Hedgehog acyltransferase occurs at the N-terminus of HhN [18-21]. Several molecules are involved in the movement, extracellular transport, and release of Hh proteins, including the transmembrane transporter-like protein Dispatched (Disp) [22-24], metalloproteases [25], the heparan sulfate proteoglycans Dally-like (Dlp) and Dally [26,27] or their regulators [28], as well as enzymes such as Sulfateless and Tout velu [29-31].

Several molecules are engaged in the reception of Hh ligands, with PTC (one PTC in fly and two PTCs in vertebrates—PTCH1 and PTCH2) as the major receptor [32]. Studies from tissue cultured cells indicate that PTC inhibits SMO at a sub-stoichiometric concentration [33]. The Hh-interacting protein (HIP) can compete with PTC to bind Hh, resulting in the negative regulation of Hh signaling [34]. On the other hand, Ihog (or its vertebrate homologues CDO and BOC), GAS1, and Glypican-3 serve as co-receptors of Hh [35-42]. It is not clear how binding of Hh proteins results in the pathway activation. It is proposed that PTC limits SMO signaling by transporting small endogenous molecules specifically targeted to SMO. Candidates of these small molecules include PI4P, lipoproteins, and pro-vitamin D3 [43-46]. However, how these molecules regulate SMO signaling is unknown.

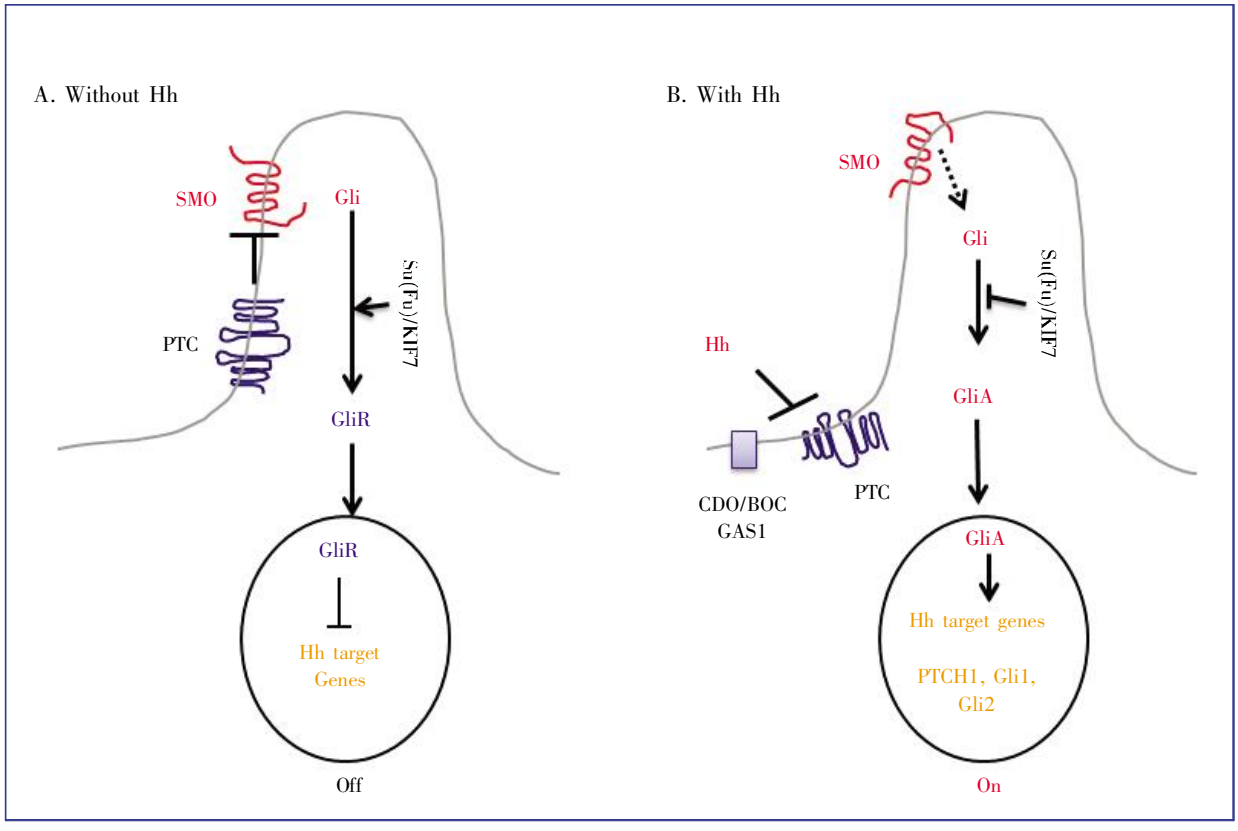

Figure 2. A simplified model for Hh signaling in mammalian cells. SMO is the key signal transducer of the Hh pathway. A, in the absence of the Hh ligands, the Hh receptor PTC is thought to be localized in the cilium to inhibit SMO signaling (via an unknown mechanism). Gli molecules are processed with the help of Su(Fu)/KIF7 molecules into repressor forms, which disable the Hh signaling pathway. B, in the presence of Hh, PTC is thought to be shuttled out of cilium and is unable to inhibit SMO. Co-receptors of Hh ligands include CDO, BOC, and GAS1. Hh reception promotes SMO conformational change, facilitating Gli activation (GliA), stimulating Hh target gene expression. This process can be inhibited by KIF7 and Su (Fu). (Positive regulators are in red, negative regulators are in blue and target genes are in orange.)

