Implications of hedgehog signaling antagonists for cancer therapy

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The hedgehog (Hh) pathway, initially discovered in Drosophila by two Nobel laureates, Dr. Eric Wieschaus and Dr. Christiane Nusslein-Volhard, is a major regulator for cell differentiation, tissue polarity and cell proliferation. Studies from many laboratories, including ours, reveal activation of this pathway in most basal cell carcinomas and in approximately 30% of extracutaneous human cancers, including medulloblastomas, gastrointestinal, lung, breast and prostate cancers. Thus, it is believed that targeted inhibition of Hh signaling may be effective in treating and preventing many types of human cancers. Even more exciting is the discovery and synthesis of specific signaling antagonists for the Hh pathway, which have significant clinical implications in novel cancer therapeutics. This review discusses the major advances in the current understanding of Hh signaling activation in different types of human cancers, the molecular basis of Hh signaling activation, the major antagonists for Hh signaling inhibition and their potential clinical application in human cancer therapy.

Keywords hedgehog; smoothened; PTCH1; human cancer therapy; basal cell carcinoma; antagonist

The hedgehog (Hh) gene was identified by two Nobel laureates through genetic analysis of segmentation of fruit fly Drosophila [1]. In the early 1990s, three homologs of the Hh gene were identified in vertebrates [2–6]. As an essential developmental signaling pathway, the Hh pathway is critical for maintaining tissue polarity and stem cell population. Inactivation of this pathway causes developmental defects such as holoprosencephaly [7]. Hyperactivation of this pathway is found in most basal cell carcinomas (BCCs) and many extracutaneous cancers [8–10]. The emerging role of Hh signaling in human cancer further emphasizes the importance of studying this pathway.

Current Understanding of Hh Signaling Mechanisms

Overall, the general signaling mechanisms of the Hh pathway is conserved from fly to human [11]. The seven transmembrane domain containing the protein smoothened (SMO) serves as the key player for signal transduction of this pathway. However, the pathway's function is inhibited by another transmembrane protein, patched (PTC), in the absence of Hh ligands. In the presence of active Hh ligands, binding of Hh to its receptor PTC releases this inhibition, allowing SMO to signal downstream to Gli transcription factors. As transcription factors, Gli molecules can regulate target gene expression by directly associating with a specific consensus sequence located in the promoter region of the target genes [12,13]. Fig. 1 shows the simplified diagram of Hh signaling in the presence or absence of Hh.

Hh proteins [one Hh in Drosophila and three Hhs in vertebrates: sonic hedgehog (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh)] are secreted molecules, functioning both on nearby and distant cells in developing tissues. Following translation, Hh proteins enter the secretory pathway and undergo autoprocessing and lipid modification reactions that produce a signaling peptide modified at both ends by palmitoyl (N-terminus) and cholesteryl (C-terminus) adducts [14–16]. The movement of Hh proteins is regulated by several molecules: Dispatched (Disp), the transmembrane transporter-like protein for release of Hh from secreting cells [11–14]; Dally-like (Dlp) and Dally, heparan sulfate proteoglycans for extracellular transport of Hh protein [15]; and enzymes, such as sulfateless and tout-velu, for heparan sulfate biosynthesis [17–19].
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PTC [one PTC in flies and two PTCs in vertebrates: patched homolog 1 (PTCH1) and patched homolog 2 (PTCH2)] is the major receptor for Hh proteins [20]. Several molecules are involved in regulating Hh reception. Hh-interacting protein (HIP) can compete with PTC in binding Hh, thus preventing Hh signaling [21]. Recent studies indicate that two additional molecules, Cdo and Gas1, are also required for Hh binding [22–28]. It is still not entirely clear how binding of Hh proteins results in pathway activation. One hypothesis is that, in the absence of Hh, PTC normally inhibits the function of SMO. Binding of Hh proteins to the receptor PTC releases PTC-mediated inhibition on SMO, thus SMO can signal to downstream molecules.

