Jingwu Xie *Editor*

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Chapter 1
Overview of Hedgehog Signaling Pathway

Chi-chung Hui and Jin Jiang

Introduction

Initially discovered in *Drosophila* and later found in all vertebrate model organisms, the Hedgehog (Hh) family of secreted proteins plays critical roles in both embryonic development and adult tissue homeostasis [41, 84]. Numerous human genetic disorders and cancer have been associated with aberrant Hh signaling activity [41, 63, 84].

Hh acts through a conserved pathway to influence the balance between activator and repressor forms of the Gli family of zinc finger transcription factors (Gli^A and Gli^R; Fig. 1.1). While *Drosophila* has only one Hh and one Gli protein, Cubitus interruptus (Ci), mammals have three Hh family members (Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog) and three Gli proteins (Gli1, Gli2 and Gli3). In mice, Gli^R function is mainly derived from Gli3, whereas Gli^A function is primarily contributed by Gli2. Gli1 is a transcriptional target of Hh signaling and acts in a positive feedback to reinforce Gli^A activity. The reception of Hh signals is mediated by a 12-span transmembrane protein Patched (Ptc) that binds directly to Hh, and a 7-span transmembrane protein Smoothened (Smo) that transduces the signal into the cytoplasm. Ptc blocks Smo activity in the absence of Hh, allowing the production of Gli^R/Ci^R that represses a subset of Hh target genes. Binding of Hh to Ptc activates Smo, which blocks Gli^R/Ci^R production and promotes Gli^A/Ci^A activation. The fundamentals of *Drosophila* and mammalian Hh

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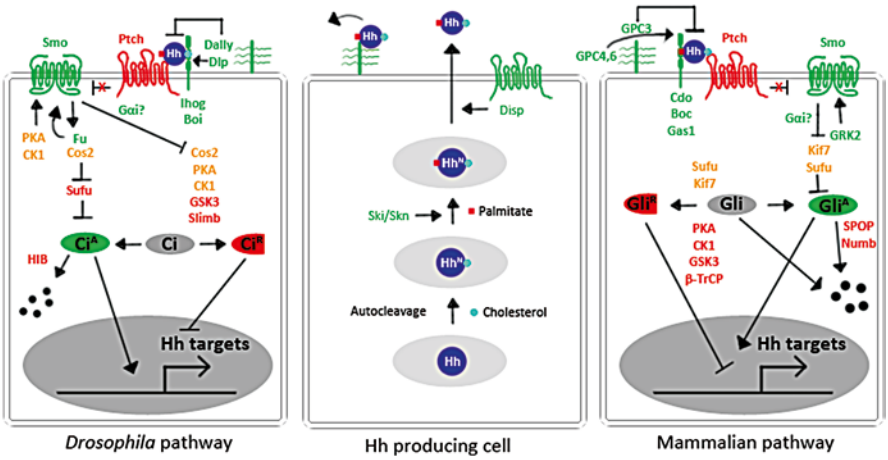

Fig. 1.1 Sending and transducing the Hh signal. In Hh-producing cells, full-length Hh is autocatalytically cleaved to generate an N-terminal fragment (HhN) modified by cholesterol. HhN is palmitoylated by Ski/Skn. Secretion of dual lipid-modified Hh is mediated by Disp. HSPGs facilitate Hh movement. Hh signal reception is facilitated by Ihog/Boi in *Drosophila* and Cdo/Boc/Gas1 in mammals, functioning as essential coreceptors. Dally and its mammalian HSPG counterpart GPC3 inhibit Hh pathway activity, whereas Dlp and related molecules GPC4 and GPC6 promote Hh signaling. In the absence of Hh, Ptc blocks Smo and full-length Ci/Gli2/Gli3 is phosphorylated by multiple kinases and subsequently targeted to ubiquitin/proteasome-mediated proteolysis through Slimb/βTRCP to generate a truncated repressor form (Ci^R/Gli^R). In *Drosophila*, efficient phosphorylation of Ci requires the kinesin-like protein Cos2, which acts as a molecular scaffold to bridge Ci and its kinases. Hh-binding to Ptc blocks its inhibition on Smo. In *Drosophila*, Ptc inhibition triggers Smo phosphorylation by PKA and CKI, leading to the cell surface accumulation and activation of Smo. Smo then recruits Cos2-Fu to activate Fu and dissociates Cos2-Ci-kinase complexes to inhibit Ci phosphorylation and processing. Furthermore, high levels of Hh stimulate Ci^A via Fu-mediated antagonism of Sufu. Hh signaling induces the expression of nuclear HIB that targets Ci^A for degradation. Fu-Cos2 is also involved in a feedback regulation of Smo phosphorylation. In mammalian systems, Kif7 is the mammalian Cos2 homolog but it does not interact directly with mSmo. mSmo phosphorylation requires GRK2. In mammals, Fu homolog is not required for Hh signaling and Sufu is a key negative regulator of Hh signaling. Kif7 and Sufu seem to play dual roles in positive and negative regulation of the Hh pathway. In addition to SPOP, which targets full length Gli2 and Gli3 for degradation, Numb is involved in Gli1 degradation. This figure is adapted from [41]

signal transduction pathways are similar, though major difference can be found in several regulatory steps. There is accumulating evidence suggesting that Hh signaling can also exert Gli-independent non-transcriptional effects [94]. In this chapter, we review the basics of the Hh signaling pathway and highlight some of the recent findings in the field.

Hh Signal Transduction

Lipid Modification and Multimerization of Hh

In Hh-producing cells, full-length Hh precursor undergoes autocleavage to release an N-terminal fragment (HhN) with a cholesterol moiety covalently linked to its C-terminus (Fig. 1.1) [70]. HhN is then palmitoylated near its N-terminus by the acyltransferase Skinny Hedgehog (Ski/Skn) [9]. While cholesterol modification increases the affinity of Hh for cell membranes and restricts its free dispersal [7, 49], dual lipid modifications facilitate the formation of large multimeric Hh complexes, allowing Hh to move over a long distance ([98] and references therein). HhN forms nanoscale oligomers with heparan sulfate proteoglycans (HSPGs), and disruption of HhN oligomerization and HSPGs interaction compromises specifically long-range signaling [86]. Dispatched (Disp), a transmembrane protein structurally related to Ptc, is required for the secretion of lipidated Hh to the extracellular space [2, 7, 55]. A recent study suggested that Disp might also act with Ptc1 to mediate the transport of Shh through tissues [29].

Heparan Sulfate Proteoglycans Regulate Hh Signaling

Genetic studies in *Drosophila* have shown that members of the glypican subfamily of HSPGs, Dally and Dally-like (Dlp), modulate the transport and reception of Hh signals [95]. Mutations in these genes as well as those affecting the biosynthesis of HSPGs impede the spread of Hh signals and reduce Hh pathway activity [50]. HSPGs seem to affect Hh signaling in many different ways (Fig. 1.1). In the absence of HSPGs, cell surface Hh diminishes, suggesting that HSPGs contribute to the stability of Hh. HSPGs appear to be required for Hh movement as a narrow strip of HSPG-deficient cells is sufficient to completely block Hh signaling in wild-type cells behind the mutant clone. In addition, Dlp is critical for Hh signaling activity and may act as an essential coreceptor [96]. A recent study suggested that there are two functional families of glypicans in *Drosophila* and mammals [92]: Dlp and its mammalian counterparts, including GPC4 and GPC6, constitute a group that acts positively and cell-autonomously for Hh signaling, whereas Dally and other glypicans, such as GPC3, form another group that inhibits Hh response. Consistent with this, GPC3 competes with Ptc for Hh binding in vitro and inhibits Hh signaling during mouse development [8]. It is important to note that HSPGs also regu late other signaling molecules, including Wg/Wnt and Dpp/TGF-β [95], and thus these extracellular matrix proteins likely exert differential effects on multiple signaling pathways during development and tumorigenesis.

Modulation of Pathway Activity by Multiple Hh-Binding Proteins

In addition to Ptc, there are multiple Hh-binding proteins identified in *Drosophila* and mammals. Some of them might act as coreceptors of Hh (Fig. 1.1). Genetic analysis in *Drosophila* revealed that the Ihog family of immunoglobin/fibronectin repeat-containing proteins, Ihog (Interference hedgehog) and Boi (Brother of Ihog), are essential for Hh pathway activity [96, 105]. Mammalian homologs of Ihog/Boi, Cdo and Boc, are also positively involved in Shh signaling [81, 97, 102]. The Ihog/Cdo family proteins bind Hh through fibronectin domains [81, 97], and Ihog can enhance Hh binding to Ptc [97], suggesting that they act as Hh coreceptors. Indeed, Ihog promotes surface presentation of Ptc, and both Ihog and Ptc are required for high-affinity Hh binding, supporting the notion that Ihog and Ptc constitute the Hh receptor in *Drosophila* [105].

Hip1 and Gas1 are two vertebrate-specific Hh-interacting proteins. *Hip1* encodes a membrane-bound glycoprotein that acts as a negative regulator of Hh signaling by competing with Ptc for Hh binding [18]. *Hip1* expression is induced by Hh signaling and restricts Hh signaling through a negative feedback mechanism [17, 36]. On the contrary, *Gas1* encodes a GPI-anchored membrane protein that promotes Shh signaling [1, 58]. Since Gas1 acts cooperatively with Cdo in the positive regulation of Hh response [1], it might function as a coreceptor of Hh.

Ptc Inhibits Smo Catalytically

Being the core Hh-binding receptor, Ptc paradoxically functions as an inhibitor of Hh signaling and blocks pathway activation in the absence of Hh. The precise mechanism by which Ptc regulates Smo remains a mystery. Recent studies suggest that Ptc and Hh reciprocally regulate Smo subcellular localization and conformation. Ptc and Smo are largely segregated in *Drosophila* imaginal discs [22] and they do not form stable protein complexes [43, 79]. Cultured cell experiments suggested that Ptc inhibits Smo at a substochiometrical concentration [79]. Ptc is homologous to the resistance-nodulation-division (RND) family of prokaryotic proton-driven transporter, and might function by transporting an endogenous small molecule Smo agonist or antagonist across membranes, as conserved residues in RND-like transporters are essential for Ptc function [79]. Indeed, Ptc regulates trafficking of lipoproteins through endosomes [44]. Several natural and synthetic small molecules can inhibit or activate Hh pathway at the level of Smo [10, 11]. In cultured cells, Ptc induces the secretion of pro-vitamin D3, and both pro-vitamin D3 and vitamin D3 inhibit Hh signaling at high concentrations [6]. Oxysterols, which lie downstream of vitamin D3 in the cholesterol biosynthetic pathway, act as positive regulators of Hh signaling at a level upstream of Smo [21, 25]. Whether oxysterols or related molecules function as physiological Smo regulators remains to be determined. A recent genetic study in *Drosophila* suggested that the phospholipid, phosphatidylinositol-4 phosphate (PI4P), is a target of Ptc action. In *Drosophila* cells, PI4P promotes Smo accumulation and Hh pathway activation, and Ptc restricts the production of PI4P by

regulating its kinase/phosphatase directly or indirectly [98]. Exactly how Ptc regulates PI4P levels and whether oxysterols or lipoprotein-derived lipids are linked to the effects of PI4P on Smo await further investigation.

Regulation of Smo Trafficking and Conformation

In *Drosophila*, Ptc restricts Smo cell surface expression by promoting endocytosis and degradation of Smo. Hh induces opposite changes in the subcellular distribution of Ptc and Smo, with Smo accumulating on the cell surface and Ptc entering the cytoplasm [22, 39, 106]. How Hh and Ptc reciprocally regulate Smo trafficking is not clear, but it is mediated at least in part by Smo phosphorylation. Phosphorylation-deficient Smo variants fail to accumulate on the cell surface in response to Hh, whereas phospho-mimicking Smo variants constitutively accumulate on the cell surface [39, 104].

A similar reciprocal trafficking relationship is observed for mammalian Ptc1 and Smo but this occurs in the primary cilium, a microtubule-based cell surface protrusion present in most mammalian cells (Fig. 1.2). Genetic studies in mice have implicated primary cilia as essential cellular organelles for mammalian Hh signaling.

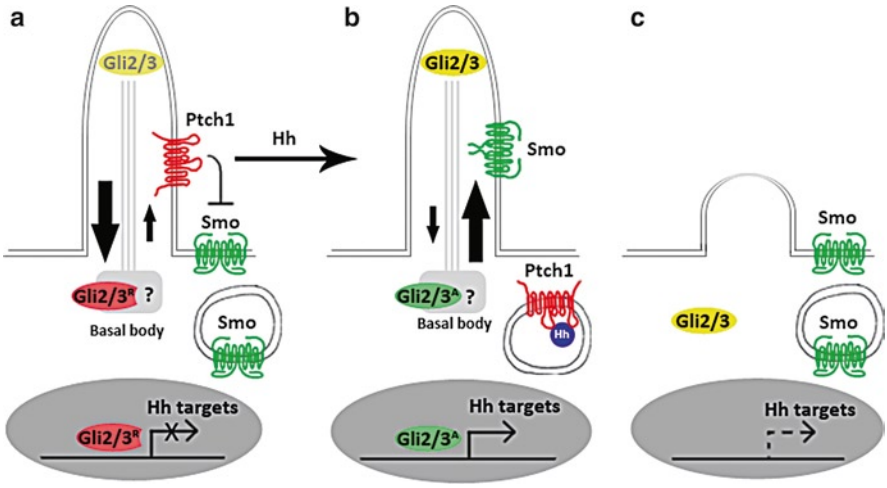

Fig. 1.2 Hh signaling and primary cilia. (**a**) In the absence of Hh, Ptch1 localizes to the primary cilia and inhibits Smo from entering primary cilia. Due to high retrograde transport activities, little or low levels of full length Gli2 and Gli3 are detected at the ciliary tip. Gli3 and Gli2 (to a lesser extent) are processed to form truncated repressors, which enter the nucleus to inhibit a subset of Hh target genes. (**b**) Hh binding of Ptch1 leads to the elimination of Ptch1 from the primary cilia and, subsequently, the entry of Smo into primary cilia. Full length Gli2 and Gli3 are found at the ciliary tip probably due to high anterograde transport activities. By ill-defined processes, Gli2 and Gli3 are converted into active forms, which promote the transcription of Hh target genes. (**c**) Deletion of primary cilia abolishes the processing as well as activation processes of Gli2 and Gli3. In addition to regulating Smo ciliary localization, Hh also induces a conformational change of Smo essential for its activation. See text for details

Mutations affecting the intraflagellar transport (IFT) machinery or other components that are involved in the assembly and function of cilia affect Hh signaling in several developmental contexts [30]. In the absence of Hh, Ptc localizes to cilia and prevents Smo from accumulating in the cilia; binding of Hh to Ptc triggers reciprocal trafficking of Ptc and Smo, with Ptc moving out of and Smo accumulating in the cilia [20, 72]. Ciliary localization of Smo correlates with Hh pathway activation: both an oncogenic Smo mutation and Smo agonists, such as SAG and oxysterols, promoted accumulation of Smo in the cilia [20, 72], and mutation of a conserved ciliary localization motif in Smo prevented its ciliary accumulation and abolished its signaling activity [20]. How Ptc restricts Smo ciliary accumulation is not clear. Smo may constantly move in and out of the cilia in equilibrium by binding to anterograde and retrograde IFT motors and Ptc may tilt this balance. In support of this model, β-arrestins promote Smo ciliary localization by mediating its association with the anterograde IFT motor kinesin-II in response to Hh [48], and Smo is enriched in the cilia of cells defective in retrograde transport [46, 64]. However, recent studies indicated that ciliary entry of Smo does not require microtubule-dependent cytoplasmic motors [46], and that Smo moves through a lateral transport pathway from the plasma membrane to the ciliary membrane [62].

Ciliary localization of Smo is not sufficient for its activation [3, 46, 73, 89], and Smo activation at the cilia likely may involve additional steps including conformational change [104]. FRET analysis demonstrated that both *Drosophila* and mammalian Smo proteins undergo a conformational change in response to Hh [104]. In response to Hh stimulation or Ptc inhibition, *Drosophila* Smo is phosphorylated by protein kinase A (PKA) and casein kinase I (CK1) at its C-terminal tail (C-tail) [4, 22, 39, 99], which triggers a conformational switch and increased proximity of two Smo C-tails within a Smo dimer [104]. Mechanistically, these phosphorylation events activate Smo by counteracting multiple Arg clusters that maintain Smo in a closed inactive conformation [104]. Mammalian Smo (mSmo) C-tail does not harbor PKA/CK1 sites, but does contain a long stretch of basic residues that inhibits its activity; and mSmo undergoes a similar conformational change upon Shh stimulation [104]. mSmo is phosphorylated either directly or indirectly by the G protein-coupled receptor kinase GRK2, which positively regulates Hh signaling [14, 59, 69], raising the possibility that GRK2 and related kinases may substitute for PKA and CK1 to regulate Smo conformation and trafficking in vertebrates.

Downstream of Smo: G Protein and Cos2/Kif7-Ci/Gli Signaling Complex

G protein $G\alpha_i$ is activated by Smo in both *Drosophila* and mammalian cultured cells [66, 71], and $G\alpha_i$ is required for the expression of Hh target gene *decapentaplegic* (*dpp*) in *Drosophila* wing imaginal discs [66]. However, whether $G\alpha_i$ plays a physiological role in Shh signaling is not clear, as inhibition of $G\alpha_i$ activity had minimal effects on Hh-dependent ventral neural tube patterning in chick embryos [53].

Smo likely signals through both $G\alpha_i$-dependent and -independent mechanisms. In *Drosophila*, Smo directly interacts with a multi-protein signaling complex containing Ci, the kinesin-like protein Costal 2 (Cos2), and the Ser/Thr protein kinase Fused (Fu) [38, 54, 65, 75]. Cos2 serves as a molecular scaffold to bring Ci and Fu together with PKA, GSK3, and CK1, leading to efficient phosphorylation and proteolytic processing of Ci [103]. Activated Smo attenuates Cos2-Ci-kinase complex formation, thus inhibiting Ci phosphorylation and processing [74, 103].

mSmo does not interact directly with the vertebrate Cos2 homologs Kif7 and Kif27 [83]. However, recent studies demonstrated that Kif7 is a functional homolog of Cos2. Kif7 forms complexes with Gli proteins and its deletion or mutation leads to aberrant regulation of Hh signaling [16, 26, 51]. Cos2 can move along the microtubules and its motor activity appears to be required for Ci processing [28]. Similarly, Kif7 function is dependent on intact IFT machinery and Hh signaling promotes ciliary localization of Kif7 [26]. Furthermore, Gli3 processing is compromised in *Kif7* null embryos [16, 26, 51]. In *Drosophila*, $G\alpha_i$ is associated with Cos2 upon Hh stimulation [66]. It remains to be determined whether $G\alpha_i$ or related proteins serve as a link between Smo and Kif7.