Significant progress has been made toward our understanding of SMO signaling, with recent reports linking SMO to G protein coupling [47-50]. In particular, a study in *Drosophila* provides direct evidence for SMO-coupling to Gαi in the regulation of Hh pathway activation [48]. The physiological relevance of G protein coupling to SMO in Hh signaling during carcinogenesis is unknown. In *Drosophila*, SMO function is stimulated through protein phosphorylation by PKA and Casein kinase I at the C-terminus [51,52]. SMO mutants lacking these phosphorylation sites are defective in Hh signaling. However, these phosphorylation sites are not conserved in vertebrate SMO, indicating a different mechanism for SMO signaling in higher organisms [52]. There are two important events during mammalian SMO signaling. First, the SMO protein undergoes a conformational change favoring SMO signaling[53], although the regulatory mechanism underlying this conformational change is unclear. Second, the ciliary translocation of mammalian SMO protein is critical for Hh signaling (see below).

Accumulating evidence indicates that primary cilia play an important role in the Hh pathway [54-59]. The function of primary cilium is regulated by protein complexes involved in intra-flagellar transport (IFT), which functions in retrograde and anterograde movement of cargo within the primary cilia [60]. Mutations in IFT protein involved in predominantly primary cilium anterograde transportation are shown to result in mice with Hh loss of function phenotypes[55,61]. Gli3 processing is the most significantly affected event in IFT mutants[56,57,61]. The presence of several Hh components upon Hh stimulation, including SMO and Gli molecules at the primary cilium, further supports the relevance of cilium in Hh signaling[62-65]. It has been shown that a SMO mutant lacking a ciliary translocation signal cannot mediate Hh signaling[54]. However, the translocation of SMO to cilium is not sufficient to activate Hh signaling [64,65]. Using tissue-specific gene knockout, recent studies have revealed dual roles of cilium (via knocking out cilium component *Kif3a*) in Hh signaling-mediated carcinogenesis in mice [66,67]. Whereas the *Kif3a* gene is required for activated SMO-mediated tumor formation, and knocking out *Kif3a* accelerates Gli2-mediated carcinogenesis. How SMO is translocated to the cilium in response to Hh signaling and how SMO activates downstream effectors are unclear. However, β-arrestin 2 can regulate ciliary localization of SMO [68]. The role of cilium for Hh signaling downstream of SMO is less clear, as not all of the signaling events occur in cilium. For example, cilium is not required for Su(Fu)-mediated regulation of Gli functions[69,70].

Several molecules have been identified to be genetically downstream of SMO signaling in *Drosophila*, including COS2 and Fused. How their vertebrate homologues function in Hh signaling is yet to be established. Recent *in vivo* studies support that a COS2 homologue KIF7 functions in the Hh pathway, but no direct interaction between SMO and KIF7 is detected[71,72], suggesting that the function of COS2 in vertebrates may be replaced by a few molecules. The phenotype of vertebrate Fused knockout mice is not similar to that observed in Shh null mice [73-75] and no changes of Hh signaling are observed in Fused null mice, suggesting that Fused is not critical for Hh signaling during early embryonic development of vertebrates.

In addition to the *Drosophila* homologues, mammalian cells have several novel cytoplasmic regulators of Hh signaling, including Rab23 [76] and tectonic [77]. Rab23 and tectonic are negative regulators downstream of SMO, but their exact mechanism of action remains to be elucidated. Unlike many Rab proteins, Rab23 is localized in the nucleus and cytoplasm [78], suggesting that Rab23 may have other uncharacterized functions apart from membrane trafficking. Through siRNA-based screenings, several additional molecules are identified to be involved in Hh signaling in mammalian cells[79,80] but their exact functions are unclear.