Very little is known about signaling events immediately downstream of SMO. In Drosophila, several laboratories have shown that SMO accumulation is promoted through protein phosphorylation at the C-terminus by protein kinase A (PKA) and casein kinase I [29,30]. 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 [30].

Accumulated evidence from several groups indicates that the primary cilia found on most vertebrate cells play an important but undefined role in the Hh pathway [31–35]. Functions of the primary cilium are regulated by large protein complexes involved in intraflagellar transport (IFT), which functions in retrograde and anterograde movement of cargo within the primary cilia [36]. A number of mutations encoding IFT proteins involved in the primary cilium anterograde IFT have been described, resulting in mice with Hh loss of function phenotypes [32]. Several Hh components, including SMO and Gli molecules, are also present at the primary cilium upon Hh stimulation [37]. A SMO mutant lacking ciliary translocation blocks Hh signaling [31]. Gli3 processing is significantly affected by IFT mutants [33,34], suggesting that SMO activates downstream molecules at the cilium. However, it is not clear how SMO is transported to the cilium in response to Hh signaling and how SMO activates downstream effectors. Evidence suggests that SMO is endocytosed and can be degraded in the lysosomes [38]. In cultured mammalian cells, both SMO and PTCH1 are internalized and localized to endosomes, and Hh induces segregation of SMO-containing vehicles from Hh-PTCH1 complexes destined for lysosomal degradation [38]. It is not clear how SMO endocytosis is regulated.

Based on studies of Drosophila, there are several molecules, including COS2 and Fused, genetically downstream of SMO signaling, but the functions of their vertebrate homologs in Hh signaling remains to be established. Inactivation of vertebrate homologs of COS2, KIF27 and KIF7, do not affect Hh signaling in cultured mammalian cells [39], which suggests that KIF7 and KIF27 may not be required for Hh signaling. Because the homology between COS2 and KIFs is very low, it is possible that a few molecules replace the function of COS2 in vertebrates. Alternatively, SMO signaling in vertebrates may utilize a distinct mechanism. Additional evidence from knockout mice with each of these KIF genes should provide insight into the in vivo roles of these COS2 homologs. Another surprise is that knockout of the vertebrate homolog of Fused can survive for up to two weeks but die of hydrocephalus [40,41]. No change of Hh signaling is seen in these knockout mice, suggesting that Fused is not critical for Hh signaling in early embryonic development. Based on these studies, however, one can not ignore the possibility that Fused is only partially involved in Hh signaling.

Several novel cytoplasmic regulators of Hh signaling, including Rab23 and tectonic [42,43], have been identified as being unique to mammalian cells. Both Rab23 and tectonic are negative regulators of Hh signaling downstream of SMO, but the exact interacting partners are not clear. Unlike many Rab proteins, Rab23 expresses both in the...
nucleus and cytoplasm (our unpublished observation), suggesting that Rab23 may have other functions besides membrane trafficking.

The negative regulatory functions of suppressor of Fused [Su(Fu)] in vertebrates, in contrast, are enhanced in mammals. Su(Fu) in Drosophila was originally identified genetically by its ability to suppress active Fused mutations, but it is not itself required for pathway activity. Several recent studies suggest that Su(Fu) plays a key negative regulatory role in Hh signaling. Su(Fu) null mouse mutants not only fail to repress the pathway [44], but have similar phenotypes as inactivation of the other key negative regulator acting upstream, PTCH1. Moreover, Su(Fu) null MEFS and wild-type cells treated with Su(Fu) short interfering RNAs display Hh pathway activation, supporting a central role in pathway repression [44]. The skin phenotype of Su(Fu)+/− mice is as severe as the PTCH1+−/− mice, the latter is a classic model for tumor suppressor function in the Hh pathway. At the molecular level, Su(Fu) is shown to associate directly with and to inhibit Gli molecules, though the details are unclear [45].