Control of Gli Protein Degradation and Processing

Ci/Gli activity is regulated by multiple mechanisms, including phosphorylation, proteolysis, and cytoplasmic/nuclear shuttling. In the absence of Hh, full-length Ci/Gli protein can be proteolytically processed into a truncated repressor (Ci, Gli3 and, to a lesser extent, Gli2) or degraded (Gli1 and Gli2). Hh signaling blocks the production of the truncated repressor, and stimulates nuclear translocation and activation of accumulated full-length Ci/Gli. Ci/Gli processing requires the activities of PKA, GSK3, and CK1 as well as the F-box protein Slimb/β-TRCP of the SCF ubiquitin ligase complex [42]. PKA, GSK3, and CK1 sequentially phosphorylate multiple sites in the C-terminal region of Ci/Gli, resulting in the recruitment of Slimb/β-TRCP [40, 76, 80, 87]. A *p*rocessing *d*eterminant *d*omain (PDD) located between the Zn-finger DNA-binding and Slimb/β-TRCP-binding domains of Ci/Gli appears to be critical for proteasome-mediated degradation that selectively removes its C-terminal half. Deletion of this domain from Ci blocks the production of Ci^R [61] and renders complete degradation of Ci [77]. Gli3 is processed more efficiently than Gli2 into a truncated repressor form probably due to a more potent PDD, and Gli1 lacks a PDD and does not exhibit repressor activity [68].

In mammalian cells, Gli2 and Gli3 are localized to the tip of primary cilia in an Hh-dependent manner (Fig. 1.2; [13, 33, 46, 90]). Upon Hh stimulation, Gli2 shifts from a predominantly cytoplasmic localization to the distal tip of the cilium and within the nucleus [51]. While $Gli3^R$ is predominantly nuclear and not found at the ciliary tip [33, 90], Hh stimulation leads to its disappearance and accumulation of full-length Gli3 ($Gli3^{FL}$) at the tip of the cilium as well as in the nucleus [103]. Importantly, Hh signaling also promotes degradation of full-length Ci/Gli2/Gli3 through an ubiquitin ligase containing HIB/SPOP [13, 45, 88, 90, 100, 101], and this mechanism serves as a negative feedback loop to tune down Hh signaling

activity in *Drosophila* [45, 100]. Gli1 is not a strong substrate for SPOP [13, 101] and its degradation involves Numb, which acts in conjunction with the E3 ubiquitin ligase Itch [23]. $Gli3^R$ is also degraded by the proteasome but this likely utilizes a different ubiquitin ligase system [90]. Control of Gli protein degradation might play a central role in preventing tumorigenesis [23, 35].

The exact locations for phosphorylation and proteasomal degradation/processing of Ci/Gli proteins are not known. As the proteasome is enriched at centrosomes that give rise to the basal body underneath the primary cilia [91], Gli proteins might be phosphorylated at primary cilia and then targeted to the centrosome-associated proteasomes for proteolysis. A recent study showed that, in the presence of Shh, the inactive catalytic subunit of PKA is enriched in the cilium base of proliferative cerebellar granular neuronal precursors and that this localization of PKA is essential for Shh-induced proliferation [5]. These observations raise an intriguing possibility that the cilium base might serve as the prime site for phosphorylation and degradation/processing of Gli proteins. In the absence of Hh, the primary cilium may act as a "cAMP gun" to locally activate PKA. Smo might activate $G\alpha_i$ in the ciliary membrane, which in turn represses the adenyl cyclase in the cilium, leading to a local drop of cAMP level and PKA activity. This model is consistent with the genetic data that Gli^A and Gli^R levels are affected in various mutant backgrounds with defective IFT and/or ciliogenesis. How Gli proteins in the cilium are linked to the transcriptional activation of Hh target genes in the nucleus remains unknown. A recent study has highlighted the involvement of cytoplasmic microtubules in ciliary entry of Gli2, but not of Smo [46]. Full-length Gli proteins may need to be "activated" at the cilia before they translocate to the nucleus to activate Hh target genes.

Sufu: A Key Regulator of Mammalian Hh Signaling

A striking difference between *Drosophila* and mammalian Hh signal transduction is the divergent roles of Fu and Sufu [41]. In *Drosophila*, *fu* is a positive regulator essential for Hh signaling, whereas *Sufu* is a genetic suppressor of the *fu* mutation, but its loss does not elicit ectopic Hh signaling and has minimal effects on development. However, in mice, Fu is not involved in Hh signaling [12, 60] and loss of Sufu has profound effects on Hh signaling with ectopic pathway activation [19, 78, 83]. Sufu may have assumed a major inhibitory function in the mammalian Hh pathway due to the existence of multiple Gli proteins. To inhibit Gli^A function, Sufu could impede Gli nuclear localization [24] or suppress Gli activity by recruiting a corepressor complex [15]. Recent studies indicate that Sufu plays a major role in Gli3 processing [13, 34, 37, 47]. Furthermore, Sufu also plays a positive role in mammalian Hh signaling through stabilization of Gli2, in part through counteracting the activity of SPOP [13, 101]. Why Fu kinase is not involved in mammalian Hh signaling? One possibility is that the role of Fu kinase in *Drosophila* Hh signaling is replaced by other protein kinases in mammals. Indeed, multiple protein kinases, including DYRK1a, DYRK2, MAP3K10, ULK3 and Cdc2l1, have been identified to influence Gli activity in mammalian cultured cells [27, 56, 57, 82].

Unresolved Questions in Mammalian Hh Signaling

Numerous studies have revealed the differential utilization of Gli^A and Gli^R in various developmental systems during mammalian embryogenesis [41]. While Gli^A levels are central to cancer formation [35], the involvement of Gli^R has been implicated by several recent reports linking primary cilia to Hh pathway-dependent tumorigenesis [32, 93]. Though deletion of primary cilia blocks the ability of an oncogenic form of Smo (SmoM2) to induce tumorigenesis, it promotes tumorigenesis induced by activated Gli2, Gli2ΔN. Since primary cilia are essential for Gli3 processing, these results suggest that reduction of Gli^R levels may accelerate Gli2ΔN-induced tumorigenesis. Sufu and Kif7 have different functional requirements for IFT or primary cilia [13, 26, 37]. It is possible that they function in separate processes downstream of Smo and deletion of primary cilia may disrupt Kif 7 function, leading to increased tumor incidence in the above studies. Further studies will be needed to decipher the distinct as well as potentially overlapping functions of Sufu and Kif 7 in Hh signaling during development and tumorigenesis.

Several genomic scale studies on Gli target genes revealed that though many target promoters contain a consensus related to the sequence TGGGTGGTC, other target genes may not require this consensus sequence for Gli-dependent transcriptional regulation [31, 85]. Whether Gli^A or Gli^R regulates these genes through interactions with other transcription factors or cofactors remains to be determined. Furthermore, there is increasing evidence that Hh exerts its effects through Gli-independent non-transcriptional mechanisms [52, 67, 94]. However, the involvement of this Gli-independent Hh signaling in development and tumorigenesis has not been studied. As detailed in the rest of this book, Hh signaling plays major roles in a wide variety of tumors and it can act via both autocrine and paracrine mechanisms. Importantly, the requirement of Hh pathway activity in tumor formation and growth seems to differ largely in a context-dependent manner. Further understanding of Hh signal transduction mechanisms at different levels along the pathway will certainly be rewarding to current efforts in targeting the pathway for cancer therapy.

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Chapter 2
Regulation of the Hedgehog Morphogene Gradient*

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Keywords Hedgehog • Secretion • Trafficking • Morphogenetic gradient • Extracellular matrix • Heparan sulfate proteoglycans

Introduction

The development of a multicellular organism is controlled by a genetic program that manifests itself in proliferation, cell differentiation, and apoptosis, leading to the formation of functional organs. A small number of secreted molecules work as "instructors" during these processes [1, 2]. Among them are the Hedgehog (Hh) family of proteins, which act from their source of production at short and long range. They trigger cell fate decisions by inducing a signaling cascade in the ligand-receiving tissues of invertebrates and vertebrates. Dramatic developmental abnormalities are observed in human embryos with compromised Hh signaling, and while great effort is being made to understand and manipulate the signaling cascade downstream of Hh receptor activation, less attention has been payed to the secretion and release of the Hh ligand itself. In this review, we will discuss recent progresses in the understanding of Hh ligand packaging and dispatch from producing cells and its consequences for gradient formation.

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Atypical Biosynthesis of Active Hh Signaling Peptide

An evolutionarily conserved feature of Hh family proteins resides in their hydrophobic lipid modifications. Immature Hh protein enters the secretory pathway where proteolytic autoprocessing and lipidation produce an N-terminal active Hh peptide modified on the C-terminus by a cholesterol moiety, both on *Drosophila* Hh and on its vertebrate counterpart Sonic Hedgehog (Shh) [3] (Fig. 2.1). Moreover, active Hh is also modified by the attachment of a stable amide-linked palmitic acid at the opposing N-terminal end [4, 5]. This acylation is catalyzed by the Skinny Hedgehog protein (Skn or Ski, also called Rasp, Central missing and Sightless) [5–8] that belongs to the family of membrane bound O-acyl-tranferases (MBOAT) [9]. The importance of these dual lipid modifications is underscored by the fact that removal of these modifications interferes with the biological activity of Hh in vivo. Notably, mutations that affect the human Shh autocatalytic processing are associated with holoprosencephaly [10].

Routing of Hh to the Plasma Membrane

The presence of lipid moieties has a significant effect on Hh solubility and is, therefore, bound to affect intracellular trafficking to the plasma membrane in producing cells. Strikingly, although Hh lipidation is essential for its activity, the exact intracellular compartments in which these modifications take place during Hh secretion are not known. Nevertheless, we do know that the dependence of correct Hh intracellular trafficking on the cholesterol moiety has been demonstrated, as a mutant form of Hh lacking the cholesterol adduct shows significantly altered subcellular distribution [11]. In addition, a hydroxyl-oxygen present within the cholesterol is responsible for the cleavage of Hh [3], and as both autoproteolysis and cholesterol modification are linked, they likely occur in the same compartment. Sensitivity of the uncleaved Shh precursor to digestion with the EndoH glycosidase suggests that the autoprocessing reaction occurs before the medial Golgi [10]. In addition, two mutant forms of Shh (point mutations) that present a temperature-dependent misfolding and retention in the ER do not undergo autoprocessing cleavage. It is thus likely that cleavage and autoprocessing occur in a post-ER pre-medial Golgi compartment, such as the *cis*-Golgi. This would also be consistent with the availability of cholesterol as a critical determinant for cleavage, as cholesterol is found at only low levels in ER membranes and at higher levels in Golgi membranes [12]. This, however, must be experimentally determined, membranes and cleavage might alternatively be required for a unique or specific Hh ER-exit route.

The site of the second Hh modification, palmitoylation, is also an open question, as mouse tagged-Skn protein is mainly localized to the ER in CHO and HeLa cells [13, 14], whereas *Drosophila* Ski was found in the Golgi apparatus in insect Schneider cells [15]. It is also not known whether palmitoylation is conditional to

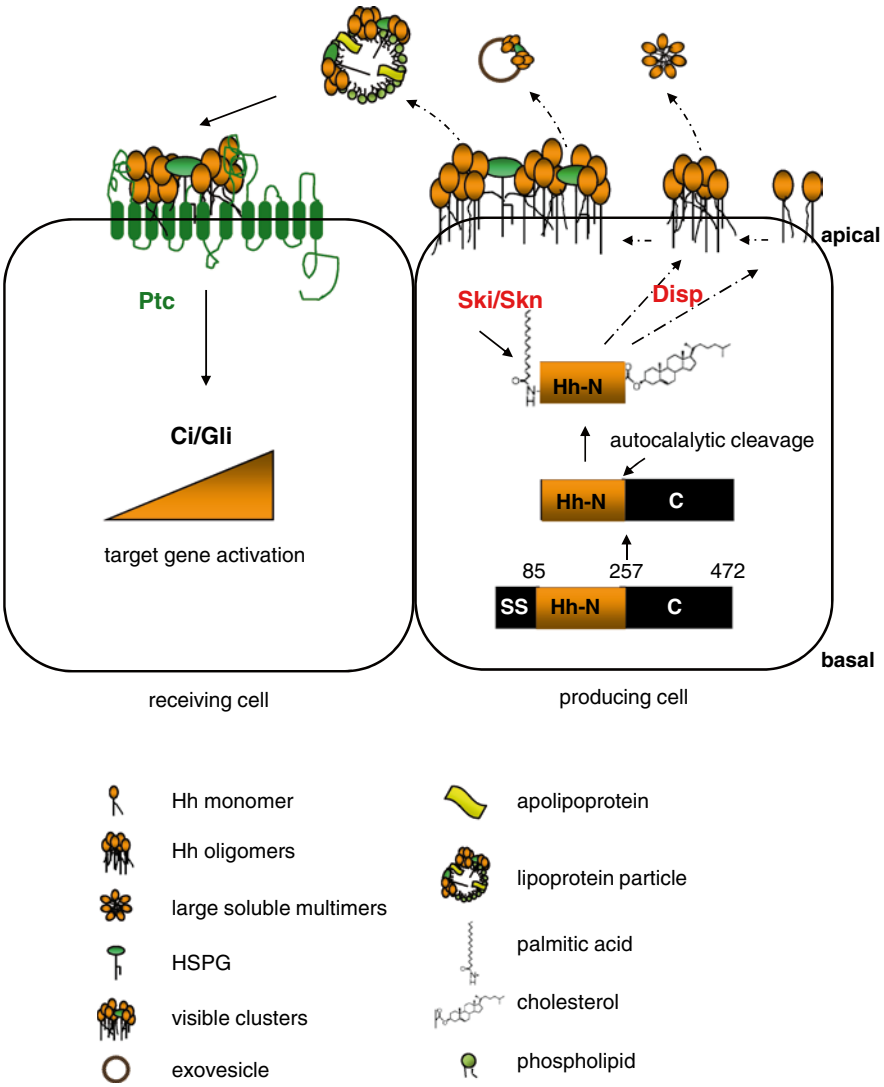

Fig. 2.1 A schematic diagram of Hh protein biogenesis, secretion, release, and transport to target cells. In Hh-producing cells, the immature Hh protein enters the secretory pathway where proteolytic autoprocessing and lipidation produce a N-terminal active peptide modified on its C-terminus by a cholesterol moiety. Additionally, a stable amide-linked palmitic acid is attached to its N-terminus by the acyltransferase Ski/Skn. The active dually lipid-modified Hh is denoted as Hh-N. The destiny of the C-terminal fragment after cleavage is not known. The transmembrane protein Dispatched (Disp) promotes the secretion of monomers and/or oligomers of Hh-N to the apical cell membrane. Monomers of Hh-N could self-associate spontaneously to form large soluble multimers and can be released in the extracellular lumen. Conversely, Hh-N oligomers at the cell surface could be selectively enriched in visible clusters along with heparan sulfate proteoglycans (HSPGs). Upon release from producing cells, the visible clusters may be transported across several cell diameters from the Hh-producing cells either into exovesicles and/or as an integral component of lipoprotein particles. The transport of Hh-N by one or by the combination of the diverse carriers leads to transcriptional activation of Hh target genes (via the transcriptional activator Ci/Gli) in receiving cells upon its association with the transmembrane protein Patched (Ptc; Hh receptor). Activation of the Hh pathway can also be triggered by a membrane-associated Hh (not shown)

cholesterol modification, as contradictory results have been offered. Analysis of recombinant human and rat Shh produced in a cell-based assay showed that all purified forms of Shh are cholesterol-modified, with a variable percentage of dually lipidated protein as if palmitoylation was subsequent to cholesterol modification [4]. On the other hand, an uncleavable as well as a mutated form of Shh, both lacking the cholesterol modification, have been found to carry [3H]-palmitic acid or ^{125}I-iodo palmitate when metabolically labeled in cultured cells [13, 16]. Similarly, a non-cholesterol-modified *Drosophila* Hh transgene displayed in vivo activity that depends on its palmitate, suggesting that cholesterol addition of Hh was not a prerequisite for palmitoylation [11].

Finally, one could speculate that acylation affects the routing of Hh secretion, as it was recently demonstrated that acylation of a similar morphogen, Wnt-3a protein, with a palmitoleic acid is required for appropriate trafficking from the ER [17]. However, in vivo analysis of a form of Hh lacking only the palmitic acid modification showed that it could reach and activate target cells as if Hh secretion was independent of palmitoylation [5]. Nevertheless, one must take these studies with caution, as they are hampered by the fact that this variant was expressed at a non-physiological level possibly causing misrouting within producing cells.

How the activity of the acyl-transferase Skn is regulated is also unclear, although another mammalian member of the MBOAT familly, Gup1, has been shown to negatively regulate the N-terminal palmitoylation of Shh, very likely by competing with Skn rather than catalyzing depalmitoylation [14]. This is consistent with biochemical studies showing that Shh palmitoylation does not follow cycles of palmitoylation–depalmitoylation and is thus not reversible [16]. In addition, palmitoylation or other N-terminal hydrophobic modifications of Shh greatly increase its activity in a cell-based assay that does not require secretion or transport without affecting its ability to bind Patched (Ptc) receptor [18]. This suggests that the possible role of palmitoylation is to modulate Hh activity by increasing its stability or by changing its affinity for associated cofactors.

Secretion and Release of Lipid-Modified Hh

During embryonic development, Hh activity can exert an effect over large fields of cells, for example up to 50 μm in *Drosophila* wing imaginal disc and 300 μm in vertebrate neural tube or limb bud [2]. The long-range activity of Hh raises the question of how a dually lipidated protein can escape the membranes of producing cells to directly activate cells distant from its source of production.

Earlier studies have shown that the cholesterol moiety attached to Hh plays an important role in the protein's membrane retention as it causes strong membrane binding (reviewed in [3]). Indeed, in vitro engineered forms of Hh lacking cholesterol have been found to freely dissociate from cells after secretion, indicating that the cholesterol adduct can function as an anchor that restricts the mobility

of the signal. Then how is cholesterol-modified Hh released from the lipid bilayer? A biophysical study revealed that cholesterol alone anchors proteins to membranes with significant strength and revealed a spontaneous desorption half-time of several hours [19], suggesting that a specific cellular activity or machinery is necessary for Hh release in a shorter time lapse.