Several evidences indicate that Suppressor of Fused [Su(Fu)] functions as a tumor suppressor gene in mammalian cells. Su(Fu) is originally identified genetically in *Drosophila* by its ability to suppress active *fused* mutations, but itself is not required for pathway activity. Unlike in *Drosophila*, *Su(Fu)* null mouse mutants fail to repress the pathway [81] and have some phenotypes similar to *Ptch1* inactivation. *Ptch1*$^{+/-}$ mice are predisposed to developing medulloblastoma, rhabdomyosarcoma, and basal cell carcinomas [82-84], whereas *Su(fu)*$^{+/-}$ mice predominantly develop basaloid epidermal proliferation. Su(Fu) plays a central role in pathway repression, as indicated by data derived from *Su(Fu)* null MEFs and wild-type cells treated with Su(Fu) siRNA[81], where loss of Su(Fu) results in the activation of Hh signaling. At the molecular level, Su(Fu) associates with and inhibits Gli molecule function, and is required for Gli3 processing[85,86]. One potential molecular basis by which Hh signaling releases the suppressing activity of Su(Fu) is the enhanced Su(Fu) protein degradation upon the activation of Hh signaling[87].

Hh signaling activation ultimately activates downstream Gli transcription factors, which can regulate target gene expression by directly binding to a consensus binding site (5'-TGGGTGGTC-3') in the target gene promoter [13,14,88,89]. The activity of Gli transcription factors can be regulated at several levels. First, the nuclear-cytoplasmic shuttling of Gli molecules is tightly regulated[85,90-92]. Protein kinase A can retain Gli1 protein in the cytoplasm via a PKA site in the nuclear localization signal domain [90], whereas activated Ras signaling induces Gli nuclear localization[92]. Second, ubiquitination, acetylation, and protein

degradation of Gli molecules is regulated by several distinct mechanisms, including β-TRCP–, cul3/BTB- and numb/Itch-mediated Gli ubiquitination [93-98]. In addition, Gli3 (Gli2 to a less extent) can be processed into transcriptional repressors, which may be mediated by the β-TRCP E3 ligase[95]. Defects in the retrograde motor for IFTs can affect Gli3 processing [99]. Furthermore, the transcriptional activity of Gli molecules is tightly regulated. Su(Fu) prevents nuclear translocation of Gli molecules and inhibits Gli1-mediated transcriptional activity[100].

Several feedback regulatory loops exist in this pathway, maintaining the level of Hh signaling in a given cell. PTC, HIP, GAS1, and Gli1 are components as well as target genes of this pathway. PTC and HIP provide negative feedback regulation, whereas Gli1 forms a positive regulatory loop. On the other hand, GAS1 is down-regulated by the Hh pathway but it is a positive regulator for Hh signaling. Alterations of these feedback loops would lead to abnormal signaling of this pathway, such as inactivation of PTCH1 in basal cell carcinoma (BCC).

The Link of Hh Signaling to 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 (also called Gorlin syndrome) [101-103]. Gorlin syndrome is a rare autosomal genetic disease with two distinct sets of phenotypes: a predisposition to develop cancer such as BCC and medulloblastoma, and developmental defects such as bifid ribs and ectopic calcification. The tumor suppressor role of *PTCH1* is demonstrated in knockout mice, where *Ptch1*$^{+/-}$ mice develop tumors in addition to other features observed in patients with Gorlin syndrome, such as spina bifida occulta[83,84,104]. A variety of cancers are associated with the activation of hedgehog signaling (Figure 3 and Table 1)[105,106].