Ultimately, Hh signaling is transduced to downstream Gli transcription factors, which can regulate target gene expression by direct association with a consensus binding site (5′-TGGGTTGTC-3′) located in the promoter region of the target genes [12,13,46,47]. There are several ways to regulate the activity of Gli transcription factors. First, nuclear-cytoplasmic shuttling of Gli molecules is tightly regulated [45,48–50]. For example, PKA is shown to retain Gli1 proteins in the cytoplasm (through a PKA site in the nuclear localization signal peptide) [48], whereas active Ras signaling promotes Gli nuclear localization [50]. Second, ubiquitination and protein degradation of Gli molecules is also regulated by several distinct mechanisms, including TrCP, Cul3/BTB and Numb/Itch [51–55]. In addition to protein degradation, Gli3 and Gli2, to a lesser extent, can be processed into transcriptional repressors, which may be mediated by the TrCP E3 ligase [53]. Defects in the retrograde motor for IFT are also shown to affect Gli3 processing [56]. Fourth, transcriptional activity of Gli molecules is also tightly regulated. It is reported that EGF can synergize with Gli transcription factors to regulate target gene expression [57]. Su(Fu) not only prevents nuclear translocation of Gli molecules, but it also inhibits Gli1-mediated transcriptional activity [58]. Table 1 summarizes the major components of the Hh pathway in vertebrates.

There are several feedback regulatory loops in this pathway. PTC, Hh-interacting protein (HIP), Gas1 and Gli1, which are components of this pathway, are also the target genes. PTC and HIP provide negative feedback mechanisms to maintain the pathway activity at an appropriate level in a given cell. In contrast, Gli1 forms a positive regulatory loop. Gas1 is down-regulated by the Hh pathway, but it is positively involved in Hh signaling. Alteration of these loops, such as loss of PTCH1 in BCCs, likely results in abnormal signaling of this pathway.

**Activation of the Hh Pathway in Human Cancers**

The major breakthrough in our understanding of Hh signaling in human cancers came from the discovery that mutations of the human homolog of the Drosophila patched gene (PTCH1) are associated with a rare hereditary form of BCC: basal cell nevus syndrome (also called Gorlin syndrome) [59–61]. PTCH1 is the receptor for Hh proteins, and previous studies have indicated that PTCH1 mainly functions in embryonic development.

**Mutations of PTCH1 in basal cell nevus syndrome**

Loss-of-function mutations of PTCH1 are the cause of basal cell nevus syndrome, the clinical features of which were originally identified by Dr. Robert Gorlin. This autosomal dominant disorder is distinguished by the development of benign and malignant tumors, including multiple BCCs, medulloblastomas and ovarian fibromas, and less frequently fibrosarcomas, meningiomas, rhabdomyosarcomas and cardiac fibromas. The disorder is also characterized by developmental defects such as pits of the palms and soles, keratocysts of the jaw and other dental malformations, cleft palate, calcification of the falx cerebri, spina bifida occulta and other spine anomalies, and bifid ribs and other rib anomalies [62–64].

Analysis of the distribution of BCCs in affected individuals in multiple families suggests that the underlying defect might be a mutation in a tumor suppressor gene. This gene was later mapped to chromosome 9q22-31, which is also frequently deleted in sporadic BCCs [65]. Positional cloning and candidate gene approaches identified the human homolog of Drosophila patched as a candidate gene for therapeutic strategies [59,60,66]. Making PTCH1 a good candidate gene for basal cell nevus syndrome, vertebrate patched was known to function in the development of organs, such as neural tube, somites and limb buds [67], with abnormalities. Screening of the patched coding region in basal cell nevus syndrome patients revealed a wide spectrum of mutations, the majority of which were predicted to result in premature protein truncation. PATCH mutations are mainly clustered into two large extracellular loops and a large intracellular loop [68]. Kindreds with
identical mutations differ dramatically in the extent of their clinical features, suggesting that genetic background or environmental factors may have an important role in modifying the spectrum of both developmental and neoplastic traits [69].