Hh may be released from specialized membranes as lipidation of Hh allows its association with sterol-rich membrane microdomains, such as lipid rafts in *Drosophila* and in mammalian cells [13, 20]. Interestingly, optical imaging using tagged forms of Hh in *Drosophila* identified nanoscale oligomers at the cell surface [21]. Moreover, a higher order of Hh clustering was also observed and shown to involve ligand interactions with heparan sulfate proteoglycans (HSPG, see below), resulting in visible clusters of the Hh ligand at the cell surface. Mutants of Hh that are impaired in oligomerization lose their long-range activity, suggesting that nanoscale organization of Hh at the plasma membrane is essential for Hh to move a distance [21] (see below).

Why is this so? Within membrane microdomains, Hh may encounter proteins important for its release and efficient spreading. For example, it appears that packaging of Hh, destined for long-range movement, partially requires the cytoplasmic membrane-scaffolding proteins Reggie1/flotillin2 [22], typical members of lipid rafts [23]. Interestingly, overexpression of Reggie1 in the wing imaginal disc of *Drosophila* increased the level of extracellular Hh and its spreading, as well as the activation of the pathway at long range [22]. Reduction of Reggie in the wing disc causes a weak reduction of long-range Hh targets, without affecting short-range signaling [22], indicating that diverse routes may be taken by different pools of Hh (i.e. long vs. short range). In fact, several other proteins have been shown to be specifically required for long-range, but not short-range, bound Hh. This is discussed below.

Some insight into the process of Hh release from producing cells came from the identification of the dispatched (disp) gene that is predicted to encode a 12 transmembrane protein, containing a sterol-sensing domain (SSD), and is required in both *Drosophila* and mouse Hh-producing cells to transport the lipid-modified Hh protein [24, 25]. Importantly, autocrine activity of Shh in the notochord and juxtacrine Hh activity in imaginal discs were not affected in disp mutant animals, but no long-range signaling was observed [24, 26, 27]. These observations suggest that Hh secretion, release, and its long-range activity are closely linked to lipid modification and likely employ a novel intracellular machinery to secrete and release the membrane-anchored Hh protein.

While the exact function of Disp remains unknown, it has been suggested to act as a proton gradient-driven transporter of the Resistance-Nodulation-Division family, as mutations in conserved residues impair its activity [27]. These permeases use a proton electrochemical gradient to function as antiporters in driving out substrates such as hydrophobic drugs, heavy metals, and endogenous compounds. From this homology, it is not clear whether Disp acts by changing the membrane microenvironment around Hh favoring clustering or whether it promotes the release

of lipid-modified proteins, as it has been shown for another transmembrane transporter of the bacterial ATP binding cassette family, the LolCDE complex from *Escherichia coli* [28].

It is intriguing that Disp contains a SSD, similar to the one present on proteins which are involved in cholesterol homeostasis or trafficking, including Niemann–Pick C1 (NPC1) [29]. Because only the cholesterol-modified form of Hh is sensitive to Disp activity, Disp-dependent Hh secretion could involve its SSD domain. This domain has been shown to be important for vesicular trafficking regulated by cholesterol level. Because association has been shown between the SSD domain of NPC1 and free cholesterol [30], it is possible that Hh secretion depends on the interaction between the SSD domain of Disp and the cholesterol moiety of Hh.

Based on these studies, and on the fact that in disp mutants, Hh protein production, and processing are not affected, several possible nonexclusive roles have been proposed: (1) Disp may be involved in the intracellular trafficking of Hh to the appropriate membrane microdomain dedicated to its secretion. Indeed, we have previously shown that in the absence of Disp function, cholesterol-modified Hh remains localized to the basolateral membrane of polarized embryonic epidermal secreting cells while it is apically localized in wild-type siblings [11]. However, no specific abnormal apical/basal localization of Hh was observed in disp mutant epithelial cells of the wing imaginal disc [24] (D'Angelo and Thérond unpublished observation); (2) Hh is also secreted under its monomeric form, however, its long-range activity was shown to be dependent on the clustering of Hh multimers with HSPG [21] and on the packaging into lipoprotein particles [31]. Interestingly, visible clusters of Hh are absent in disp mutant animals suggesting that it may control the oligomerization of Hh [11, 32]. It is thus possible that Disp directly promotes the clustering of Hh which is then sequentially associated with other proteins; and (3) Disp may facilitate a direct release of Hh from the plasma membrane into the extracellular space. However, secretion of an active form of Shh was observed from Disp null fibroblast, arguing against this model [25]. In summary, we believe that Disp functions in the formation of an active fraction composed of Hh oligomers and other proteins (HSPGs, lipoprotein particles, see below) dedicated to long-range transport.

Hedgehog Spreading: A Dilemma in Motion

After release, several modes of Hh movement have been suggested including (1) transcytosis (where proteins are passed cell-to-cell by alternating endo- and exocytosis events) [33], or (2) movement on various vehicles through the extracellular matrix (ECM). However, various studies in *Drosophila* have illustrated that blocking dynamin – the "motor" involved in many cell processes, including endo- and exocytosis – has no effect on the spreading of Hh in either the wing disc [34, 35] or in the fly embryo [36]. Thus in flies, the first model has been generally rejected

in favor of the second. This prediction is certainly bolstered by numerous studies which have found that secreted or membrane-attached proteins which make up the ECM can regulate the spreading or signaling of Hh. One group of these proteins attracting much interest is the HSPGs.

Proteoglycans are proteins which have long, unbranched glycosaminoglycan (GAG) chains (sugar polymers) attached to specific serine residues on their protein core. HSPGs have heparan sulfate (HS)-containing GAG chains. Originally viewed as important ECM components, recent evidence has highlighted the critical role of these sugar-modified proteins in morphogenesis and development. Accordingly, mis-regulation of the different proteins involved in HSPG biosynthesis manifests in a large variety of phenotypes [37].

A number of studies have suggested that HSPGs, more specifically the glypicans – a subtype of HSPGs anchored to the plasma membrane though a glycerophosphatidylinositol (GPI) motif – play an important role in the cells which secrete or spread Hh. Glypicans can accumulate in membrane microdomains and appear to be important for the recruitment of Hh into visible clusters (as discussed above) [21]. In addition, HSPGs at the plasma membrane may be involved in Hh stability, as loss of *tout velu* (*ttv*) or *sister of tout velu* (enzymes of the EXT family involved in the GAG chain polymerization) causes a noticeable reduction in Hh levels in the wing imaginal disc [38]. Indeed it has been demonstrated that glypicans recruit lipophorin (a Hh vehicle – see below) to *Drosophila* Hh secreting cells [39]. Thus, the GPI-anchored glypicans appear to be involved in Hh organization at the plasma membrane of secreting cells, Hh stability, and perhaps even Hh vehicle recruitment and loading.

Glypicans also have the ability to move within tissues. Eugster et al. [39] showed that glypicans are commonly shed by wing imaginal discs, moving into surrounding tissues where they are internalized. Furthermore, both fly glypicans Dally-like (Dlp) and Dally appeared to fractionate with Hh and lipoproteins in density-gradient experiments, through interactions with their GPI and the GAG chains [39], suggesting that they can move with these Hh carrying particles. Moreover, the fact that released glypicans, Hh, and lipoproteins are co-localized in endosomes in receiving cells indicates that they may be internalized together [39]. Altogether, fly glypicans do not only play a role in Hh secretion, but also appear to be associated and move with Hh carriers with which they are probably also internalized in receiving cells. This suggests that glypicans could also play an active role in the spreading of morphogens such as Hh.

Extracellular Gradient Formation by HSPGs

Control of Hh movement and regulation of extracellular Hh gradient formation by HSPGs is an attractive hypothesis and has been researched in various labs. Hh has a high affinity for HSPGs that reside at the cell surface [40]; however, Hh is unable

to enter or cross a field of mutant cells which are deficient in HSPG assembly, as is observed in mutants for proteins which are involved in HSPG biosynthesis like *ttv* and *sulfateless* (a sulfotransferase involved in GAG chain modification) [34]. In these cases, Hh is found accumulated at the edge of the mutant tissue, proximal to the source, [41–44] and cannot spread to activate signaling within or past the mutant field [44]. Furthermore, the dependence of Hh movement toward HSPGs is specific to the cholesterol modification as contrary to fully modified Hh, non-cholesterol or non-palmitoyled forms of Hh can spread through a field of cells deleted for *ttv* activity. This is mimicked in cells where glypicans themselves (Dally and Dlp) are lost in the embryo, where Hh mobility is repressed [34]. Therefore, in addition to regulating Hh stability or membrane localization in secreting cells, glypicans are thought to be involved in an active process of Hh movement from one cell to the next, perhaps by passing the morphogen from GAG chain to GAG chain.

Recent evidence from flies has suggested that one glypican, in particular, may play an important role in Hh movement. In *Drosophila*, *dally* mutant wing imaginal discs show weakly decreased Hh signaling, and data indicate that Dally is involved in posterior Hh stability and recruitment of lipophorin [39]. Indeed, Dally itself appears to be present in high levels at the apical pole, and boosts Hh levels at the apical membrane of disc cells. Moreover, Dally release may aid in Hh secretion at this pole and long-range spreading through the ECM found in the apical lumen [45]. Certainly, overexpression of secreted-Dally ectopically activates Hh signaling far from the Hh source, something which is not seen when a transmembrane-tethered form of Dally is expressed [45, 46].

The protein Notum has been studied in both flies and mammals due to its HSPG-related phenotypes. In flies, it has been suggested that Notum acts negatively on the Wnt pathway through its regulation of Dally [47, 48], and studies in mammalian cells have confirmed the ability of Notum to act as a PLC-like lipase and cleave GPI-anchor proteins, including various glypicans [49]. In flies, recent genetic studies have shown that Notum, like Dally, specifically regulates long-range signaling of Hh. Indeed, Dally is unable to augment Hh movement in the absence of Notum, suggesting a link between Notum-dependent release of Dally and Hh spreading [45].

How Dally and Dally-like release promotes long-range Hh spreading is not clear. However, it is possible that Dally augments the release of Hh on specific vehicles such as lipophorin [39] or allows it to spread in a controlled manner without being lost within the surrounding lumenal space. It is also feasible that Dally GAG chains could protect Hh from protease-mediated degradation within the ECM, and along these lines the levels of sulfatation on HSPGs were shown to be an important factor in ADAM protease-mediated Hh cleavage [50] (see below). In conclusion, in *Drosophila*, HSPGs are required for the stable retention of Hh on the cell surface, which results in the restricted and controlled movement of Hh through the surface of the epithelium. Whether glypicans mediate similar processes in vertebrates is an interesting question for future research.

A Similar Spreading Mechanism from Flies to Mice?

In vertebrate models, like in the fly, Hh ligands can act at long range [2]. Given that the requirement of dynamin for Hh spreading has not been tested in vertebrate systems, it remains to be seen whether Hh moves through the ECM in a similar manner as is seen in *Drosophila* epithelial cells. Indeed, recent findings from the Roelink lab, showing the mammalian protein Disp may be important in Hh receiving cells for continued spreading, has led to the speculation that Hh may be recycled in receiving cells to be released for transcytosis [51]. This would, thus, mean that Hh movement in these tissues could be subject to regulation by a different set of proteins to those in invertebrates. However, the fact that several HSPGs and members of the EXT family, as well as other ECM proteins, can still regulate the non-autonomous signaling of Shh suggests at least some role of ECM in this process.

The specific roles of Hh-HSPG interaction have been tested in mice carrying mutations within a sequence essential for Shh–proteoglycan interactions (the Cardin–Weintraub sequence) [52]. These mutant mice presented growth abnormalities of the CNS, but were free of the patterning abnormalities usually associated with Shh reduction. Intriguingly, it was found that interaction of Shh with proteoglycans is essential for its role in precursor proliferation in the external granule layer of the cerebellum, and illustrated that in this model proteoglycan–Shh interaction altered the cellular response to Shh, as a different profile of target genes and transcriptional activators (Gli) was detected in cells with and without this interaction [52]. Within the developing brain, the extracellular proteoglycans worked to localize Shh to the proliferating zone and, once there, they regulate the cellular response to Shh which favors the activation of a specific subset of proliferation-activating target genes. Thus, proteoglycans, such as the glypicans, appear to be important in controlling Hh signaling by reducing spreading of the ligand. This is at odds with a role in Hh movement, but indicates that at least some forms of Hh may move through the ECM in vertebrate systems, where they encounter ECM proteins such as glypicans.

Various additional results have indicated that glypicans can adopt several different roles within the Hh pathway. For example, null-mice for the vertebrate glypican GPC3 have increased Hh signaling [40, 53], as GPC3 competes with Ptc for the Hh ligand and thus inhibits signaling. It does this by inducing Hh internalization and degradation, thereby reducing its availability to Ptc [40]. Thus in tissues such as the bones, where Indian Hh, a vertebrate member of the Hh family, stimulates the development of the endochondral skeleton, KO mice for GPC3 have increased Ihh levels and thus increased signaling, leading to bone overgrowth [54].

Consistent with the idea that Hh moves through the ECM, several other secreted glycoproteins have been implicated in extracellular Hh stability, and control the non-autonomous signaling of Hh. In *Drosophila* mutant animals for the ECM protein Shifted (Shf), most Hh target genes have reduced expression [55, 56]. Shf associates with both Hh and other ECM proteins, such as HSPGs, and its loss decreases Hh stability, and therefore, signaling [55, 56]. Shf may also play a role in

Hh movement as an Hh-GFP fusion protein does not appear to spread as far when Shf is lost, and although this could arguable be due to reduced stability, experiments where Hh levels have been restored to normal by Hh overexpression in a *shf* mutant background still show a reduced zone of target gene activation [55], indicating that both stability and movement are affected. Additionally, like many other pathway members, Shf action is specific only to cholesterol-modified Hh [55]. Akin to Shf, in Zebrafish, the vertebrate-specific Scube2 protein, an EGF and CUB domain protein, has been implicated in Hh specification of myogenic tissue [57, 58]. Scube has been described to have a cell non-autonomous requirement in the Hh receiving cells and may thus be involved in the formation of the Hh gradient, like its EGF-like domain containing fly cousin, Shf. In conclusion, several ECM proteins, whether secreted or plasma membrane tethered, control Hh stability and gradient formation in vertebrates, indicating ECM-mediated Hh movement as a common mechanism among different phyla.

Hh Uses Multiple Carriers for Its Travels

In flies and in vertebrates, Hh acts both at short and long range [1]. When discussing Hh carriers, one should remember that, depending on the range of its activity, Hh might associate with different carriers. Whatever the carrier, Hh needs to be able to pass through the hydrophilic environment of a tissue and its lipid moieties need to be shielded or cleaved. Several mechanisms would be capable of solubilizing hydrophobic Hh protein and evidence has been produced for some of them (reviewed in [59]). Also, recent data have led to the proposition of new and interesting mechanisms.

Could Hh simply be released from the cell surface by cleavage? This theory has been tested in *Drosophila*, in which two transgenic fly lines have been generated that either contain a transmembrane anchored form of Hh, Hh-CD2, or a Hh-GPI that anchors Hh via a glycosyl-phosphatidylinositol attachment to the outer leaflead of the plasma membrane. Interestingly, absence of a long-range effect of those forms was concluded, strongly arguing that in *Drosophila* wing disc Hh is not simply released from the cell surface by cleavage. However, in mammals, the picture might be different, as Dierker et al. speculated that proteases, or lipases, could cleave and release Shh from membranes once it has been directed there. Indeed, they recently found that metalloproteases of the ADAM family function as the so-called "sheddases" in cells [50]. In particular, the action of secreted ADAM proteins, but not restricted to ADAM 17, leads to the removal of plasma membrane-bound Hh from mammalian Bosc23 cells. Interestingly, this group shows that an active form of Shh can be obtained from the medium that is devoid of both N- and C-terminal lipid modifications. Whether such mechanism also exists in intact tissues or in other species still needs to be validated.

For short-range transport, Hh could be handed over to the neighboring cell via direct cell-cell contact. ECM components such as HSPGs may be pivotal in this process.

Dally and Dally-like, as discussed above, have been widely implicated in Hh binding, and given that cholesterol-modified Hh cannot cross a field of cells depleted of HSPGs, an involvement in lateral diffusion has been suggested in *Drosophila* [48]. Although cleaved Dally participates in Hh long-range spreading [45], it certainly remains possible that noncleaved proteoglycans also mediate short-range movement of Hh along the cell surface by transferring Hh from one HSPG on one cell to another one located next to it.

Compelling experimental evidence points to the existence of multiple Hh carriers for long-range transport of Hh. These might work in parallel and/or depend on the tissue type and developmental stage of the organism as well as on the source of Hh. Four different long-range carriers have been described:

1. Micelles formation by Hh (via self-aggregation or lipid binding proteins) has been proposed to shield the ligand's lipids attachments. Indeed, the presence of multimeric Hh complexes has been known for a long time. Closer analysis of these forms has revealed large soluble multimers of 5–6 molecules of Shh-N in conditioned media of vertebrate cells [60], and multimers of Hh-N (of 160 kDa) are also present in conditioned culture media from Hh-producing *Drosophila* Schneider cells [13, 32]. What could aid the formation of these structures? It is thought that the hydrophobic cholesterol moiety plays an essential role, perhaps by favoring micelle-like structure with interactions between lipids forming a lipophilic interior. Intriguingly, in *Drosophila*, absence of palmitoylation does not prevent Hh multimer nor visible cluster formation, but Shh that lacks palmitate fails to multimerise [13]. Additionally, the formation of these palmitate-mediated aggregates is physiologically important, as abrogation of higher molecular weight complexes leads to a loss of long-range signaling in vertebrates.

   While the interaction of Hh molecules in these complexes is of a non-covalent nature, Dierker et al. additionally described covalently cross-linked Hh oligomers [50]. The formation of these so-called "undisruptable" complexes depends on transglutaminase and heparan sulfate activity [50], and whether this form exists throughout the Hh-producing species remains to be investigated.
2. Akin to micelle, Hh could be secreted as an integral component of lipoprotein particles. Lipoprotein particles seem to be involved in the transport of systemic Hh in *Drosophila*. They are made up of cholesterol, phospholipids, and scaffolding proteins – apolipoproteins, the latter two components building a monolayer membrane. It is conceivable that Hh is anchored within lipoprotein particles via its attached lipid and/or cholesterol. Lipoprotein particles are produced in the fat body (a tissue functionally related to the liver) of the fly and are secreted into the hemolymph in which they circulate and reach other tissues. The reduction of lipophorin levels in the fat body leads to a significant remote effect on Hh spreading and signaling in peripheral tissues [31]. Lipoprotein-like particles also exist in higher vertebrates, however, whether these particles have a physiological function in the transport of Hh protein remains to be investigated.