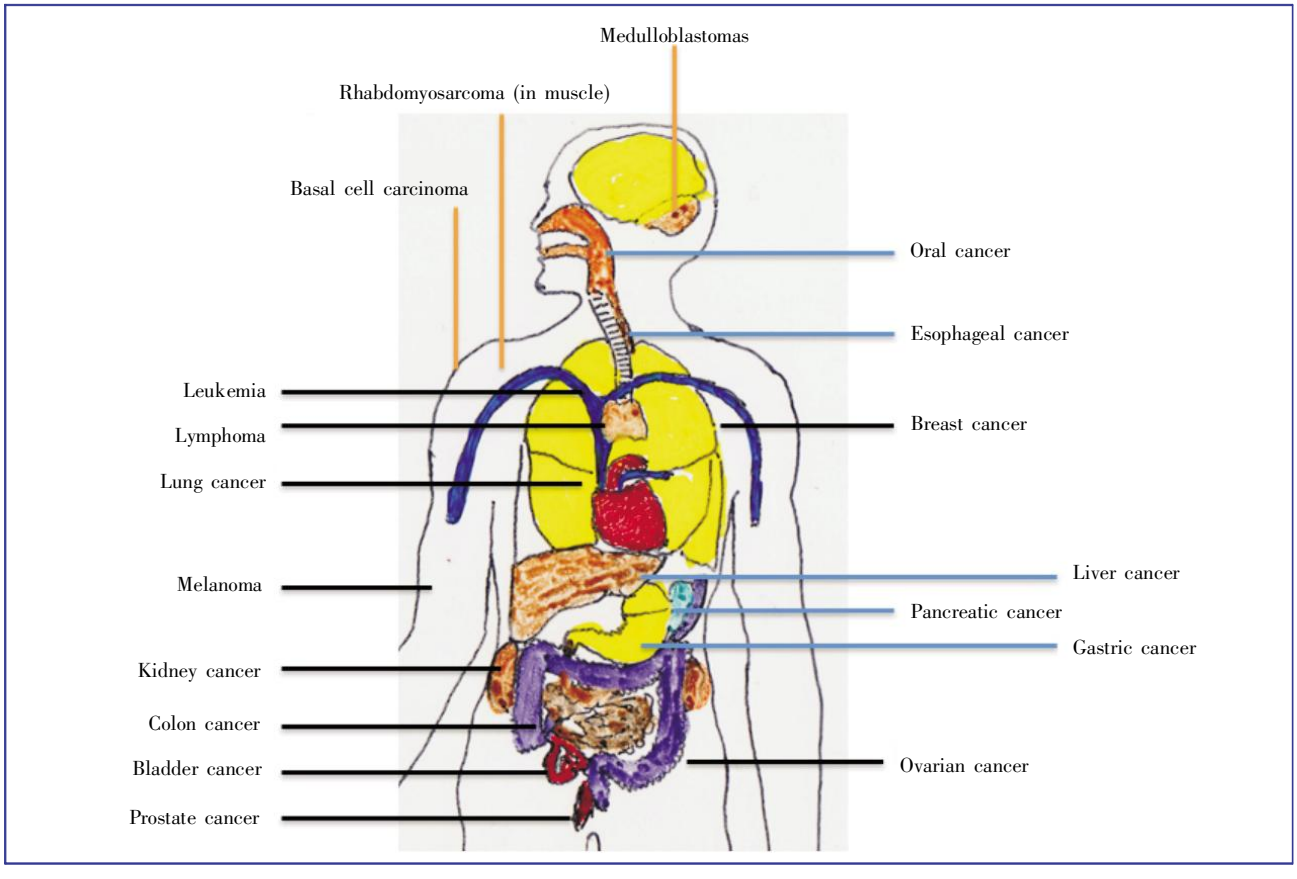

Figure 3. Activation of Hh signaling in human cancer. Following the discovery of Hh signaling activation in Gorlin syndrome, increasing evidence suggests that Hh signaling is frequently activated in human cancer. Based on current findings, we group these cancers into three groups. Group one is associated with Gorlin syndrome, including basal cell carcinomas, medulloblastomas, and rhabdomyosarcomas (in muscle) (in red). Group two includes cancer types with reproducible data of Hh signaling activation from several groups, such as oral cancer and many gastrointestinal cancers (in blue). Group three includes cancer types with limited or variable results from different groups (in black). Several common cancer types are included in group three; further investigation will provide insight as to the significance of Hh signaling in these different types of cancer.

Table 1. Activation of hedgehog signaling in human cancer

Tumor type	Gene alteration	Mouse models	Functions	Hh-based clinical trial
BCCs	PTCH1, SMO, Su(Fu) PTCH1,	Ptch1$^{+/-}$; R26-SmoM2/K14-cre; K5-SMO (or Gli2); K14-Shh Ptch1$^{fl/fl}$/cre (K6a, K14 or Mx1)	Driver	Phase II/III
Medulloblastoma	SMO, Gli2, Su(Fu), Gli1, Ren	Ptch1$^{+/-}$; SuFu$^{+/-}$/p53$^{-/-}$; R26-SmoM2/cre (Math1, hGFAP, Pax7); Ptch1$^{fl/fl}$/cre (Math1, hGFAP, Olig2)	Driver	Phase II
Rhabdomyosarcoma	PTCH1, Gli1, Su(Fu)	Ptch1$^{+/-}$; Su(Fu)$^{+/-}$/p53$^{-/-}$; SuFu$^{+/-}$/Ptch1$^{-/-}$; R26-SmoM2/CAG-CreER	Driver	
Esophageal cancer	Shh, Gli2	Surgical rat models	Unknown	
Gastric cancer	Shh	Not available	Unknown	Phase II
Liver cancer	Shh	Not available	Unknown	
Pancreatic cancer	Shh, Ihh	R26-SmoM2/CAG-CreER; orthotopic	Metastasis	Phase II
Gliomas	Gli1, Shh	PDGF-B based mouse model; xenograft	Micro-environment	
Breast cancer	Ihh, Shh	Ptch1$^{+/-}$ (transient)	Unknown	
Prostate cancer	Shh	Not available	Unknown	
Lung cancer	Shh	Xenograft	Unknown	
Melanoma	Shh	Xenograft	Unknown	
Ovarian cancer	Shh	Xenograft	Unknown	Phase II (suspended)
Colon cancer	Shh	Xenograft	Unknown	Phase II (suspended)
Osteochondromas	Gli2	Tg (Gli2;ColIIAI); p53$^{+/-}$	Unknown	
Kidney cancer	Shh	Not available	Unknown	
Endometrial cancer	Shh	Not available	Unknown	
Multiple myeloma	Shh	Xenograft	Cancer stem cell	
Chronic myeloid leukemia	Shh	Not available	Unknown	

For references, please check reviews by Yang *et al*.[105], Teglund *et al*.[106], and references therein.