The tumor suppressor role of PTCH1 has been further demonstrated in mice. Mice heterozygous for a PTCH1 null mutation exhibit the same essential features, such as tumor development (eg medulloblastomas, rhabdomyosarcomas and BCCs) and developmental defects (eg pina bifida occulta), as basal cell nevus syndrome patients [70, 72]. The mouse studies confirm that PTCH1 functions as a tumor suppressor.

**Activation of the Hh pathway in sporadic BCCs**

BCC, the most common human cancer, consistently has abnormalities of the Hh pathway and often loses PTCH1 function due to point mutations and the loss of the remaining allele. Most PTCH1 mutations lead to loss of the protein function. Mice heterozygous for a PTCH1 null mutation develop BCCs following UV irradiation or ion radiation. Currently, PTCH1−/− mice represent the most practical model for UV-mediated BCC formation [72].

The PTCH1 gene region is lost in more than 50% of human sporadic BCCs, whereas the Hh pathway is activated in almost all BCCs, suggesting alteration of additional genes in the Hh pathway in this type of skin cancer. Indeed, mutations of SMO are found in about 10% of sporadic BCCs [73−77]. Unlike wild-type SMO, expression of activated SMO molecules in mouse skin results in formation of BCC-like tumors [73]. These findings provide additional insight into the role of the Hh pathway in human cancer. It has also been reported that Su(Fu) is mutated in some BCCs [74−76]. LOH are not detected in the Su(Fu) gene region, unlike in the PTCH1 region, in sporadic BCCs, suggesting that Su(Fu) loss is not a major somatic change.

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<th>Function</th>
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<th>Knockout mouse</th>
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<td>Desert hedgehog</td>
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<td><strong>Hh regulator</strong></td>
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<td>Patched homolog (PTCH) 1</td>
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<td>PTCH2</td>
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<td><strong>Co-receptors</strong></td>
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<td>Gli1</td>
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<td><strong>E3 ligase for Gli</strong></td>
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<td>β-TrCP1</td>
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<td>Cul3/βTβ</td>
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<td>Numb/Itch</td>
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Taking all the mutation data into account, the underlying molecular basis for the activated Hh signaling still remains unknown in approximately 30% of BCCs. Thus, we predict that mutations of additional genes in the Hh pathway are yet to be discovered in sporadic BCCs.

We have shown that activated Hh signaling in BCCs leads to cell proliferation through elevated expression of PDGFR [78], whereas targeted inhibition of Hh signaling causes apoptosis via Fas induction [79].

**Activation of Hh signaling in extracutaneous tumors**

Recent studies indicate that Hh signaling is activated in many types of extracutaneous tumors, including brain, gastrointestinal, prostate, lung and breast cancers. Unlike with BCCs, overexpression of Hh ligands is believed to be responsible for activating Hh signaling in some of these tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81]. In pancreatic, esophageal and liver cancers, activation of this pathway is found in both early tumors [80,81].

Activation of Hh signaling in extracutaneous tumors is associated with cancer progression [82,87−89], suggesting that Hh signaling is required for the development and progression of melanoma, gliomas and B-cell lymphomas [91,92].

Different, and sometimes contradictory results have been reported regarding Hh signaling activation in different tumor types. There are several reasons for this. First, it is possible that the involvement of Hh signaling in human cancers may be context dependent, occurring in some tissues or cell lines but not in others. Evidence suggests that Hh signaling may be involved in maintaining cancer stem cell proliferation [93,94]. Second, tumor heterogeneity is a major factor in the analysis of Hh target gene expression by real-time polymerase chain reaction. For example, we identified activation of the Hh pathway in prostate cancer more frequently from transurethral resection of the prostate specimens than from prostatectomy specimens [88]. Third, different standards have been used to define Hh signaling activation. Some studies have used elevated expression of Gli1 as a read-out of Hh signaling activation [50], whereas others have assessed expression of several Hh target genes, such as Gli1, PTCH1, sFRP1 and HIP [82,83,85,90,95]. Similarly, though most studies have used multiple approaches, some have only involved immunohistochemistry to detect Hh signaling activation [96]. Therefore, it is imperative to establish a unified standard for detecting Hh signaling activation in human cancer. As the research in this area progresses, we will gain a clearer picture about Hh signaling activation in human cancers. Table 2 provides a summary of current data on Hh signaling activation in human cancers.