3. A third possibility is that Hh could be directed to secretory multivesicular bodies (MVBs). External vesicles, the so-called exosomes, have recently been suggested as carriers for Hh-related peptides in *Caenorhabditis elegans*, although a direct prove has not yet been demonstrated [61]. Nevertheless, the membrane-bound V0 sector of the vacuolar H^+-ATPase (V-ATPase), an integral membrane component of MVBs, acts along this pathway leading to the apical secretion of exosomes containing Hedgehog-related peptides [61]. It is an intriguing possibility that similar mechanisms exist in other organisms in order to release Hh. For instance, Shh has been observed in extracellular vesicular structures called nodal vesicular particles (NVPs) located at the surface of mouse ventral node during embryonic development [62]. It has been speculated that NVPs, which consist of a membrane sheath and a lipid core, enable transportation of Shh over vast distances without solubilization [62]. Further investigation into the nature and origin of these vehicles is merited.
4. Membrane-associated Hh may also provide another active form of the ligand. Although it is generally accepted that Hh is released in a soluble form, up to 90% is found in *Drosophila* in a membrane-containing fraction including the plasma membrane [31]. This cell surface associated Hh in producing cells could feasibly be extracted by adjacent receiving cells. The Hh receptor Ptc on those cells might internalize Hh which then results in Hh signaling pathway activation [63]. Interestingly, in *Drosophila* imaginal discs, long cellular protrusions, called cytonemes, are found to extend toward certain morphogen-expressing boundaries [64]. Although no direct evidence for their involvement in Hh signaling has been demonstrated, the morphogen Decapentaplectic (Dpp) is found in cytonemes through which it is directed to its receptor Thickveins in Dpp-responsive cells [65]. Whether Hh uses this mode of transport as a bridge to reach its receptors over several cell diameters is still elusive, but has recently become under intense scrutiny.

From these data, we can conclude that Hh is transmitted by a variety of diverse carriers, each of which may provide unique properties to Hh, allowing differential stability, signal duration, and/or range of activity, through dissimilar biophysical characteristics and composition of these carriers. However, one must reiterate that controversies still exist about how these numerous Hh forms exert a combined signaling activity on ligand-receiving cells, and that where and how these vectors are assembled also presents a "black-box" in our understanding. Inventive and highly technical investigations are needed to shed light on these intriguing questions.

While it has been well established that constitutive activation of the Hh pathway (by loss of function of the Hh receptor Ptc) is responsible for certain kind of cancers (medulloblastomas, rhabdomyosarcomas and basal cell carcinomas), the growth of other tumor types has been shown to be regulated by an autocrine or juxtacrine manner involving secretion of Hh from these tumors. More recently, a new paracrine model has been proposed in which tumor cells produce Hh that stimulates surrounding stromal cells to produce growth factors that in turn support tumor growth [66]. This model conforms much more to Hh developmental activities,

which in most cases are typically paracrine [67]. In addition, the level of Hh and/or its carriers secreted from these tumor cells may correlate with their severity, as it has been shown in tumor biopsies from prostate cancer [68]. Thus, research focused on Hh secretion and spreading, and proteins involved in these processes is essential in the battle against Hh-related tumors.

Conclusions

While great effort is being made to understand and manipulate the signaling cascade triggered by the Hh ligand, less attention has directed toward understanding the role of aberrant amounts of secreted Hh might have on surrounding responsive tissue. This issue is of great importance as locally concentrated Hh triggers a strong and direct gene response. Although very little is known about (1) where within the producing cells Hh processing and the addition of its unique lipid modifications occur, (2) what intracellular route Hh takes to reach the plasma membrane, (3) what constitutes the cellular machinery that leads to secretion, and (4) what kind of vehicle Hh uses to exert particular activities, emerging evidence strongly suggests that these may occur in a very specific way. It is these unique features of this molecule that might provide the Achilles tendon for therapeutic targets in the fight for a wide variety of syndromes caused by abnormal endogenous secretion of Hh ligand.

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Chapter 3
Smoothened Signaling Through a G-Protein Effector Network

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Keywords Hedgehog • Cancer • Smoothened • G-protein

Introduction

The important role the Hedgehog (HH) signaling pathway plays in cancer was first revealed by patients diagnosed with the familial disorder known as Gorlin Syndrome, who harbor loss-of-function mutations in the HH receptor Patched (PTCH) [1–3]. Besides numerous developmental abnormalities, consistent with disruption of this important developmental signaling pathway, individuals afflicted with this disorder have an inherited predisposition to medulloblastoma, basal cell carcinoma, and rhabdomyosarcoma [2]. Similar mutations found in sporadic cases of these same tumor types implicated *PTCH* as an important tumor suppressor in human cancer [4]. Other components of the HH pathway, such as the gene encoding the seven-transmembrane (7TM) protein Smoothened (SMO), are also found mutated in sporadic forms of these same malignancies [5, 6]. More recently, constitutive activation of the HH pathway has been implicated in other human cancers including those of the breast, prostate, pancreas, and lung, where HH is thought to play a role as a tumor-survival factor [7]. Combined, it has been estimated that approximately 25% of all human tumors harbor a constitutively active HH signaling pathway [8]. As such, considerable effort has gone into identifying novel small-molecule inhibitors of HH signaling. Consistent with the rate-limiting role SMO plays in HH signaling, the vast majority of HH inhibitors isolated from numerous small-molecule screens appear to target SMO [9]. A number of these compounds are currently in clinical trials as anti-cancer agents, targeting tumors that are dependent on HH pathway activity [10]. Thus, a

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clear understanding of the mechanisms by which SMO communicates with downstream pathway components, and how such inhibitors affect these processes, will directly impact human health.

Hedgehog Signal Transduction

Much of what is understood about HH signaling originates from studies of this signaling pathway during the development of the fruit fly *Drosophila melanogaster* [11]. It is now well accepted that the major components, and how they communicate with each other, are highly conserved from Drosophila to man. Although there are significant differences in the importance of some of the signaling components across phyla, it is not yet clear if these variations are due to specific contextual differences or to evolutionary divergence. Thus, in this chapter we generalize about the HH signaling pathway from work derived from numerous animal models, mentioning specific biological contexts only where necessary to illustrate a particular point.

HH is produced and secreted by discrete compartments within a developing field of cells, where it elicits both short- and long-range effects on target cells [12]. The receiving cells interpret the level of HH activation through poorly defined, indirect interactions between the HH receptor PTCH and the signal transducer SMO. PTCH inhibits the activity of SMO in a manner that appears to be catalytic, whereas SMO is constitutively active in the absence of PTCH [13, 14]. One of the mechanisms by which PTCH inhibits SMO activity involves PTCH-dependent trafficking of SMO to lysosomes [15, 16]. In response to HH, PTCH is removed from the cell surface, thereby allowing SMO phosphorylation, stabilization, and accumulation at the plasma membrane [15, 17]. Ultimately all signaling downstream of SMO coalesces to regulate the stability and activity of the GLI/CI family of transcription factors [18]. In the absence of HH, these proteins exist as proteolyzed transcriptional repressors. HH blocks this proteolytic conversion, and stabilizes full-length transcriptional activators. The degree of HH a cell is exposed to ultimately determines the ratio of GLI/CI repressor and activator forms to regulate a spectrum of transcriptional targets that is thought to correlate with the concentration of the initial HH signal.

While the general flow of information through the HH signaling cascade is known, the direct effectors of SMO, and the mechanism(s) by which it communicates with them are still being characterized. The first clue as to how SMO transduces the signal from the plasma membrane to the intracellular effectors came with the observations that Drosophila SMO directly associates with the kinesin-related protein Costal2 (COS2) [19–22], and that mammalian SMO binds the COS2 ortholog KIF7 [23]. The functional consequence of KIF7-SMO binding in mammalian systems is not yet clear. However, in Drosophila, COS2 serves as a scaffold upon which a complex containing CI and the protein kinases Fused (FU), cyclic-AMP (cAMP)-dependent protein kinase A (PKA) and casein kinase 1 (CK1) assembles [24, 25]. As such, a direct association between COS2 and SMO

connects the membrane signaling components with the cytoplasmic effectors. Subsequent to this finding, a direct association between SMO and FU that drives a feed-forward loop to facilitate high-level signaling was described [26]. We recently demonstrated that the intracellular molar concentration of SMO is significantly lower than that of CI, COS2, or FU, suggesting that direct association between SMO, COS2, and FU is unlikely to facilitate all aspects of HH signaling [27]. It is, therefore, likely that multiple pools of intracellular effectors exist; some that are in direct contact with SMO, and some that are regulated through the use of G-proteins and/or second messengers.

Smoothened as a G-Protein-Coupled Receptor

Much of what we know about G-protein-coupled receptor (GPCR) structure and function has resulted from studies of the prototypical GPCR rhodopsin, the first GPCR to be fully sequenced and to yield high-resolution structural data [28–30]. Sequence analysis of rhodopsin suggested the existence of several distinct functional domains, including seven predicted alpha-helical transmembrane segments, an extracellular amino-terminal domain, three extracellular loops, a carboxyl-terminal domain with multiple phosphorylation sites, and three intracellular loops [29]. Structural analysis of rhodopsin, and more recently of the β_2-adrenergic receptor and A_{2A} adenosine receptor, confirm the existence and conservation of these domains, underscoring their importance in GPCR function [28]. As such, proteins possessing these well-established functional domains in their primary amino acid sequence or predicted tertiary structure are classified as members of the GPCR superfamily, which is estimated to encompass more than 1% of all human genes [30].

SMO was originally identified as a gene necessary for proper organization of the early Drosophila embryo [31]. Subsequent genetic and molecular characterization of SMO revealed it to be a requisite component of the HH signal transduction cascade [32]. Primary sequence comparisons revealed that SMO and the Frizzled (FZ) family of GPCRs are quite similar across distinct functional domains: 37% similarity across the extracellular amino-terminal domains, and 52% similarity across the seven predicted transmembrane domains [32]. As such, SMO has been classified as a member of the FZ family of GPCRs. The specific contributions of conserved GPCR functional domains to SMO-mediated regulation of HH pathway activity are discussed below (Fig. 3.1).

Cysteine-rich domain (CRD). A conserved CRD is situated in the extracellular amino terminus of all FZ family GPCRs [33–35]. Disulfide bonds between amino-terminal cysteine residues and/or cysteine residues in the extracellular loops of FZ drive receptor conformations that are necessary for its ligand binding and ligand-induced dimerization [34, 36]. Like FZ, SMO possesses multiple cysteines in its amino terminus and extracellular loops that are positionally conserved across species (http://www.gpcr.org). In vitro studies in mammalian cell culture suggested that the amino-terminus of SMO, which encompasses the CRD, is not required for GLI activation [37]. However, genetic analyses in both Drosophila and zebrafish

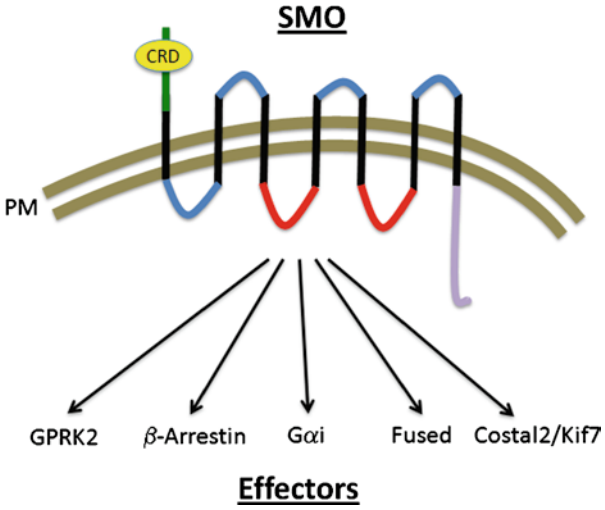

Fig. 3.1 Domains and effectors of smoothened (SMO). A schematic depicting the predicted topology and domains of SMO in the plasma membrane is shown. The seven predicted transmembrane domains of SMO are shown in *black*. The amino-terminal domain is shown in *green* and the cysteine-rich domain (CRD) in *yellow*. Three extracellular and one intracellular loops are shown in *blue*, but intracellular loops 2 and 3 – which are thought to couple to G-proteins – are shown in *red*. The carboxyl-terminal domain is shown in *purple*. The various known direct effectors of SMO are indicated below it

support that conserved cysteine residues in the CRD are critical for SMO signaling and/or subcellular localization [38–40], suggesting that the CRD is a requisite functional domain. Further studies are needed to more clearly define contributions of the CRD to SMO signaling.

Transmembrane domains and intracellular loops. The topology of the 7TM regions dictates the activation state of a GPCR by directing the conformation of its intracellular loops and cytoplasmic tail [41]. In response to ligand, the TM domains of the receptor shift to allow changes in conformation of the intracellular portions of the protein that facilitate receptor phosphorylation and/or G-protein selectivity, docking, and activation [41]. The importance of SMO TM sequence/structure is underscored by known oncogenic SMO mutations, all of which are localized to predicted TM segments [6, 42]. It is likely that these mutations lock SMO TM and intracellular domains in an activated state, which is insensitive to PTCH-mediated inhibition.

The cytoplasmic tail along with intracellular loop 3 (ic3), and to a lesser extent ic2, constitute the G-protein docking site on the vast majority of GPCRs [43]. While extensive structure/function analysis of the SMO intracellular loops has not been reported, chimeric studies in cultured fibroblasts reveal a critical role for ic3 in activation of the signaling cascade [37]. These findings are supported by a loss-of-function *SMO* mutation in Drosophila of a highly conserved Arg residue localized to the carboxyl-terminal end of loop ic3 [39]. The importance of the intracellular

loops is further supported by a study demonstrating that introduction of peptide analogs of either ic2 or ic3 into cultured cancer cell lines, which have an activated HH signaling pathway, attenuates their proliferation [44]. Further studies are needed to identify binding partners of SMO ic2 and ic3, and to determine whether these domains constitute a binding site for a partner G-protein.

Carboxyl-terminal intracellular tail. Multiple phosphorylation sites, which have been shown to be critical for pathway activation, have been identified in the SMO carboxyl-terminal tail [45–47]. Phosphorylation of such sites in response to ligand is a well-characterized event in canonical GPCR signaling, which generally serves to recruit various adaptor proteins and signaling effectors to the activated receptor [48]. Accordingly, HH-stimulated phosphorylation of SMO by PKA and G-protein regulated kinase 2 (GRK2) triggers both adaptor protein recruitment and SMO multimerization [49–51]. Mutations that prevent phosphorylation of any of these characterized phosphorylation sites compromise the ability of SMO to signal [45–47].

SMO Signaling Through Heterotrimeric G-Proteins

We recently demonstrated that *Gαi* overexpression in Drosophila triggers activation of HH target genes and wing patterning defects consistent with excessive HH signaling [52]. These phenotypes correlated with the activation state of the expressed *Gαi* transgene, as overexpression of a *Gαi* mutant that cannot bind GTP resulted in no observable phenotype. Conversely, overexpression of wild-type Gαi triggered modest gain of function phenotypes, and overexpression of a transgene encoding constitutively active *Gαi* resulted in strong HH gain of function phenotypes.

Activation of heterotrimeric G-proteins of the Gαi family frequently serves to decrease intracellular pools of cAMP through Gαi-mediated inhibition of adenylate cyclase (AC) [53]. Accordingly, in our study, we observed a SMO- and Gαi-dependent reduction in total intracellular cAMP within 5–10 min of HH stimulation [52]. Modulation of cAMP appears to be critical for in vivo HH signaling, as a mutant allele of the cAMP phosphodiesterase *DUNCE* [54] enhanced the HH loss-of-function phenotype induced by expression of a dominant negative SMO mutant in the Drosophila wing [52]. The ability of cAMP to modulate HH pathway-dependent patterning events is further supported by studies demonstrating that overexpression of an anthrax virulence factor, which functions as a potent bacterial AC, triggers wing phenotypes similar to HH loss-of-function mutations [55]. Further, modulation of cAMP by Sonic HH (SHH) has also been demonstrated in vertebrate systems: retinal ganglion cell axons exposed to recombinant SHH reduce their intracellular pools of cytoplasmic cAMP [56], while frog melanophores exposed to SHH aggregate their melanosomes, a process favored by low concentrations of intracellular cAMP [57]. Taken together, these studies support that one mechanism by which SMO initiates HH signal transduction is to regulate cAMP production through the activation of Gαi family heterotrimeric G-proteins.

Gαi as a context-specific modulator of HH signaling. In vitro studies on vertebrate SMO support the ability of SMO to activate a subset of heterotrimeric G-proteins, with strongest effects on those of the Gαi family, for which SHH-induced GTP binding has been demonstrated [57–60]. Activation of Gαi in these systems fulfills the requirements of canonical HH pathway induction, as it can be inhibited by PTCH and/or small-molecule SMO inhibitors, and can be activated by SHH stimulation [57, 60]. SHH target gene induction in cultured fibroblasts is sensitive to pertussis toxin (PTX), a potent Gαi inhibitor, further supporting that Gαi is engaged by vertebrate SMO in response to ligand stimulation [60].

The above studies provide support for involvement of Gαi in HH signal transduction. However, studies performed in differing developmental or cellular contexts failed to identify a role for Gαi in the HH pathway [37, 61–63]. RNAi screens in cultured Drosophila cells did not implicate Gαi as a component of the HH signaling pathway [62, 63], while studies performed in cultured 10T1/2 cells failed to detect changes in intracellular cAMP following SHH stimulation [37]. The latter might be explained by findings that the bulk of HH signaling in vertebrate cells appears to occur in the primary cilium, a small sensory organelle that is present on most vertebrate cell types [64]. Because the volume of the primary cilium is negligible when compared to the body of the cell, localized changes in ciliary cAMP may be undetectable in whole cell lysates.

Conflicting results have also been obtained from in vivo studies examining the role of Gαi in HH signaling. Uncoupling of SMO from Gαi by expression of the PTX catalytic subunit in chick neural tube did not demonstrate compromised SHH-dependent neural cell type specification, suggesting that Gαi is not required for SHH patterning events in this developmental context [61].