Activation of the Hedgehog Pathway in Human Cancer

BCC and medulloblastoma

Almost all BCCs have activated Hh signaling derived from *PTCH1* (50%), *SMO* gene mutations (10%), or other genetic alterations[107-111]. Unlike wild-type SMO, expression of SmoM2, an activated SMO mutant identified in human BCCs, in mouse skin results in the formation of BCC-like tumors[107]. *Su(Fu)* is also mutated in a few BCCs[109]. From the compiled data, the genetic alteration of the Hh pathway is detected in about 70% of BCCs. Since most BCCs have activated Hh signaling, we predict that alterations in other Hh signaling molecules or related molecules may be responsible for Hh pathway activation in 30% of sporadic BCCs. At molecular level, activated Hh signaling in BCCs leads to cell proliferation through elevated expression of PDGFRα[112], whereas targeted inhibition of the pathway causes apoptosis via the induction of Fas[113].

About one third of medulloblastomas have activated Hh signaling. Like BCCs, loss-of-function mutations of PTCH1 are often responsible for the pathway activation. Mutations in *SMO* and *Su(Fu)* are found only in a few cases. In addition, non-canonical activation of Gli2 via ATOH1 and Yap1 has been detected in medulloblastomas. Hh signaling is activated both in the desmoplastic form (more often) and the classic form of medulloblastomas.

Activation of Hh signaling in cancers not associated with Gorlin syndrome

Accumulating data supports the activation Hh signaling in many types of human cancer, including those associated or not associated with Gorlin syndrome. It is estimated that over 30% of human cancers demonstrate activated Hh signaling to a given

extent, including brain tumors, melanomas, leukemias, lymphomas, gastrointestinal, prostate, lung, and breast cancers. Unlike the situation in BCCs and meduloblastomas, which are associated with Gorlin syndrome (type I cancer), gene mutation is not primarily responsible for activated Hh signaling in those cancers not associated with Gorlin syndrome (type II cancer)[114,115]. The current understanding is that Hh signaling activation in type II cancers is caused by ligand-dependent mechanisms or non-canonical Hh signaling activation. The association of ligand-dependence (or ligand-independence) with a specific cancer type, tumor morphology or tumor stage has not yet been established.

The Role of Hh Signaling in Cancer Initiation, Progression, and Metastasis

Increasing evidence suggests that Hh signaling is involved in a specific stage of carcinogenesis in a given cancer type. In Barrett's esophagus, an early precursor of esophageal adenocarcinomas, both Shh and Ihh are highly expressed in the epithelium, which is associated with stromal expression of Hh target genes *Ptch1* and *BMP4* [116]. *Sox9*, a target gene of *BMP4*, is highly expressed in the epithelial lesion [116]. These results indicate that Hh signaling plays an important role in the initiation of esophageal adenocarcinomas. In pancreatic cancer, activation of this pathway is found in (prostatic intraepithelial neoplasia, PIN) lesions as well in metastases [117-120], indicating that Hh signaling plays a significant role in pancreatic cancer. However, transgenic mice with pancreatic-specific expression of *SHH* or *GLI2* develop undifferentiated pancreatic tumors which differ from pancreatic ductal adenocarcinomas (PDAC)[120-122], suggesting that sole activation of Hh signaling is not sufficient to drive PDAC development. In other tumors, such as gastric and prostate cancers, the activation of Hh signaling is associated with cancer progression[123-127]. Consistent with these findings, the inhibition of Hh signaling in prostate and gastric cancer cells reduces cell invasiveness[124,127,128] (our unpublished data). Reports also suggest that Hh signaling is required for development and progression of melanoma, glioma, breast cancer, ovarian cancer, leukemia, and B-cell lymphoma [129-134]. However, the role of Hh signaling in each cancer type has not been completely established. It is suggested that Hh signaling plays an important role for cancer stem cells in several cancer types, such as glioma, medulloblastoma, and possibly breast cancer (see more discussion below).