**Small Molecule Modulators of Hh Signaling**

**Cyclopamine**

Cyclopamine, a plant-derived steroidal alkaloid, binds directly to the transmembrane helices of SMO and inhibits Hh signaling [97]. The discovery of small molecule antagonists of SMO such as cyclopamine has opened up exciting new prospects for molecularly targeted therapy for and prevention of human cancers associated with Hh signaling.

Oral cyclopamine can block the growth of UV-induced BCCs in PTCH1+/− mice by 50%, perhaps by increasing Fas-induced apoptosis [79]. Furthermore, cyclopamine treatment in this mouse model prevents the formation of additional microscopic BCCs, implying a potential use of cyclopamine in BCC prevention. Cyclopamine administration reduced BCCs, but not SCCs or fibrosarcomas, in these mice, highlighting the specificity of cyclopamine for the Hh pathway [79]. Using murine BCC cell lines derived from this mouse model, cyclopamine is shown to inhibit cell proliferation, possibly through down-regulation of growth factor receptor PDGFR. Similarly, cyclopamine is effective in reducing medulloblastoma development in PTCH1+/− mice as well as tumor growth of many cancer cell lines in nu/nu mice [50,85,90,98,99].

**Synthetic SMO antagonists**

Other synthetic SMO antagonists, such as CUR61414 from Curis/Genentech, have also been found to be effective in reducing BCCs in PTCH1−/− mice. Using an ex vivo model of BCC, CUR61414 caused the regression of UV-induced basaltic lesions in punch biopsies taken from PTCH1−/− mice [100]. Since that study, a topical formulation of this compound has been tested against sporadic BCCs in a phase I clinical trial. However, for unknown reasons, the compound did not appear to affect Hh target gene expression in this clinical trial. Additionally, several other synthetic compounds differing structurally from cyclopamine have been identified for their ability to bind directly to SMO [101,102].
**Other Hh signaling modulators**

A few small molecule inhibitors for Gli1 functions are identified through chemical library screening. Two such inhibitors act in the nucleus to block Gli function, and one of them interferes with Gli1 DNA binding in living cells [103]. Importantly, the discovered compounds efficiently inhibited in vitro tumor cell proliferation in a Gli-dependent manner and successfully blocked cell growth in an in vivo xenograft model using human prostate cancer cells harboring downstream activation of the Hh pathway [103]. The growth of these tumors can not be inhibited by cyclopamine or its analogs, raising the possibility that these Hh antagonists may have broad uses in cancer therapeutics. Clinical application of these compounds, however, awaits additional preclinical studies in defined tumor models.

Recent studies indicate that vitamin D3, the secretion of which can be facilitated by PTCH1, can inhibit SMO signaling through direct binding to SMO. This finding raises the possibility that BCCs may be treated with nutritional supplements [104].

Since abnormal expression of Shh is very common in several human cancer types, neutralizing antibodies for Shh have demonstrated effectiveness in reducing cell proliferation in cancer cells with activated Hh signaling [83]. Future clinical application of Shh neutralizing antibodies will require additional preclinical studies.

In addition, several synthetic SMO agonists are available for functional studies of Hh signaling in human cancer [101]. With appropriate optimization, it is possible that these Hh agonists may be used to treat human conditions with reduced Hh signaling, such as holoprosencephaly. Table 3 shows currently known small molecule inhibitors of Hh signaling.

**Summary**

In summary, rapid advances in our understanding of Hh signaling have provided great opportunities for developing novel therapeutic strategies for human conditions with altered Hh signaling, particularly cancer. Optimized use of Hh signaling antagonists will make these therapies feasible. The challenges for therapeutic application of Hh signaling inhibitors include identification of the right tumors for therapeutic application; reliable and reproducible animal models for testing these compounds; and optimization of drug dosages to minimize the side effects.
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