Taken together, these seemingly conflicting results raise the possibility that Gαi is required only in certain cellular or tissue contexts during development. This suggestion is supported by the observation that while chick retinal ganglion axon explants are sensitive to SHH-mediated cAMP modulation and growth suppression, chick neural tube explants are not [56]. Signaling redundancy in specific tissues and/or at distinct developmental time points may also account for the apparent lack of Gαi involvement in HH signaling in some in vivo systems. This possibility is supported by work in both Drosophila and cultured vertebrate cells, which show multiple activating signals and feed-forward loops originating from SMO following ligand stimulation [26, 65, 66]. Further studies are required to ascertain if these additional SMO signals are dominant, or can compensate when Gαi is compromised.

A SMO-Dependent G-Protein Signaling Network

Based on a series of elegant biochemical reconstitution experiments, heterotrimeric G-proteins are proposed to function as ligand-gated switches [53]. Ligand stimulation triggers the GPCR to serve as a guanine nucleotide exchange factor (GEF) for its partner heterotrimeric G-protein, allowing the GDP-bound Gα subunit to bind

GTP and become activated. Upon activation, Gα was originally thought to dissociate from its Gβγ subunits and interact with its effector(s) through random collision along the plasma membrane. Initially the only known effector of G-proteins was adenylate cyclase (AC), the enzyme that converts ATP into cAMP. As such, the Gα subunit responsible for *s*timulating AC, and driving cAMP production was named Gαs, while the Gα subunit that inhibited AC activity to lower cAMP production was named Gαi [53]. Gα subunits were originally believed to be attenuated by their own intrinsic GTPase activity to return the Gα to its inactive GDP-bound form, thereby allowing it to reassociate with its partner Gβγ subunits.

The identification of additional G-proteins, the advent of molecular biology, and the subsequent investigation of G-protein function in vivo culminated to show that more regulators of the G-protein GTPase cycle were required than initially predicted by the in vitro model [67, 68]. These regulators consist of non-receptor GEFs that promote GDP release, novel inhibitors of GDP release (GDI), *r*egulators of *G*-protein *s*ignaling (RGS) that significantly increase the GTPase activity of the Gα subunit, and GRKs that function to desensitize the activated GPCR and/or propagate receptor signaling [69]. Numerous Gα and Gγ subunits have now been described and demonstrated to be capable of signaling themselves, regulating their own spectrum of specific effectors [69].

The discovery that SMO can signal as a *bona fide* GPCR has the potential to quickly expand the number of signaling proteins regulated by SMO, to include those that act as part of a SMO regulated G-protein signaling network (SGN). Thus, we anticipate that like other GPCRs SMO might regulate a large network of signaling proteins, including other G-proteins, and modifiers and effectors of these G-proteins. We discuss below evidence for such a network of regulators, and what some of the novel components of this SGN might be.

G-protein modulators. Although overexpression of activated Gαi in vivo resulted in strong HH gain of function phenotypes, we found that attenuation of Gαi function triggered only mild HH loss of function phenotypes [52]. These weak phenotypes might indicate that another *Gα* gene product, of which there are five in Drosophila, functions in a redundant manner with *Gαi* during Drosophila development. A likely candidate gene for this redundant function is *Gαo*, a member of the *Gαi* family that can function redundantly with Gαi in other systems [53]. Further, the mammalian homologue of the Drosophila Concertina α subunit, Gα12/13, has been implicated in SHH-mediated regulation of the small GTP-binding protein Rho [58]. Although our survey of three *Gα* gene products did not implicate Gαs in HH pathway regulation, a genome-wide screen in cultured Drosophila cells showed that knocking down *Gαs* could enhance HH signaling activity [63]. Further work is needed to determine if Gαs might represent a feedback mechanism that resets the basal level of cAMP after HH induces a decrease in cellular cAMP concentration via Gαi.

Another group of G-proteins we anticipate will serve as novel SMO effectors are the Gβγ subunits of its partner heterotrimeric G-protein(s). At a minimum, these proteins could function as negative regulators of Gαi by acting as GDIs [69]. The Gβγ subunits might also have the capacity to regulate their own novel set of effectors in the HH signaling cascade and/or modulate effectors that they share with

their Gα subunits. One such effector, AC, is stimulated by Gβγ proteins [69]. Therefore, like Gαs, Gβγ subunits activated in response to HH might be utilized to reverse Gαi-induced decreases in intracellular cAMP.

GPCR kinases and arrestins. As discussed above, phosphorylation of GPCRs on the cytoplasmic carboxyl-terminal tail is a common event following ligand stimulation. Phosphorylation regulates wide-ranging events including receptor subcellular localization, association with downstream pathway effectors, and commonly serves to recruit β-arrestin type adaptor proteins, which can propagate receptor signaling and/or drive receptor internalization and desensitization [70, 71]. Phosphorylation of activated receptors is driven primarily by the GRK family of kinases. GRK2 regulation of SMO follows a well-established GPCR paradigm: GRK2 phosphorylation triggers β-arrestin recruitment, which drives SMO to clathrin-coated pits where it undergoes activation-dependent internalization [72]. Interestingly, rather than desensitizing SMO to attenuate transduction of the HH signal, GRK-mediated phosphorylation and subsequent β-arrestin recruitment appear to regulate positive steps in HH signaling. Co-expression of GRK2 with SMO in cultured C3H10T1/2 cells enhances SMO-dependent activation of GLI, while GRK knockdown in cultured HEK293 cells attenuates SMO signaling in response to the SMO agonist SAG [50, 73]. An in vivo requirement for GRK2 in HH signaling was confirmed through studies analyzing zebrafish and mice lacking GRK2 function. In both cases, these animals demonstrated developmental phenotypes consistent with HH loss of function [73].

Drosophila GRK2 (dGRK2) has been demonstrated to be both a positive regulator of SMO signaling as well as a HH target gene, suggesting that it functions in a ligand-induced feed-forward loop [74]. As is the case in vertebrate systems, phosphorylation of SMO by dGRK2 results in both β-arrestin recruitment and SMO internalization in HH receiving cells [49]. dGRK2 appears to function only on activated SMO that has transduced a signal in response to ligand, as dGRK2 overexpression in wing discs triggers the removal of SMO from the plasma membrane without attenuating HH target gene induction. dGRK2-mediated internalization of SMO is independent of PTCH-driven removal of SMO from the plasma membrane in cells not receiving the HH signal, further suggesting that dGRK2 functions solely to regulate activated SMO [49]. This supports that SMO plasma membrane localization is regulated in a manner similar to numerous other GPCRs: in the absence of ligand stimulation, SMO undergoes a tonic endocytosis that is regulated by PTCH [75], while ligand stimulated SMO is internalized by the combined activity of dGRK and β-arrestin [49, 72]. As is the case with vertebrate SMO, assembly of the dGRK/β-arrestin complex on dSMO appears to be a positive regulatory event, despite it resulting in the eventual removal of SMO from the plasma membrane [49, 72]. Taken together, these studies highlight the importance of an evolutionarily conserved regulatory complex that assembles in response to ligand-induced GRK phosphorylation of SMO, of which β-arrestin appears to be paramount.

Protein kinase A. PKA was originally identified as a cAMP stimulated protein kinase, consisting of two regulatory subunits and two catalytic subunits [76].

The regulatory subunit inhibits the activity of the catalytic subunit, and this repression is released when the regulatory subunit binds to cAMP. PKA phosphorylates a broad spectrum of substrates, resulting in many diverse biological outputs. The various functions of PKA are thought to be spatially distinct, with PKA binding to its substrates and regulators on scaffolding proteins called A kinase anchoring proteins (AKAPs) [77]. AKAPs cluster relevant GPCRs, G-proteins, kinases, and other downstream effectors to discrete localizations within a cell [69, 77]. COS2 has been demonstrated to associate with SMO, downstream HH effectors, as well as with PKA and CK1 [20, 21, 24, 25]. As such, we hypothesized that COS2 might act as a nexus for HH signaling in a manner akin to that of AKAP proteins. We tested this hypothesis and noted that Gαi and COS2 do associate, and that this association was enhanced by HH [52]. It is, therefore, likely that COS2 acts as a scaffolding protein to recruit SMO, Gαi, and PKA, and likely, analogous to how AKAPs function, might also act to locally modulate the levels of cAMP.

PKA was initially shown to function as a negative regulator of HH signaling, phosphorylating CI in order to convert it to its repressor form [78, 79]. It was later identified as a positive regulator of HH signaling, through its ability to phosphorylate and stabilize SMO to result in SMO enriching at the plasma membrane in a highly active form [25, 46, 80]. Thus, PKA plays two seemingly opposite roles in HH signaling – in the absence of HH it acts to keep the HH pathway in its off-state and in the presence of HH functions to convert SMO into its active form. Consistent with the important role PKA plays in HH signaling, *Costal1 (COS1)* mutations, which enhance the phenotype of *COS2* mutations, were recently shown to encode mutations in both the regulatory and catalytic subunits of PKA [81].

It has been suggested that the role PKA plays in HH signaling is cAMP independent [78, 79]. This hypothesis was presented to explain the observation that a mutant mouse PKA catalytic subunit was able to rescue a *PKA* null mutation in Drosophila. These experiments assumed that the mutant PKA catalytic subunit would be unable to associate with the regulatory subunit of Drosophila PKA. However, recent demonstrations of SMO coupling to Gαi and regulating cAMP levels suggests that a cAMP independent role of PKA in HH signaling may not be correct [52, 60, 65]. Moreover, the identification of a *COS1* mutation encoding a PKA regulatory subunit, which modulates HH signaling, is consistent with a cAMP-dependent activation of PKA [81].

Small-Molecule Modulators of SMO

Mice engineered to lack SHH die shortly after birth and exhibit a wide range of developmental defects, including cyclopia [82]. A similar phenotype was observed in offspring of livestock that ingested the corn lily *Veratrum californica*. Two groups recognized the similarities between these phenotypes and tested the hypothesis that a chemical derived from this plant, cyclopamine, functioned as an inhibitor of HH signaling [83, 84]. Cyclopamine turned out to be a potent inhibitor

of HH signaling, in vitro and in vivo, and was subsequently shown to bind directly to the heptahelical bundle of SMO to functionally antagonize its signaling capability [85]. Consistent with SMO facilitating a rate-limiting step in HH pathway activation, numerous small-molecule screens for novel HH inhibitors have identified distinct SMO antagonists [9]. Many of these SMO inhibitors act in a competitive manner with cyclopamine for binding to SMO, supporting that they bind the 7TM segments. However, some of these SMO modulators bind to SMO in a non-competitive manner and/or activate SMO, suggesting that SMO may have a number of different small-molecule binding sites, as is the case with numerous GPCRs [43].

One basic tenet of pharmacology is that drugs themselves do not possess intrinsic biological properties, but rather can only act to modify existing biological processes [86]. Thus, the identification of small-molecule modulators of SMO implied the existence of endogenous SMO modulators. Furthermore, it has been known for a number of years that the HH receptor PTCH has significant homology with a family of physiological pumps in bacteria, leading to the speculation that PTCH functions to regulate the concentration of such an endogenous SMO modulator [13, 87]. Consistent with this homology, a recent study using a mixed-cell culturing system provided evidence for a lipophilic molecule being pumped into the culture medium in a PTCH-dependent manner [88]. This molecule was identified as the oxysterol, pro-vitamin D3, which was demonstrated to bind SMO in manner similar to that of cyclopamine. Purified pro-vitamin D3 inhibited HH activity, both in vitro and in vivo, with a potency similar to that of cyclopamine [88]. This was one of the first identifications of an endogenous SMO modulator, in this case an antagonist. Two other groups subsequently identified oxysterol molecules that functioned as HH activators [89, 90], suggesting that, like numerous GPCRs, SMO activity is controlled by endogenous small-molecule ligands.

Future Directions

As the critical role(s) that HH signaling plays in tumor growth and progression continues to emerge, and the clinical use of SMO antagonists increases, the impact of on-target adverse effects is likely to become evident. For example, one SMO antagonist was recently demonstrated to have significant efficacy against medulloblastoma [91], but to induce growth defects when administered to young mice [92]. This study was performed to reveal potential problems that may be encountered by inhibiting a developmentally relevant signaling pathway in a pediatric population, the most common class of patients presenting with medulloblastoma [93]. Long bones of the limbs of animals exposed to this compound during early development were found to be significantly shortened, an effect likely due to specific inhibition of Indian HH-regulated bone growth [92]. This observation underscores the importance of delineating all the signaling events immediately downstream of SMO, as one could anticipate that various classes of small-molecule SMO inhibitors

might affect distinct signaling arms. Moreover, classes of SMO antagonists that only inhibit a distinct subset of SMO effectors, such as the SGN effectors, might be used clinically for specific classes of cancer patients. Ideally, such compounds would inhibit the effectors relevant to tumor growth while having minimal impact on effectors more relevant to the role HH plays in tissue homeostasis. Such drugs would be particularly useful to medulloblastoma patients, whose ability to take at least a subset of the SMO antagonist currently in clinical trials would be severely compromised by on-target developmental defects.

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Chapter 4
Kinases and Phosphatases in Hedgehog Signaling

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Keywords Smo • Ci/Gli • PKA • CK1 • CK2 • GSK3 • Fu • PP4 • PP2A

Since the nineteenth century, phosphates have been known to be bound to proteins. In 1954, an enzyme responsible for the transfer of a phosphate to another protein was discovered; this biological reaction is referred to as phosphorylation [1]. The enzyme catalyzing phosphorylation was known as a protein kinase. A year later, it was demonstrated that a protein involved in glycogen metabolism was regulated by the addition or removal of a phosphate, suggesting that reversible phosphorylation could control the activity of a specific protein [2, 3]. The study of phosphorylation was then brought into the limelight of medical research. In 1992, Fischer and Krebs received the Nobel Prize in medicine for their pioneering work. Today, it is well-known that phosphorylation/dephosphorylation is one of the most important posttranslational events in cell biology. It has been shown to be involved in many aspects of regulation such as increasing or decreasing the biological activity of a protein, facilitating the sorting of intracellular proteins, mediating protein–protein interactions, as well as labeling proteins for degradation. Importantly, the reception of a signal on the surface of a cell often results in a change in cellular phosphorylation patterns with various proteins being phosphorylated or dephosphorylated, which might eventually cause changes in cellular behavior.

Protein phosphorylation in Hedgehog (Hh) signaling has been extensively studied. Like the proteins in other signaling pathways such as Wnt and NFkB, various Hh pathway components are phosphorylated at multiple residues and most of these phosphorylation events are regulated by the upstream signal [4]. Understanding how the phosphorylation events in Hh signaling are normally controlled may provide new avenues for developing therapeutics aimed at preventing and treating human cancers

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due to aberrant Hh signaling. We begin this chapter with a discussion of the functions of protein phosphorylation in various situations of Hh signal transduction. We will then review the balance between phosphorylation and dephosphorylation of the critical components that are involved in transducing the upstream signal. After that, we will discuss the idea of differential phosphorylation mediating the Hh signaling activity gradient in animal development.

Phosphorylation Events in Hh Signaling

Hh exerts its biological influence through a highly conserved signal transduction cascade. The mammalian Sonic Hedgehog (Shh), as well as the *Drosophila* Hh, can function as morphogens and regulate cell proliferation, embryonic patterning, and organ development. As discussed in the previous chapters, the Hh signal is transduced through a reception system that includes the 12-span transmembrane protein, Patched (Ptc), and the 7-span transmembrane protein, Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo signaling activity by a mechanism that is still not clear, even though studies indicate that small molecules, such as lipids, could be involved [5, 6, 7]. The presence of Hh relieves the inhibition of Smo by Ptc, activating Smo and allowing Smo to regulate the downstream signaling components. The main outcome of Hh signaling is the modulation of transcriptional responses via the cubitus interruptus (Ci)/Gli family of zinc-finger transcription factors.

The *Drosophila* system is an ideal model system with which to study the Hh pathways because of the fruit fly's well-established genetics, small genome size, minimal genetic redundancy, and ease of handling. Studies with *Drosophila* have provided invaluable information on Hh signaling. In *Drosophila*, Hh induces cell-surface accumulation and phosphorylation of Smo [8]. Recent studies identified protein kinase A (PKA) and casein kinase 1 (CK1) as two of the kinases that phosphorylate Smo and increase its cell-surface localization and signaling activity [9–11]. We discuss the regulation of Smo phosphorylation in a separate section below. In addition to Smo, other pathway components, such as Fused (Fu), Costal2 (Cos2), and Suppressor of Fused (Sufu) are phosphorylated upon Hh stimulation [12, 13]. Fu was first identified as a serine/threonine (Ser/Thr) kinase that regulates segmental polarity in *Drosophila* [14, 15]. Fu is composed of a catalytic domain and a carboxy-terminal regulatory domain and has been shown to be essential for Hh pathway activation [16]. Fu is in a non-phosphorylated, inactive state in the absence of Hh and a phosphorylated active state in the presence of Hh [17, 18]. The responsible for kinase Fu phosphorylation is unknown, although it was proposed that Fu activity is autoregulated through an intramolecular mechanism [19], suggesting that Fu could be auto-phosphorylated. In addition, mutational analysis of Fu identified Thr158 in its activation loop to be a critical residue that is phosphorylated upon Hh stimulation [20]. The identification of the kinase that phosphorylates and thus activates Fu, and the biological significance of this phosphorylation event await further investigation.

Cos2, a kinesin-like protein, exists in a large protein complex with Fu and the full-length Ci [21, 22]. Cos2 mainly acts as a negative regulator in Hh signal transduction as loss of function mutations in *cos2* results in accumulation of high levels of full-length Ci (Ci^{FL}) and activation of Hh target genes [22, 23]. Cos2 inhibits the transcriptional activator activity of Ci^{FL} by inhibiting its nuclear translocation [24–27]. Cos2 also acts as a scaffold protein to bring PKA, CK1, and glycogen synthase kinase 3 (GSK3) to Ci^{FL}, thus promoting sequential phosphorylation of Ci^{FL} by these kinases [28, 29], which leads to proteasome-mediated Ci^{FL} processing to generate its repressor form Ci^{REP} (see below section). In addition to the role in Ci processing, Cos2 exerts a negative effect on Smo, which blocks the phosphorylation and activation of Smo likely by masking its phosphorylation sites [30]. Other than its negative roles in Hh signal transduction, Cos2 has a positive role in Hh-responding cells, and this correlates to its ability to form a complex with the C-terminal intracellular tail of Smo [18, 26, 31, 32]. In addition, Fu is diminished in cells lacking Cos2 [18, 33], which might explain, at least in part, why Hh signaling activity is attenuated in *cos2* mutant cells adjacent to the A/P boundary where there is Hh stimulation [34] in *Drosophila* wing discs. Interestingly, Cos2 is also phosphorylated in response to Hh and this phosphorylation depends on Fu kinase activity [18, 35]. Phosphorylation of Cos2 at Ser572 by Fu disassociates the Cos2–Smo–Ci complex, which promotes Cos2 degradation, activates Smo, and stabilizes Ci [30, 36].