Increasing evidence indicates that Hh signaling is critical for cancer stem cell maintenance and function[135-137]. For example, leukemia stem cell maintenance and expansion is dependent on Hh signaling[135,136]. Alteration of the Hh pathway is reported to affect the hematopoietic stem cell (HSC) population in some studies, but does not change HSC in other studies[136,138-141]. Based on the cancer stem cell theory, it is anticipated that the activation of Hh signaling will exert resistance to cancer chemotherapy and radiotherapy [142]. Indeed, several studies have shown that the activation of Hh signaling is associated with resistance to chemotherapy and radiotherapy [143-145]. The Hh signaling inhibitor IPI-926 enhances the delivery of the chemotherapeutical drug gemcitabine in a mouse model of pancreatic cancer [144]. Further investigation is certainly warranted to determine the role of Hh signaling in the cancer stem cells of solid tumors.

Upon reviewing the literature on Hh signaling in human cancer, conflicting results concerning the activation of Hh signaling are often reported for the same cancer type. These discrepancies may arise due to the following reasons. First, the function of Hh signaling in human cancers may be context dependent, occurring in some tissues or cell lines but not in others. For example, accumulating data suggests that Hh signaling functions in maintaining cancer stem cell proliferation[135-137], but not the proliferation of all cancer cells. The percentage of cancer stem cells varies greatly among tumor types. Second, heterogeneity in tumor tissue often accounts for differences in the analysis of Hh target gene expression by real-time PCR. For example, prostate cancer specimens can be obtained from prostatectomy or transurethral resection of the prostate (TURP). Whereas the prostatectomy specimens contain only 5%–10% of tumor cells in the tissue, the TURP specimens generally have more than 70% of tumor cells. Thus, the data from these two types of specimens may differ due to the percentage of cancer cells in each tissue [127]. Laser microscope captured tissues will also have a significant amount of non-cancerous cells, with the percentage varying between operators. Third, a standard defining the activation of Hh signaling is required. Some investigators use the increased expression of Gli1 as the read-out[92,130], whereas others examine the expression of several Hh target genes, such as Gli1, PTCH1, sFRP1, and HIP [120,124,125,146,147], or only use immunohistochemistry to detect the activation of Hh signaling[133,148]. In all, however, most studies use multiple approaches. Thus, the literature must be accepted with caution. Particular attention should be paid to the methodology used in the studies and the reproducibility of the results. In our view, employing immunohistochemistry to detect activation of the Hh signaling pathway for one Hh target gene is unreliable.

Animal Models for Hh-Mediated Carcinogenesis

It is widely accepted that correlation of Hh target

gene expression with the tumor specimens is not sufficient to claim a role of Hh signaling in cancer. Establishing animal models using tissue-specific activation of Hh signaling is critical for understanding Hh signaling in carcinogenesis. Currently, mouse models for BCC and medulloblastoma are well established, whereas mouse models for other Hh-signaling mediated types of carcinogenesis need improvement. Table 1 summarizes the major mouse models for Hh signaling-mediated carcinogenesis[105,106].

Mouse models for BCCs

Wild-type mice never develop BCCs, even after treatment with carcinogen, UV or ionizing radiation. Ptch1$^{+/-}$ mice are susceptible to BCC development following UV irradiation or ionizing radiation[104]. The frequency of BCC development under these conditions is around 50% with 1 or 2 tumors per mouse[113,149]. Due to the embryonic lethality of Ptch1$^{-/-}$, tissue-specific knockout of Ptch1 has been generated[150]. By combining conditional gene knockout and the inducible activity of the keratin 6a promoter, Krt6a-cre:Ptch1$^{neo/neo}$ mice develop BCC following stimulation with retinoic acid[151]. In addition to the Ptch1 knockout mouse model, transgenic mice expressing Smo using Krt5 or Krt14 promoter also develop BCC-like tumors[107,152]. However, these transgenic mice eventually lose the expression of Smo by an unknown mechanism. Using conditional knock-in technology, skin-specific SmoM2^{YFP} (Krt14-creER: R26-SmoM2^{YFP} or Krt14-cre: R26-SmoM2^{YFP}) knock-in mice develop multiple microscopic BCCs at a very early age, providing an easy genetic assay for Hh signaling downstream of Smo[153]. Su(Fu)$^{+/-}$ mice develop skin lesions resembling skin hyperplasia but not BCC-like tumors[154]. Several transgenic mice have been developed using downstream transcriptional factors Gli1, Gli2, and Shh[155-157]. The inducible expression of Gli2 in the skin results in BCCs after a few weeks. These mouse models provide a rich resource for furthering our understanding of Hh signaling-mediated BCC development.

Mouse models for medulloblastomas

A small portion of Ptch1$^{+/-}$ mice (10%–30%) develop medulloblastomas and rhabdomyosarcomas[83,84]. The synergy between the p53 pathway and Hh signaling is clearly shown in the medulloblastoma model. Whereas p53 null mice do not develop this type of tumor, all Ptch1$^{+/-}$p53$^{-/-}$ mice develop medulloblastomas[158]. On the other hand, Ptch2$^{+/-}$ mice do not develop medulloblastoma per se, but Ptch1$^{+/-}$ mice have an increased incidence of medulloblastoma[159,160]. Su(Fu)$^{+/-}$ mice develop skin phenotypes similar to Gorlin syndrome but are generally not tumor prone[154]. However, Su(Fu)$^{+/-}$ mice with a p53 null background frequently develop medulloblastomas characterized by Hh signaling alterations[161]. Although Ptch1$^{+/-}$:Su(Fu)$^{+/-}$ mice are more likely to develop medulloblastoma than Ptch1$^{+/-}$ mice, the difference is not statistically significant[162]. In addition to the loss of tumor suppressor genes, transgenic mice expressing SmoM2 mutant under the control of neuroD2 promoter results in medulloblastoma [163]. Tissue-specific activation of Hh signaling via Ptch1 knockout or SmoM2 expression using granule neuron precursor lineage specific promoters (Math1, GFAP, Oligo-2, TLx3), but not the purkinje neuron specific promoter, leads to the formation of medulloblastoma[164,165], indicating that granule neuron precursors are the source for the development of medulloblastoma. Further analysis shows that CD15 is the medulloblastoma stem cell marker[137,166].

Mouse models for Hh signaling-mediated carcinogenesis in other organ sites

Postnatal induction of an activated allele of Smoothened (*R26-SmoM2*) using a ubiquitously expressed, inducible Cre transgene (*CAGGS-CreER*) has been used to explore the role of Hh signaling-mediated carcinogenesis in mice [153]. In this model, all mice developed rhabdomyosarcoma and basal cell carcinoma, with 40% also developed medulloblastoma. In addition, pancreatic lesions resembling low-grade mucinous cystic neoplasms in humans and diverticular harmartomatous lesions in both the intestine and stomach are observed. However, no other tumor types are observed in this mouse model, suggesting that activation of Hh signaling is not sufficient to initiate tumor development in the prostate, lung, breast and gastrointestinal tract.

Similar data are observed in other studies. For example, it is shown in orthotopic mouse models that Hh signaling is necessary for pancreatic cancer metastasis[167] (also our unpublished data). Pancreatic tissue-specific deletion of *Smo*, on the other hand, did not affect the formation of pancreatic ductal adenocarcinomas (PDAC), whereas *GLI2* expression (CLEG2:Pdx1-cre mice) or Shh expression only lead to formation of undifferentiated pancreatic tumors [120-122,168]. These results indicate that activation of Hh signaling alone is not sufficient to drive PDAC formation, but is essential for tumor progression and metastasis. In consistent with this theory, PDAC development in Kras$^{+/G12D}$:Pdx1-cre mice is not affected by the removal of the *Smo* gene, and Pdx-1-driven expression of SmoM2 does not result in PIN lesions despite that paracrine Hh signaling is observed in the pancreatic tissue[122,168].

A recent study indicates that Shh expression in the epithelium of Barrett's esophagus can lead to stromal expression of Hh signaling target genes[116]. Using a Shh

transgenic mouse model, it is shown that epithelial expression of Shh can lead to stromal expression of the Hh target gene BMP4, its target gene Sox9 in the epithelium and a columnar phenotype of mouse esophageal epithelium, resembling a feature in human Barrett's esophagus. These data suggest that the activation of Hh signaling can drive the formation of some features resembling Barrett's esophagus in mice.

For investigating the role of Hh signaling in other cancer types, the major models are based on xenografts in immunodeficient mice (nude or SCID mice)[106]. With potential implications of Hh signaling inhibitors for clinical cancer treatment, more established mouse models will be needed. Because modeling cancer metastasis is a challenge, we anticipate an increase in the use of orthotopic mouse models for studying Hh signaling in cancer progression and metastasis in the foreseeable future.

Small Molecule Modulators of Hedgehog Signaling

More than 50 compounds have been identified to have inhibitory effects on Hh signaling. Of these, 4 are being used in clinical trials. There are 3 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, Cyc-T, and synthetic compounds GDC-0449, Cur61414, XL-139, and LDE-225); and Gli inhibitors (HPI-1, HPI-2, GANT-56, and GANT-61)[105]. We can divide Hh signaling inhibitors into three groups: natural products (cyclopamine and its derivatives, and other natural products), synthetic small molecules, and Hh signaling modulators. Table 2 lists major current Hh signaling inhibitors[46,113,125,169-186].

Natural products (cyclopamine, its derivatives, and others)

Cyclopamine, a plant-derived steroidal alkaloid, inhibits Hh signaling through direct binding to the transmembrane helices of SMO[171]. Identification of specific, small molecule antagonists of SMO has revealed exciting new prospects for the targeted therapy of human cancers associated with Hh signaling.