Sufu was first identified in *Drosophila* and its name came from the ability to suppress the wing vein defect of the *fu* mutant [14]. In *Drosophila* Hh signaling, Sufu forms a large protein complex with Cos2, Fu, and Ci and is reported to regulate Ci subcellular localization and transcriptional activity in the nucleus [26, 37]. However, *Drosophila* embryos lacking Sufu display intact Hh signaling and develop into viable and fertile adults [38]. The role of Sufu in mammalian Hh signaling is strikingly different. Loss of Sufu in mammals leads to ectopic Hh pathway activation, suggesting that Sufu is the major intracellular inhibitor of Gli activity [39]. It has also been speculated that Sufu might have adopted other functions during evolution [39]. Interestingly again, *Drosophila* Sufu is phosphorylated in response to Hh and this phosphorylation event depends on Fu kinase activity [18, 40], although there is no evidence to indicate that Fu is a direct kinase for Sufu; it is possible that other kinase(s) are involved. In addition, further studies are necessary to characterize the consequence of Sufu phosphorylation in response to Hh stimulation.

Kinases Regulating the Transcriptional Factor, Ci/Gli

Hh signaling regulates the balance between the transcriptional activator and repressor forms of Ci/Gli [4, 12], which is mediated by phosphorylation. Ci plays dual roles that are performed by two distinct forms. In the absence of Hh, Ci^{FL} undergoes proteolytic processing to generate a truncated form (Ci^{REP}). Ci^{REP} functions as a repressor to block the expression of Hh responsive genes such as *decapentaplegic* (*dpp*) [41, 42], which encodes a member of the TGFβ/BMP family of secreted

proteins that functions as a long-range morphogen to control the growth and patterning of the wing [43, 44].

What triggers Ci proteolytic processing? The initial discovery came from a study of *Drosophila* cAMP-dependent PKA. It was reported that loss-of-function mutations in the catalytic subunit of PKA led to constitutive Hh signaling [45–48]. Subsequent work indicated that PKA phosphorylates Ci at multiple Ser/Thr residues in its C-terminal region, which is essential for the processing [49–51]. Later, GSK3 and CK1 were identified and characterized as two other kinases that act cooperatively with PKA to promote Ci processing [52, 53]. GSK3 and CK1 phosphorylation of Ci are primed by PKA phosphorylation at nearby Ser/Thr residues; these sites form critical phosphorylation clusters within the C-terminal region of Ci and this hyperphosphorylation promotes Ci processing [54]. Thus, Ci processing requires phosphorylation by multiple kinases including PKA, GSK3, and members of CK1 family.

How does phosphorylation of Ci regulate its processing? Early in 1998, an F-box/WD40 containing protein, Slimb, was identified to act downstream of PKA to promote Ci processing [55]. The vertebrate homolog of Slimb, βTRCP, functions as a substrate recognition subunit of the so-called SCF (*S*kp1, Cullin, and *F*-box) ubiquitin ligase complex that normally targets phosphorylated substrates, such as Iκ-B and β-catenin, for ubiquitination, and subsequent proteasome-mediated proteolysis [56]. The phosphorylation of Ci at multiple phosphorylation clusters creates binding sites for the SCF complex [57, 58], leading to partial degradation of Ci thus generating the truncated repressor form Ci^{REP} [5, 59].

How does Hh inhibit Ci processing? An elegant study by Zhang et al. describes a precise mechanism by which Hh inhibits Ci phosphorylation [28]. Ci^{FL} exists in a large protein complex including Cos2 and Fu. Complex formation impedes nuclear translocation of Ci^{FL} through microtubule-dependent and independent mechanisms [60]. Interestingly, Cos2 directly associates with PKA, CK1, and GSK3 and acts as a scaffold between these kinases and Ci^{FL} to facilitate Ci^{FL} phosphorylation [28]. An in vivo experiment performed in this study demonstrated that a Kinesin-Cos2 chimeric protein, in which the microtubule-binding domain of Cos2 is replaced by a canonical kinesin motor domain, carried Cos2-interacting proteins to the microtubule plus end. Accumulation of Kinesin-Cos2 at the plus end of microtubules allowed for a discrete intracellular localization that allowed the enrichment of PKA, CK1, and GSK3 at the same position. In addition, Cos2 was shown to be required for Ci phosphorylation by these kinases. Importantly, Hh signaling appears to inhibit Ci phosphorylation and processing by dissociating the Cos2-Ci-kinase complex [28].

In vertebrate species, three Gli proteins, Gli1, Gli2, and Gli3, are the transcriptional mediators of Hh signaling. Genetic and biochemical studies suggest that Gli2 and Gli3 are the primary mediators of Hh signaling. Gli1 is a transcriptional target of Hh signaling and provides positive feedback to reinforce the Hh signaling activity [61–68]. Gli2 mainly functions as an activator and Gli3 as a repressor, although in some developmental contexts the repressor activity of Gli2 and the activator activity of Gli3 have also been detected [65, 69–72]. Consistent with their ability to function

as both an activator and repressor, Gli2 and Gli3 are proteolytically processed to truncated forms, and there is evidence that the processing Gli2 and Gli3 are inhibited by Hh [73–76]. The multiple clusters of phosphorylation sites found in Ci are also present in Gli proteins [4], suggesting that Gli processing could also be regulated by multiple kinases and Slimb/β-TRCP in a similar manner to the aforementioned regulatory mechanisms for Ci processing. Indeed, studies have shown that the processing of Gli3 requires the binding of βTRCP to phosphorylated Gli3 [77, 78].

Kinases Mediating the Activation of Smo in Response to Hh

As discussed in early chapters of this book, the Hh signal is transduced through a reception system at the plasma membrane that includes the receptor complexes Ptc–Ihog and the signal transducer Smo [4, 12, 79]. Binding of Hh to Ptc–Ihog relieves the inhibition of Smo by Ptc, which allows Smo to activate Ci/Gli transcription factors. Regulation of Smo activity remained poorly understood until recently. Smo has homology to G-protein-coupled receptors (GPCRs) [80], and a recent study revealed that Gαi, a G-protein in *Drosophila*, is essential for full activation of Hh signaling [81]. However, Smo is not a typical GPCR because it does not directly bind to Hh ligand and, upon Hh stimulation, it accumulates on the cell surface (see below) and recruits downstream components [18, 31–33, 85]. In *Drosophila*, Hh induces cell-surface accumulation and phosphorylation of Smo [8]. Recent studies from several labs identified PKA and CK1 as two of the kinases that directly phosphorylate Smo and regulate its cell-surface expression and activity [9–11]. Blocking PKA or CK1 activity prevents Hh-induced Smo accumulation and attenuates pathway activity, whereas increasing PKA activity promotes Smo accumulation and pathway activation [9]. Interestingly, similar to the phosphorylation clusters in Ci, Smo is phosphorylated by PKA and CK1 at three clusters of residues in its intracellular domain both in vitro and in vivo [9–11]. Phosphorylation-deficient forms of Smo in which PKA or CK1 sites are mutated to alanine (Ala) are defective in Hh signaling. Phosphorylation appears to be sufficient to activate Smo as phosphorylation-mimicking Smo variants in which PKA and CK1 sites are converted to acidic residues, such as aspartate (Asp) and glutamate (Glu), exhibit constitutive signaling activity [9, 11].

It is interesting that the same set of kinases plays opposing roles and phosphorylates distinct substrates depending on the signaling states. In the *Drosophila* Hh pathway, PKA and CK1 phosphorylate Ci in the absence of Hh to inhibit pathway activation. In the presence of Hh, PKA and CK1 phosphorylate Smo to stimulate its signaling activity. Similarly, in the Wnt signal transduction, the absence of Wnt allows GSK3β and CK1 to phosphorylate β-catenin, thus inhibiting Wnt signaling. Upon Wnt stimulation, GSK3β and CK1 phosphorylate the Wnt coreceptor LRP5/6 to activate signaling [82, 83]. These observations raise the important question of how different substrates are chosen by the same kinases in different signaling states. One solution, as suggested by the studies on the Wnt pathway, is that each pathway

may utilize two distinct pools of kinases, one in proximity to the transcription factor/effector and the other to the membrane reception system [82, 83].

What is the consequence of Smo phosphorylation? Like many GPCRs, Smo forms homodimers and undergoes a confirmational change when it is phosphorylated by PKA and CK1 upon Hh stimulation [83]. Zhao et al., using fluorescence resonance energy transfer (FRET) analysis, observed a basal level of FRET when using the donor cyan fluorescent protein (CFP) and the acceptor yellow fluorescent protein (YFP) to be fused to the C-terminal tail of Smo. They further found that this basal FRET was dramatically increased by Hh treatment. In addition, CFP- and YFP-tagged Smo N-terminal part exhibited a high level of FRET even in the absence of Hh treatment, suggesting that Smo forms a constitutive dimer/oligomer through its N-terminal region while the C-tails are within a dimer and are situated at a distance but are brought together in response to Hh through a conformational switch [84]. The dimerization model explains why the phosphorylation-deficient forms of Smo, as well as a chimeric Smo variant with its C-tail replaced with that of the Wingless (Wg) receptor Frizzled 2 (Fz2), inhibited the activity of endogenous Smo [9, 10, 85]. How does Hh induce a conformational switch in the C-tail of Smo? The degree of conformational change in the C-tail of Smo, monitored by FRET, correlates with the level of its phosphorylation that can be manipulated by substitution of various Ser/Thr residues into either Ala or Asp [84]. Thus, a series of Smo conformational states rely on the level of Hh activity and Smo phosphorylation. More interestingly, stretches of positively charged arginine (Arg) residues residing adjacent to the phosphorylation clusters keep the C-tail of Smo in the inactive conformation. Phosphorylation of Smo at adjacent PKA and CK1 sites neutralizes the positive charges conferred by the Arg motif, leading to Smo dimerization and activation [84]. In addition to inducing a conformational change, phosphorylation also promotes Smo accumulation on the cell surface [8, 9].

How does Hh regulate Smo phosphorylation and cell-surface accumulation? Although Smo phosphorylation promotes its cell-surface accumulation and signaling activity, the mechanisms leading to its cell-surface accumulation are still unknown. Fu and Cos2 exist in a large protein complex [21, 22]. Smo transduces the Hh signal by physically interacting with the Cos2-Fu complex [18, 31–33, 85]. Cell-surface recruitment of an intracellular signaling complex through accumulated Smo is thought to cause dissociation of the Cos2-Ci-Kinase complex and hence inhibition of Ci processing [28]. Thus, the intracellular signaling complex containing Fu and Cos2 was thought to transduce the Hh signal downstream of Smo. Surprisingly, Liu and colleagues identified a mechanism of feedback regulation of Smo by the Fu–Cos2 protein complex [86]. They found that Fu is essential for Hh-induced Smo phosphorylation and cell-surface accumulation because Smo is inhibited in *fu* mutant clones or by expressing a dominant-negative (DN) form of Fu, and such inhibition is alleviated by removal of Cos2. Conversely, overexpressing Cos2 blocks Smo accumulation, which is reversed by coexpressing Fu. In addition, Fu antagonizes Cos2 by phosphorylation at Ser572, which attenuates the Cos2-Smo interaction and promotes Cos2 degradation. The authors further provided evidence that Fu and Cos2 control Smo cell-surface accumulation by regulating Smo phosphorylation [86].

Consistently, the observations by Claret et al. demonstrated that Fu plays a positive role in Smo activation [87]. These data suggest that Hh signaling proceeds through an amplification step mediated by Smo and its downstream components. In addition to regulating the phosphorylation of Smo, Hh may regulate the dephosphorylation of Smo that is mediated by its phosphatase (see the section "Phosphatases in Hh signaling" below).

Is there another kinase involved in regulating Smo? The answer to this question is yes. It has been shown that the C-tail of Smo has 26 Ser/Thr residues that are phosphorylated in response to Hh [11]. Several phosphorylated sites in the C-tail of Smo match the consensus sequence for casein kinase 2 (CK2); however, the link between CK2, Smo phosphorylation, and Hh signaling is lacking. We found that RNAi of CK2 attenuates Hh-induced Smo accumulation and downregulates Hh target gene expression, whereas increasing CK2 activity by overexpressing CK2 increases Smo accumulation and induces ectopic expression of Hh target gene. Mutating the CK2 consensus residues attenuates the ability of Smo to transduce high-level Hh signaling activity (unpublished). There is also the possibility that G-protein-coupled receptor kinase 2 (GRK2) phosphorylates Smo because GRK2 plays a conserved role in modulating the Hh pathway [5, 39], and GRK2 has been shown to regulate Smo in *Drosophila* [88, 89] and in mammals [90, 91]. Besides, a genetic screen in *Drosophila* also identified a candidate kinase that could possibly regulate Smo (Holmgren, meeting abstract). Above all, we have learned that multiple kinases are involved in Smo phosphorylation that is triggered by the upstream signal.

Non-conserved Mechanisms of Regulation by Kinases

Vertebrate Smo proteins lack PKA and CK1 phosphorylation consensus sites found in *Drosophila* Smo, which implies that vertebrate Smo is not regulated by direct PKA and CK1 phosphorylation. However, GRK2 and perhaps other kinases may regulate the conformation of Smo in vertebrates. In support of this hypothesis, in response to Hh stimulation, vertebrate Smo proteins exhibit a similar conformational switch to that utilized by *Drosophila* Smo [84]. Interestingly, a long stretch of Arg residues, which is conserved among vertebrate Smo proteins, negatively regulates mouse Smo likely by keeping it in an inactive conformation [84]. Additionally, there is evidence that mammalian Smo is phosphorylated in cultured cells by GRK2 [90]. It is also tempting to speculate that Hh-induced phosphorylation of Smo through GRK2 alters the intracellular cellular localization of Smo and/or the transport of Smo into cilia. Further investigation is needed into whether GRK2-mediated phosphorylation of Smo promotes vertebrate Smo activity by inducing a similar conformational change.

The function of the Ser/Thr kinase Fu is also divergent among species from *Drosophila* to human. As discussed above, it is critically involved in *Drosophila* Hh signaling but it has no effect on mouse Hh signal transduction [92, 93].

One possible explanation could be due to gene duplication. Understanding how Fu evolved divergent functions in distinct cellular processes is one of the topics that await further investigation. Recently, researchers have been trying to identify the kinases involved in vertebrate Hh signaling as the functions of Fu, PKA, and CK1 are divergent. So far, some kinases have been identified, such as DYRK2 [94], unc-51-like kinase 3 (ULK3) [95], and protein kinase C (see Chap. 6). However, the underlying molecular mechanisms by which these newly identified kinases are involved in Hh signaling are unclear.

Phosphatases in Hh Signaling

Levels of cellular protein phosphorylation are often modulated by the opposing action of protein kinases and phosphatases. Phosphatases are typically classified into two main groups: Ser/Thr protein phosphatases (STPs) and protein tyrosine phosphatases (PTPs). STPs can be subdivided into the phosphoprotein phosphatase (PPP) comprising PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7, and the protein phosphatase Mg^{2+}- or Mn^{2+}-dependent (PPM) family, comprised primarily of PP2C [96]. In the Hh signaling cascade, multiple Ser/Thr kinases are involved, including PKA, GSK3, and CK1 family members as discussed above. Are there any phosphatases involved in Hh signal transduction? In 2005, Nybakken et al. carried out a genome-wide RNAi screen in cultured *Drosophila* cells, in which they identified hundreds of potential new regulators of Hh signaling, including *microtubule star* (*mts*) that encodes the catalytic subunit of protein phosphatase 2A (PP2A) [97]. They showed that RNAi of Mts reduced Hh signaling. PP2A is a multimeric enzyme that consists of the catalytic subunit Mts, a regulatory A subunit (encoded by CG33297 in *Drosophila*) and a regulatory B subunit. PP2A functions are mediated largely by the regulatory B subunit that directs the phosphatase to distinct substrates. In this study, Nybakken et al. found that dsRNAs targeting Widerborst (Wdb), a B subunit, reduced Hh signaling by 50%. This indicated that Wdb is likely to be the B subunit that targets Mts to its substrate in the Hh signaling pathway. Consistently, it is found that B56e, the vertebrate ortholog of Wdb, regulates the Hh pathway during Xenopus eye field separation [98]. In another genetic screen for modifiers of Hh signaling in *Drosophila*, Casso et al. found that *mts* is necessary for full activation of Hh signaling [99].

Even though both of the above studies identified PP2A to be a positive regulator in Hh signaling, its relevant substrates remain undetermined. We know that regulated phosphorylation of Smo and Ci are critical events in mediating Hh signal transduction. As discussed earlier in this chapter, multiple Ser/Thr kinases regulate Hh signaling by phosphorylating Smo and Ci; however, Smo and Ci phosphatases remained unknown until an in vivo RNAi screen was carried out by Jia et al. [100]. In this study, protein phosphatase 4 (PP4) and PP2A were identified as phosphatases that influence Hh signaling by regulating Smo and Ci, respectively. RNAi knockdown of PP4 elevates Smo phosphorylation and accumulation, leading to increased

Hh signaling activity. Furthermore, the authors mapped the PP4-interacting domain in Smo and found that deletion of this domain in Smo promotes Smo phosphorylation and signaling activity. In addition, PP4 was found to regulate Hh-induced Smo cell-surface accumulation. Mechanistically, Hh was shown to downregulate the Smo-PP4 interaction that is mediated by Cos2. In the same in vivo RNAi screen, PP2A was identified to be a Ci phosphatase. Inactivating the regulatory subunit, Wdb, by RNAi or by a loss of function mutation downregulates, whereas overexpressing this regulatory subunit upregulates the level and thus signaling activity of Ci^{FL}. Furthermore, Wdb counteracts kinases to prevent Ci phosphorylation. The authors obtained evidence that Wdb attenuates Ci processing by dephosphorylating Ci. Thus, PP4 and PP2A are two phosphatases that act at different positions of the Hh signaling cascade. It is possible that other phosphatases are also involved in dephosphorylating Hh signaling components including Smo and Ci. For example, it was reported that PP1 could be an additional phosphatase for Smo (Zhu, meeting abstract), which would not be surprising since multiple kinases are involved.

Gradient Hh Signaling Activity Is Interpreted by Differential Phosphorylation of the Intracellular Components

Mammalian Shh, as well as *Drosophila* Hh, can function as morphogens and regulate cell patterning in a concentration-dependent manner [4, 101–105]. Hh proteins play broad roles in the development of appendages, such as wings, eyes, and legs [106]. One of the best systems for studying Hh signaling is the *Drosophila* wing. In the developing wing (wing imaginal disc), posterior compartment (P-compartment) cells express and secrete Hh proteins that act upon neighboring anterior compartment (A-compartment) cells adjacent to the A/P boundary to induce the expression of *dpp*. The long-range morphogen Dpp protein then diffuses bidirectionally into both the A and P compartments and functions as a morphogen to control the growth and patterning of cells in the entire wing in a concentration-dependent manner [44, 45, 107]. Hh also specifies cell patterning at the A/P boundary by activating other genes, including the short-range morphogens *engrailed* (*en*) and *ptc* [102, 108]. Low levels of Hh activity are able to induce the expression of *dpp*, whereas higher levels of Hh activity are also able to activate *ptc*. The induction of *en* appears to require the highest doses of Hh signaling activities [102]. Thus, the Hh activity levels can be monitored by expression of different responsive genes. This phenomenon is similar to the differential signal transduction of Wingless (Wg) in which different levels of Wg activity differentially regulate specific genes [109]. An important issue in developmental biology is to understand how cells perceive and transduce signal gradients, and how this results in differential cell fates.

Hh family members play a similar role in vertebrate limb development to that in *Drosophila*. *Shh* is best characterized in vertebrates and is involved in an array of developmental processes [106]. In the limb bud, Shh is expressed in the zone of polarizing activity (ZPA), which polarizes the digits along the A/P axis [110],

which is analogous to that in the *Drosophila* wing. Ectopic expression of Shh in the anterior limb bud induces additional digits in a concentration-dependent manner. In the neural tube, Shh is produced first in the notochord and then in the floor-plate; different levels of Shh activity direct the formation of multiple neuronal subtypes (V0, V1, V2, and V3 interneurons, and motorneurons) [106, 111], indicating that the protein functions to induce cell identity (see Chap. 2 for Hh morphogen gradient formation). Taken together, it is an important issue as to how different levels of the Hh morphogen generate distinct developmental outcomes.

How are the phosphorylation events modulated by gradient Hh signaling activity? Although the precise mechanism(s) of how an Hh gradient is sensed and transduced inside cells is still not well understood, it is likely that the differential phosphorylation of Smo and Ci correlate to different levels of Hh stimulation. In *Drosophila* wing discs, Hh induces cell-surface accumulation and activation of Smo through phosphorylation [4]. Interestingly, the extent of Smo phosphorylation appears to determine both the abundance of Smo at the cell surface and its signaling potency, raising an interesting possibility that different thresholds of Hh are transduced by differentially phosphorylated isoforms of Smo [10]. Low Hh only induces low levels of Smo phosphorylation and a low ratio of dimerized Smo, which suffices to activate low-threshold responses such as *dpp* expression, whereas high Hh induces high levels of Smo phosphorylation and a high ratio of Smo dimerization, which activates high-threshold responses including *ptc* and *en* expression. Indeed, it has been shown that increasing the number of phospho-mimetic mutations resulted in a gradual increase of FRET that is indicative of Smo dimerization/oligomerization [112]. It is believed that hyperphosphorylation of Smo is essential for transducing high levels of Hh signaling activity [31].

How are different thresholds of Hh activity interpreted by Ci/Gli transcription factors? In *Drosophila* wing development, threshold responses to the Hh morphogen appear to be mediated by differential regulation of the two forms of Ci. Accumulation of Ci^{FL} and expression of *dpp* occur in broader domains than activation of ptc and en, suggesting that low levels of Hh suffice to block Ci processing whereas higher levels of Hh are required to stimulate the activity of Ci^{FL}. Hence increasing the low levels of Hh activity may cause decreasing levels of Ci phosphorylation at the clusters that mediate Ci processing, thus generating less Ci^{REP} repressors. On the other hand, Ci^{FL} may also need to be activated by phosphorylation/dephosphorylation. For example, blockage of Ci processing in slimb mutant clones induces *dpp* but not *ptc* [52]; Ci^{FL} itself is inactive and only high levels of Hh stimulate the maturation of Ci^{FL} into a labile hyperactive form [113]; phosphorylation of Ci by PKA not only promotes Ci processing but also inhibits the activity of Ci155 independent of Ci processing [52].

A similar model has been proposed for regulating Gli proteins by graded Shh signals [114, 115]. In the neural tube, loss of Gli2, a major source of Gli activator, results in loss of progenitor cells of most ventral characters whereas progenitor cells of ventral-lateral and lateral characters still form [63, 64]. Ventral-lateral and lateral neural progenitors lost in *Shh* mutants are partially restored in *Shh Gli3* double mutants [116, 117]. These results suggest that high levels of Shh specify the

ventral most progenitors via stimulating the Gli2 (and perhaps Gli3) activator, whereas low levels of Shh specify ventral-lateral and lateral progenitors through inhibiting the Gli3 repressor. Hence, a gradient of Shh could generate a gradient of Gli activator activity and a reverse gradient of Gli repressor activity.

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Chapter 5
Signaling Cross-Talk of Oncogenic KRAS and Hedgehog Pathways in Pancreatic Cancer

Xiaodong Cheng

Introduction

Pancreatic tumors are highly metastatic and heterogeneous; over 90% are adenocarcinomas thought to arise from the pancreatic ducts, based on established ductal differentiation features such as cuboidal shape, ductal antigen expression, and growth into tubular structures [30]. Consistent with this idea, proliferative premalignant lesions of the ductal epithelium, termed pancreatic intraepithelial neoplasia (PanINs), are found in advanced malignant tumors with similar spatial distributions [16]. In addition, PanINs advance toward increasingly atypical histological stages with a concomitant accumulation of genetic alterations that have been identified in pancreatic ductal adenocarcinoma (PDA) [45, 49, 82]. Although it is commonly believed that PanINs are precursors of pancreatic ductal adenocarcinoma, the true cell(s) of origin for this malignancy has not been unambiguously defined. This is partially due to the enormous developmental plasticity of the pancreas, which enables phenotypic change between cell lineages (transdifferentiation). The islet-ductal, acinar-ductal, ductal-islet cell transdifferentiation has been observed both in cultures [25, 63, 84] and *in vivo* [4, 34, 57]. Therefore, ductal adenocarcinoma may arise from fully differentiated ductal epithelium, from other cell lineages such as acinar cells or islets cells, or from putative pancreatic stem cells [47, 56]. It is also possible that any of the cell lineages mentioned above is capable of giving rise to pancreatic adenocarcinoma, and specific genetic alterations determine the phenotypic endpoint of the tumor regardless of the precise cellular origin.

PDA is now one of the well-characterized neoplasms at the genetic level. Microdissection and new DNA, protein, and tissue array technologies have revealed multiple genetic alterations in premalignant lesions similar to those in PDA. There is now sufficient clinical, genetic, and pathological evidence for a tumor

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progression model for PDA, in which the pancreatic ductal epithelium progresses from normal to increased grades of proliferative premalignant lesions of the ductal epithelium to invasive cancer [30]. Accompanying the progressive morphological changes is the sequential accumulation of genetic alterations in oncogene KRAS, the tumor suppressors INK4A [10, 60, 78], P53 [6, 22, 26, 60] and SMAD4/DPC4 [8, 24, 72, 79], and telomeric structure [21, 70]. In addition to these frequent genetic abnormalities, mutations in the tumor suppressors BRCA2, TGFBR1, and TGFBR2, the serine–threonine kinases AKT2 and LKB1/STK11, and certain DNA mismatch-repair genes represent other less common genetic events in PDA. A recent comprehensive genome-wide genetic analysis of 24 PDA samples reveals that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations [37]. While this study confirms all the aforementioned frequently mutated genes uncovered by conventional strategies, the bulk of the genes that are genetically altered in PDA have not been reported previously. These alterations define a core set of 12 cellular signaling pathways and processes that are genetically altered in 67–100% of the tumors. Among these 12 implicated signaling pathways, KRAS signaling, apoptosis, cell cycle control of G1/S transition, transforming growth factor β (TGF-β) signaling, Wnt/Notch signaling and Hedgehog (Hh) signaling pathways are altered in all of the 24 PDA samples analyzed. Dysregulation of these core pathways and processes through mutation represents the major features of pancreatic tumorigenesis and have clear functional relevance to neoplasia [37]. The major focus of this review chapter is to discuss recent progresses in understanding the roles of signaling cross-talk between the KRAS and Hh pathways in PDA tumorigenesis and their implications for developing novel therapeutic treatments of PDA.

KRAS Signaling and PDA

RAS family small GTPases are key signaling molecules that controls a multitude of important cellular processes, including cell growth, differentiation, and survival. At the molecular level, RAS proteins function as a binary switch via cycling between the GDP-bound inactive form and GTP-bound active form. Under physiological conditions, the cellular activity of RAS proteins is tightly controlled. In basal or resting cells, RAS proteins exist predominantly in the GDP-bound state because they possess low intrinsic GTP hydrolysis activity. External stimulations such as binding of a growth factor to its receptor at the cell membrane lead to the activation of intrinsic receptor tyrosine kinase and subsequently a cascade of signaling events that eventually recruit SOS, a guanine nucleotide exchange factor (GEF), to close proximity to RAS on the plasma membrane. The binding of SOS to RAS leads to the dissociation of GDP and allows RAS to bind GTP, which is usually in large excess over GDP in cells. Binding of GTP activates RAS by inducing major conformational changes at switch I (amino acids 30–38) and switch II

(amino acids 59–67) regions that are important for interaction with downstream effectors. GTP-bound RAS activates a myriad of distinct effectors, among which the serine/threonine protein kinase RAF, phosphoinositide 3′-kinase (PI3K), and RalGDS are the three most well-characterized. These downstream effectors activate distinct signaling cascades, leading to the activation of transcription factors and/or other signaling molecules. Attenuation of RAS signaling is achieved via intrinsic GTP hydrolysis to GDP, a relatively slow process that can be dramatically accelerated by GTPase-activating proteins (GAPs), which enhance the GTPase activity by approximately 10^5-fold. Balance between GEFs and GAPs, two opposing forces involved in RAS regulation, is critical for maintaining normal cellular homeostasis.

Given the key regulatory roles that RAS family proteins play in essential cellular functions, it is not surprising that abnormal RAS signaling is associated with various major human diseases, particularly cancer. Aberrant RAS activation plays a critical role in tumorigenesis, as activating RAS mutations in one of three closely related RAS isoforms, HRAS, KRAS, or NRAS, are found in ~30% of all human cancers [9]. Oncogenic RAS mutations, most commonly associated with codon 12, 13, or 61, result in a disruption of both intrinsic and GAP-mediated GTP hydrolysis and therefore constitutively lock the protein in the GTP-bound, active form. Whereas HRAS, KRAS, and NRAS share very similar biochemical properties and downstream effectors, association of mutant RAS isoforms with human cancer is tissue specific, meaning that usually only the activating allele of one particular *RAS* isoform, but not the others, is found in a specific type of human cancer. For example, gain-of-function KRAS mutants are frequently found in pancreatic, colorectal, and non-small-cell lung cancers; activating HRAS mutations are usually detected in bladder, kidney, and thyroid carcinoma; and NRAS mutations are associated with melanoma, hepatocellular carcinoma, and hematologic malignancies. While it is most likely related to the isoform-specific functions and expression of RAS proteins, the mechanisms of this tissue-specific association of RAS isoforms with human cancer are not clear.

PDA has the highest incidence of activating KRAS mutations among all human cancers [3]. Activating KRAS mutations, representing the earliest genetic changes associated with the transformation of normal ductal epithelium and PDA development, have been detected in pancreatic duct lesions with minimal cytological and architectural atypia, and occasionally in histologically normal pancreas [11, 40, 46, 49, 71]. The frequency of KRAS mutations correlates with disease progression, reaching ~100% in PDA [37, 76]. In addition to the ubiquitous association of oncogenic KRAS and PDA in human patients, studies using genetically engineered mouse models have also firmly established that oncogenic KRAS indeed play a critical role in the initiation and progression of PDA. For example, targeted endogenous expression of an oncogenic *KRAS* allele in the mouse pancreas is sufficient to drive the development of PanINs and subsequently at low frequency the progression to both locally invasive adenocarcinoma and metastatic disease with sites of spread exactly as found in human PDA [1, 28].

Cross-Talk Between KRAS and Hh Signaling in Cancer

Recently, the Hh signaling pathway has been implicated as playing an important role in the progression and maintenance of PDA [7, 39, 73]. Hh signaling is essential for morphogenesis, tissue patterning, and stem cell maintenance in metazoan embryos [31]. Hh binds to its membrane receptor Patched (PTCH), releasing PTCH inhibition of a seven-transmembrane protein, Smoothened (SMO), which in turn activates downstream cytoplasmic transcription factors, the Ci protein in *Drosophila* or the mammalian homologue GLI proteins [43]. Components of the Hh signaling pathway, including the ligand and the receptors, are overexpressed in human PDA tissues and cell lines. Suppressing Hh activity using cyclopamine, a steroidal alkaloid that inhibits Hh signaling through direct interaction with SMO [13], in some PDA cells with activated Hh signaling, can inhibit cell growth *in vitro* and reduce tumor growth *in vivo* in the xenograft and orthotopic mouse model [7, 18, 39, 73].

The coincidence of uncontrolled activation of the RAS and Hh pathways in the early stages of PDA suggests that cross-talk between these two pathways may be a very important mechanism for the initiation and development of PDA. However, the causal effects between KRAS and Hh signaling in pancreatic tumorigenesis are not clear. Earlier results from *Pdx-Shh* mice had suggested that ectopic expression of Hh ligands is sufficient to activate the Ras signaling pathway by inducing a mutation in the *Kras* gene [73]. However, a recent in-depth study indicates that cell-autonomous activation of the Hh pathway is not sufficient to induce mutations in the *Kras* gene or to activate MAPK downstream of Ras [54]. In addition, while expression of endogenous level of oncogenic *Kras*, $Kras^{G12D}$, leads to PanINs identical to all three stages found in the cognate human condition and eventually PDA in mice [28], activation of Hh signaling alone is not sufficient to induce PanINs and PDA in a mouse model in which Hh signaling is activated specifically in the pancreatic epithelium [54]. Since KRAS mutation represents one of the earliest genetic alterations and occurs almost universally in PDA, we hypothesized that oncogenic KRAS promotes pancreatic tumorigenesis in part through activation of the Hh signaling pathway in PDA. To test this hypothesis, we established a KRAS oncogene-based, genetically defined human pancreatic cancer model using primary human pancreatic ductal epithelia (HPDE) immortalized by E6/E7 genes of human papilloma virus (HPV)-16 virus [20, 52]. Expression of $K\mathit{RAS}^{V12}$ in an immortalized, but nontumorigenic HPDE cell line, HPDE6-c7, using retroviral expression vector led to the transformation of the corresponding cell line. The resultant cell line, designated as HPDE6-c7-$KRAS^{V12}$, expresses an increased level of RAS, exhibits high RAS activity, and grows anchorage-independently in soft agar. Expression of $KRAS^{V12}$ in HPDE-c7 cells also led to an increased activation of its downstream effectors, such as MAPK and AKT. The phospho-MAPK and phospho-AKT levels were enhanced in the HPDE-c7-K-RAS^{V12} cells compared with the parental cells [35]. This genetically defined human pancreatic cancer model, along with several similar and independently established KRAS-based human pancreatic cancer cell models [12, 58], provides a useful tool for probing the mechanism of KRAS-mediated oncogenic transformation of HPDE. Oncogenic KRAS transformation of

human pancreatic ductal epithelial cells increases GLI transcriptional activity, an effect that is inhibited by the MEK-specific inhibitors, but not by the PI3K- and SMO-specific inhibitors. Inactivation of KRAS activity by a small-interfering RNA specific for oncogenic KRAS inhibits GLI activity and GLI1 expression in PDA cell lines with activating KRAS mutation. In addition, expression of the constitutively active form of $BRAF^{E600}$, but not myr-AKT, blocks the inhibitory effects of KRAS knockdown on Hh signaling. Suppressing GLI activity leads to a selective attenuation of the oncogenic transformation activity of mutant KRAS-expressing PDA cells [36]. These results provide direct evidence that oncogenic KRAS, through RAF/MEK/MAPK, activates Hh signaling via upregulation of GLI in PDA. Although dysregulation of Ras signaling in mice lacking p53 function in the pancreatic epithelium has recently been shown to induce Shh expression [29], our studies show that the ability of oncogenic KRAS to activate Hh signaling in the absence of Hh ligand represents another important mechanism by which oncogenic KRAS promotes tumor formation and also offers an explanation for why more than 50% of PDA cells lines with sustained Hh signaling activity are resistant to cyclopamine [73]. These findings combined with an earlier observation that sustained Hh activation activates platelet-derived growth factor receptor α and the RAS pathway [80] suggest that RAS and Hh signaling pathways can potentially form a positive feedback loop to promote tumorigenesis in pancreatic cancer.

Cooperation between the KRAS and Hh signaling pathways in pancreatic tumorigenesis has also been demonstrated *in vivo* in animal models more recently. When Shh-expressing pancreatic ductal epithelia with Ink4a/Arf and Trp53 null background were orthotopically implanted in mouse, no visible tumors developed. Transplantation of the same cell expressing KRas, or both KRas and Shh, led to the efficient formation of tumors. However, the tumors induced by the combination of Shh and KRas were significantly larger than those induced by KRas alone, indicating that KRAS and Hh cooperating in pancreatic tumorigenesis [48]. *Pdx1-Cre*; *LsL-Kras*^{G12D}; *Ink4a/Arf*$^{lox/lox}$ transgenic mice develop tumors resembled human PDA. Cancer cells from the tumor showed positive staining for Shh, while no staining was observed in surrounding stroma and non-neoplastic epithelial cells. Oncogenic KRAS signaling is believed to contribute to the observed Hh activation as expression of oncogenic $Kras^{G12D}$ in immortalized human pancreatic ductal cells leads to significant overexpression of SHH [19]. Furthermore, using *p48-Cre*/+; *LSL-Kras*$^{G12D/+}$; *Trp53*$^{F/+}$; *Smo*$^{F/+}$ mice and *p48-Cre*/+; *LSL-Kras*$^{G12D/+}$; *Trp53*$^{F/+}$; *Smo*^{F}/*Smo*^{F} mice, Hanahan and colleagues demonstrate that autocrine Shh–Ptch–Smo signaling is not required in pancreatic ductal cells for PDA progression. In PDA tumor cells, activation of Gli transcription, independent of upstream ligand-mediated signaling, is regulated by TGF-β and KRAS and is required for the KRAS-mediated transformed phenotype of cultured PDA cancer cells [51]. Expressing a green fluorescent protein alone or fused to oncogenic KRAS under the regulation of ptf1a regulatory elements in developing zebrafish pancreas allows real-time visualization of both normal and oncogenic KRAS-expressing pancreatic progenitor cells in living zebrafish embryos. Unlike normal GFP-labeled pancreatic progenitor cells, pancreatic progenitor cells expressing oncogenic KRAS underwent normal specification and migration but failed to differentiate. This block in

differentiation resulted in the abnormal persistence of an undifferentiated progenitor pool and was associated with the subsequent formation of invasive pancreatic cancer, which displayed several characteristics in common with the human disease, including abnormal Hh pathway activation [53].