The specificity of cyclopamine varies depending on the concentration used. While cyclopamine at a low concentration (< 10 μmol/L) has specific inhibitory effects on Hh signaling, high doses of cyclopamine can result in cell death without affecting Hh target gene expression[187]. In several mouse models, the *in vivo* effect of cyclopamine on tumor shrinkage has been demonstrated. Oral delivery of cyclopamine blocks the growth of UV-induced BCCs in *Ptch1*$^{+/-}$ mice by 50%[113]. The treatment in this model also prevents development of additional microscopic BCCs, implying a cancer prevention potential of cyclopamine. Similarly, cyclopamine is shown to be effective in reducing the development of medulloblastoma in *Ptch1*$^{+/-}$ mice[169] and tumor growth of cancer cell lines in nu/nu mice[92,120,124,170]. Additional modifications on cyclopamine aiming at increasing acid stability and aqueous solubility are now available, such as IPI-926 and Cyc-T[176,188]. IPI-926 is now at use in a Phase I clinical trial.

Synthetic Hh signaling antagonists

Increasing synthetic Hh antagonists are being reported in the literature, with most compounds targeting SMO. Four of these compounds are now in clinical trials (Table 2)[182,183], including GDC-0449. The successful clinical trials with GDC-0449 against human BCCs further encourage the translational investigation in this area[183]. The clinical trial of the same compound in a medulloblastoma patient led to rapid tumor shrinkage, but was complicated by drug resistance due to a SMO mutation, disabling the binding of GDC-0449 to SMO. This case report implies a need for novel alternative strategies for treatment of cancers associated with Hh signaling. There are also several small molecules targeting at Shh or Gli[185,189,190]. Due to the wide spread existence of non-conical regulation of Gli transcription factors and the potential resistance to SMO inhibitors, antagonists targeting downstream effectors of the Hh pathway constitute a valuable resource for developing chemotherapeutic strategies against Hh pathway-related cancers.

Hh signaling modulators

Recent studies indicate that vitamin D3, whose secretion can be facilitated by PTCH1, can inhibit SMO signaling through direct binding to SMO. This finding raises a possibility to treat BCCs with nutritional supplements[46]. Promising data show that the effect of tazarotene, a retinoid with retinoic acid receptor (RAR) beta/gamma specificity against BCC carcinogenesis is sustained after its withdrawal[191]. The common cooking ingredient curcumin has also been shown to block Hh signaling-mediated carcinogenesis. Several natural products, including genistein, EGCG and resveratrol, are also shown to affect Hh signaling in a mouse model of prostate cancer[192]. A commonly used antifungal agent itraconazole, is shown to affect Hh signaling[186]. The detailed molecular mechanisms of action for these signaling modulators remain elusive.

Summary

The linkage of Hh signaling activation to a variety of

Table 2. Hedgehog signaling inhibitors

Inhibitor	Other name	50% inhibition concentration (IC_{50})	*In vitro*/*in vivo* studies	References
Cyclopamine		300 nmol/L	In vivo & in vitro	[111,171,172]
KAAD-cyclopamine		20 nmol/L	In vitro cultured cells	[123,169,182]
Jervine		500 nmol/L	In vitro and cultured embryos	[183–185]
Cyc-T	Cyclopamine tartrate salt	20 nmol/L	In vitro & in vivo studies	[174]
Cur-61414		200 nmol/L	Phase I clinical trial	[186]
Sant-1,2,3,4		20–200 nmol/L	In vitro studies	[187]
Compound 5		<100 nmol/L	In vitro studies	[188]
Compound Z		<1 nmol/L	In vitro studies	[189]
2-amino-thiazole		30 nmol/L	In vitro studies	[189]
Gant-58,61		5 μmol/L	In vitro & in vivo studies	[190]
IPI-926		<20 nmol/L	Phase I clinical trial	[191]
GDC-0449	Vismodegib	<20 nmol/L	Phase I/II/III clinical trials	[175]
BMS-833923 (XL139)	XL139	<20 nmol/L	Phase I clinical trial	NCI clinical trial database
LDE-225		<20 nmol/L	Phase I clinical trial	NCI clinical trial database
Vitamin D3		100 μmol/L	In vitro	[46]
Robotnikinin		>10 μmol/L	In vitro	[192]
HPI-1, 2,3,4		<10 μmol/L	In vitro	[176]
Itraconazole		<1.5 μmol/L	In vitro and xenograft	[181]

human cancers and the discovery of novel Hh signaling inhibitors provides opportunities for developing novel cancer therapeutic strategies. However, several major challenges must be overcome before Hh signaling inhibitors are thrust into clinical application. These include a lack understanding of the molecular mechanisms for Hh signaling-mediated carcinogenesis, identifying correct tumor types for therapeutic application, the need for reliable and reproducible mouse models for testing and optimizing drug dosages to minimize side effects, and novel stra tegies to mitigate drug resistance.

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