In addition to pancreatic cancer, interaction between oncogenic RAS and Hh signaling pathways has also been reported in other systems including melanoma and gastric cancers. Study of a tyrosinase promoter-driven *NRAS*^{Q61K}; *Ink4a*$^{-/-}$ transgenic mouse model reveals that *Gli1* and *Ptch1,* but not *Shh,* are expressed in tumor samples, an indication of an active Shh-Gli pathway downstream of ligand. The expression of *Gli1* and *Ptch1* but not of the *NRAS*^{Q61K} transgene was significantly higher in lymph node metastases vs. primary skin tumors. It was further shown that endogenous RAS-MEK and AKT signaling regulated the nuclear localization and transcriptional activity of GLI1 in melanoma and other cancer cells [69]. Immunohistochemical analyses of 35 gastric carcinoma samples show a significant correlation between Hh pathway activation and phopsho-ERK1/2 levels. Expression of a constitutively active KRAS^{V12} mutant in five gastric cancer cell lines leads to increased GLI1-activity in all five cell lines. The effect of oncogenic KRAS/MEK1 was blocked by the suppressor of fused (SUFU) and the deletion of the N-terminal domain of GLI1. These results suggest that the KRAS/MEK/ERK cascade has a positive regulatory role in GLI transcriptional activity in gastric cancer [68]. A recent study showed that simultaneous activation of GLI1 and EGFR signaling induced anchorage-independent growth of RK3E rat kidney epithelial cells and human HaCaT keratinocytes, while neither expression of GLI1 nor activation of EGFR signaling alone was sufficient to elicit transformation. This EGFR- and GLI1-mediated synergistic transformation required the activation of the RAS/RAF/MEK/ERK but not of the PI3K/AKT pathway [64]. Taken together, these results suggest that collaboration of RAS and Hh signaling pathways represents a common scheme in the oncogenic transformation of multiple type cancers.

Anticancer Therapeutics Targeting KRAS or Hh Pathway

Clinically, PDA is one of the most lethal human diseases, with a 5-year survival rate of less than 5% and a median survival rate of less than 6 months. Furthermore, pancreatic cancer is resistant to most treatments, including chemotherapy, radiation, and combination therapy. Even for the 15–20% of patients with resectable nonmetastatic adenocarcinoma of the pancreatic head, the 5-year survival rate is only 20%, with a median survival time of 17–20 months [27]. Thus, the development and characterization of new therapeutic agents, especially those based on molecular targeting with high specificity, are desperately needed.

Because the paramount importance of oncogenic KRAS proteins in the initiation and development of different human cancers, considerable amount of efforts has been directed to target RAS for cancer treatments. Thus far, RAS has been proven to be “undruggable” as attempts to block RAS signaling directly have met with little successes. In the past, several strategies have been developed to block

activated KRAS, including both farnesyl transferase inhibitors (FTIs) and antisense oligonucleotides [2, 59]. FTIs have been intensively investigated in preclinical and clinical trials as a cancer therapy [67]. Although FTIs are capable of inhibiting RAS processing *in vitro* [33, 41] and in genetically engineered mouse models that harbors *HR*AS oncogene [42], several phase II and phase III clinical trials have shown that FTIs do not have significant single-agent activity in lung, pancreatic, colorectal, bladder, and prostate cancers [14, 67]. In addition, FTIs may be more effective in preventing the membrane translocation of HRAS but not KRAS and NRAS due to the fact that KRAS and NRAS proteins can be geranylgeranylated and translocated to the membrane in the presence of FTIs [32, 77]. This may partly explain the failure of FTIs in clinical trials which reflect the decreased effectiveness of FTIs toward the inhibition of the KRAS oncoprotein more commonly present in human cancers.

Synthetic lethality screening has lately emerged as a new strategy to identify agents that act on a target or targets whose functional inactivation is lethal only in the context of a specific cancer-causing mutation allele [17, 38]. Two general approaches involving the use of either small chemical compound libraries or RNA interference (RNAi) libraries for the identification of KRAS synthetic lethal agents have been explored [23, 35, 81]. Recently, efforts to identify small molecules as KRAS synthetic lethal inhibitors via high-throughput screening have been made by several research groups, and some significant progress has been achieved [17, 23, 35]. Using a genetically defined human cancer model based on oncogenic HRAS and human neonatal fibroblasts, Stockwell and colleagues pioneered the synthetic lethal chemical screening approach to search selective synthetic lethal inhibitors for their ability to kill $HRAS^{V12}$-transformed cells but not their isogenic nontumorigenic counterparts. From a high-throughput screening of a 22,550 compound library, erastin, along with several known anticancer agents, was identified as HRAS synthetic lethal compound [17]. Since KRAS is more frequently mutated in human cancers, selective toxicity in mutant KRAS-expressing cell lines would broaden the applicability of erastin as a therapeutic agent. It was determined that erastin also exerted selectively lethality to tumor cells harboring oncogenic KRAS, albeit to less extend than that of KRAS [81]. On the other hand, oncrasin-1 has been identified via HTS as a small molecule that can effectively kill KRAS mutant ovarian and lung cancer cells but not normal isogenic cells or HRAS/NRAS mutant cancer cells [23]. Further mechanistic studies revealed that apoptosis induction by this compound is blocked by knockdown of KRAS or protein kinase C iota (PKC_ζ), suggesting that oncrasin-1 is synthetic lethal to active KRAS and PKC_ζ [23]. PKC_ζ is an atypical protein kinase C that is activated by oncogenic RAS protein and is required for KRAS-induced transformation and colonic carcinogenesis *in vivo* [50], as well as pancreatic cancer cell transformation and tumorigenesis [66]. Using the genetically defined human pancreatic cancer cell model as described above, we have also developed a high-throughput screening assay for identifying small chemical inhibitors that selectively target the oncogenic KRAS-expressing cancer cell but not its nontumorigenic counterpart [35]. While these newly identified RAS synthetic lethal inhibitors hold great promise for developing novel anticancer therapeutics targeting KRAS, further structure–activity relationship analyses and new drug discovery

studies based on these chemical leads are imperative for improving the selectivity, potency, and drug-like properties.

RNAi screens using a short hairpin RNA (shRNA) library targeting protein kinases, phosphatases and cancer-related genes or a genome-wide shRNA library have led to the identification of several KRAS synthetic lethal partners, whose inactivation results in selective cell death in cancer cells contained an oncogenic KRAS allele but not in cells with wild-type KRAS status [5, 23, 44, 65]. The unveiling of these cellular targets essential for the survival of oncogenic KRAS-driven cancer cells has provided mechanistic insights into understanding the mechanism of synthetic lethality for KRAS. Moreover, these discoveries also lend opportunities for developing pharmacological inhibitors for these targets as novel cancer therapeutics targeting KRAS oncogene addiction. Interestingly, one of the identified KRAS synthetic lethal partners is PTCH2, a component of Hh signaling pathway [5]. While the significance and implication of this finding remains to be uncovered, it nevertheless suggests that cooperation between KRAS and Hh signaling may play a role in tumorigenesis.

Despite that the involvement of Hh signaling pathway in cancer is revealed much later compared to RAS, inhibitors of the Hh signaling pathway have emerged in recent years as a promising new class of potential therapeutics for cancer treatment. Numerous small molecules that target different components of the pathway have been identified and currently under clinical trials. The majority of Hh pathway inhibitors reported to date target SMO with a few other direct at upstream (Hh) or downstream (GLI). Cyclopamine, a natural alkaloid isolated from *Veratrum californicum*, was the first small-molecule inhibitor of the Hh pathway to be reported [15]. Cyclopamine inhibits Hh signaling by direct binding to the seven-transmembrane helical bundle of SMO [13]. As the first-generation Hh inhibitor, cyclopamine is of low affinity, poor oral bioavailability, and suboptimal pharmacological properties. More potent and soluble analogs with improved bioavailability have been designed and synthesized [74]. In addition, noncyclopamine-like Hh inhibitors have been developed through high-throughput screening of synthetic compound libraries [55]. For a detailed list of potential anticancer Hh inhibitors, please refer to a recent review [62]. One such compound, GDC-0449, is a new generation orally bioavailable and selective SMO inhibitor. A recent clinical trial of 33 patients with advanced basal-cell carcinoma refractory to conventional treatments has shown that GDC-0449 has excellent antitumor activity in locally advanced or metastatic basal-cell carcinoma: after a median treatment of 9.8 months, 18 patients showed objective response to the drug while 11 other patients had arrested stable diseases. Only four patients had progressive cancer during the treatment [75]. In a more dramatic case study, a 26-year-old male with metastatic medulloblastoma that was refractory to multiple therapies was reported. Genetic analyses of tumor specimens obtained before treatment suggested that abnormal activation of the hedgehog pathway is associated with the tumor, with loss of heterozygosity and somatic mutation of the *PTCH1* gene, a key negative regulator of hedgehog signaling. Treatment with GDC-0449 resulted in rapid regression of the tumor and lessening of symptoms. Unfortunately, the initial response to the treatment was transient and the cancer

relapsed in a resistant form that was not responsive to the drug after 3 months of treatment [61]. To determine the mechanism of drug resistance, the mutational status of Hh signaling genes was analyzed, an amino acid substitution at a conserved aspartic acid residue 473 of SMO was revealed in the tumor after disease progression but not in the primary tumor specimens before treatment. Functional analyses showed that the mutation had no effect on Hh signaling but disrupted the ability of GDC-0449 to bind SMO and suppress this pathway. A mutation altering the same amino acid was also found in a GDC-0449-resistant mouse model of medulloblastoma [83].

Conclusion

The recent advances in cancer genomics have provided a wealth of information for deciphering the molecular anatomy of cancer. For many types of cancers, it is conceivable that we will soon have a complete set of the individual pieces of a puzzle: a list of the genes and mutations associated with cancer development. However, our knowledge of the complex signaling networks, particularly cross-talks between cancer-related signaling molecules required to piece the puzzle together, remains sparse. The realization of signaling cross-talk between KRAS and Hh suggests that cooperation between the two universally dysregulated signaling pathways in PDA may play an important role in pancreatic tumorigenesis. This finding also has therapeutic implications for the treatment of pancreatic cancer. Because of its intrinsic heterogeneity and complexity, cancer is unlikely conquered by a single magic bullet aiming at one specific signaling molecule. Combination therapy simultaneously targeting multiple signaling pathways important for oncogenesis may represent the best hope for pinning down the elusive target and preventing the development of resistance to single agent, such as GDC-0449. Inhibiting the KRAS and Hh pathways synergistically can potentially provide an effective therapeutic strategy for treating PDA.

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Chapter 6
Hedgehog and Protein Kinase C Signaling

Jing Li and B. Mark Evers

Keywords Hedgehog • PKCα • PKCδ • MEK/ERK

Introduction

Hedgehog (HH) signaling plays an important role in human cancers through promoting cancer cell growth and proliferation of tumor stem cells. The protein kinase C (PKC) family, which comprises at least ten isoforms, has been shown to exert multiple biological functions, including adhesion, secretion, proliferation, differentiation, and apoptosis. In this review, we will summarize recent findings demonstrating crosstalk between the HH and PKC signaling pathways during development, in stem cells and in malignant and nonmalignant cells. The role of MEK/ERK pathway in this crosstalk will also be discussed. The integration of these signaling pathways in the regulation of HH signaling provides for potentially new targets in the control of HH-dependent tumorigenesis.

Protein Kinase C

The protein kinase C (PKC) family represents a group of widely distributed serine–threonine kinases [1]. Eleven PKC isoforms have been identified and divided into three major classes: the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, θ, and η), and the atypical PKCs (ζ and ι/λ) [2]. The conventional PKC isoforms have an intact C1 diacylglycerol/phorbol ester binding domain and C2 calcium-binding domain and thus require phospholipids and calcium

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for activation [2]. The novel PKCs do not require calcium for their activation [2]. The atypical PKCs can be activated in the absence of diacylglycerol and calcium [2]. Upon activation, PKC isoforms often translocate to particular subcellular compartments, including the plasma membrane, Golgi complex, nuclear membrane and nucleus [3]. PKC isoforms play important roles in signal transduction of various physiological stimuli, including growth factors, hormones, and transmitters, thus PKCs are involved in many cellular processes [4].

Functions of Different PKC Isoforms in Human Cancer

Each of the PKC isoforms is unique in its contribution to cancer development and progression. The conventional PKCs are generally considered to be predominantly antiapoptotic and principally involved in promoting cell survival and proliferation. PKCα regulates multiple biological processes, including cell proliferation, apoptosis, and cell motility [5]. However, the role of PKCα in regulating tumor growth and development is complex and highly tissue dependent. PKCα can either act as a tumor promoter or a tumor suppressor [6]. Overexpression of PKCα has been demonstrated in tissue samples of prostate, endometrial, high-grade urinary bladder, hepatocellular and breast cancers, suggesting a role of PKCα as a tumor promoter. In contrast, PKCα is down-regulated in basal cell carcinoma (BCC) and colon cancers, demonstrating a possible role of PKCα as a tumor suppressor in these tumor types. PKCβI and βII function in various signal-transducing pathways for proliferation, differentiation, metabolism, and more cell-type-specific functions [7, 8].

The novel PKCs generally have a tumor suppressor function and are regarded as pro-apoptotic proteins; however, the evidence is complex. PKCδ has been implicated both as a tumor suppressor and positively or negatively regulates cell proliferation and apoptosis [9]. For example, in breast cancer, PKCδ has shown both pro-survival and pro-apoptotic effects [10]. PKCε has been shown to behave as an oncoprotein [11]. Overexpression of PKCε increased proliferation, motility, and invasion of fibroblasts or immortalized epithelial cells. In addition, transgenic animal models have clearly shown that overexpression of PKCε is tumorigenic resulting in metastatic disease. PKCθ has been proposed as a key player in T-cell activation and an attractive therapeutic target in T-cell-mediated disease processes [12].

Atypical PKCs have been implicated in the malignant behavior of transformed human cells as well. Evidence over the past few years has shown that PKCι is a human oncogene and that the oncogenic PKCι signaling is a target for novel mechanism-based cancer therapy [13]. For example, PKCι is critical for transformed growth in human non-small cell lung cancer cells. PKCζ is involved in diverse physiological functions [14]. For example, PKCζ is involved in the control of glioblastoma cell migration and invasion by regulating the cytoskeleton rearrangement, cell adhesion, and matrix metalloprotease-9 expression. These findings suggest that PKCζ is a potential therapeutic target for glioblastoma.

Crosstalk of HH and PKC

Gli proteins, including Gli1, Gli2, and Gli3, represent a family of zinc-finger transcription factors and play critical roles in the mediation and interpretation of HH signals [15]. Fused (Fu) is a serine–threonine kinase required for HH signaling and hSu(fu), a human homologue of *Drosophila* Su(fu), is a suppressor of Fu [16]. hSu(fu) negatively regulates Gli activity [16, 17]. hSu(fu) contains conserved PKC phosphorylation sites [16, 17], indicating that it may be subject to regulation by PKC. Information demonstrating the crosstalk between HH and PKC signaling pathways was derived from nontumor cells, such as mammalian 293T and NIH 3T3 fibroblasts. These studies mainly focused on the crosstalk of PKCα or PKCδ with HH signaling. Neill et al. [18] first demonstrated that PKCα and PKCδ-mediated Gli activity in the mammalian 293T cells. When 293T cells were cotransfected with constitutively active PKCα or PKCδ and a luciferase reporter construct containing Gli1-binding sites (GBS), the constitutively active PKCα decreased Gli1 activity by over 60%, suggesting that PKCα is a potent negative regulator of Gli1 transcriptional activity. In contrast to PKCα, constitutively active PKCδ increased the activity of Gli1, indicating a positive role of PKCδ in the regulation of Gli1 transcriptional activity.

The interaction of PKC with HH was further studied in LIGHT2 cells, a HH-responsive NIH 3T3 fibroblast cell line stably transfected with a Gli-regulated luciferase reporter containing eight tandem copies of GBS (8×GBS-luciferase) [19]. Treatment of LIGHT2 cells with phorbol 12-myristate 13-acetate (PMA), a phorbol ester, increased Gli-luciferase activity that was blocked by the PKC inhibitor GF109203X, suggesting PMA activation of Gli is mediated through PKC. Treatment with PMA increased mRNA levels of PTCH 1 and GLI1, two endogenous Gli-regulated genes, which was inhibited by GF109203X. The specificity of Gli-dependent transcription by PMA in NIH 3T3 fibroblasts was further confirmed by transfecting a wild-type 8×GBS-luciferase reporter or a mutated 8×GBS-luciferase reporter harboring a point mutation that abolishes the binding of Gli. PMA-stimulated Gli-luciferase activity was only detected in cells transfected with wild-type GBS-luciferase reporter but not the mutant reporter, indicating that PMA activity is mediated through activation of GLI transcriptional activity. Furthermore, stimulation of GLI-dependent transcription by PMA is mediated through a novel PKC. When LIGHT2 cells were treated with PMA in the presence of Gö6976 (inhibitor of classical PKCs) or rottlerin (inhibitor of novel PKCs), PMA-mediated GLI transcriptional activity was prevented by rottlerin, but not by Gö6976, suggesting this effect is mediated through a novel PKC isoform, which is likely PKCδ since NIH 3T3 fibroblasts express PKCα and PKCδ, two isoforms responsive to PMA. The involvement of PKCδ was confirmed by transfection of LIGHT2 cells with a dominant-negative mutant of PKCδ which blocked the GLI-luciferase activity. Taken together, this study demonstrates that PKCα plays a negative role, whereas PKCδ plays a positive role, in the regulation of HH signaling.

Our group further established the crosstalk of PKCα or PKCδ with HH signaling [20]. As noted above, Neill et al. [18] have shown that PKCα is a negative regulator of GLI1 transcriptional activity in 293T cells. We further confirmed the specific regulation of PKCα on GLI activity in NIH 3T3 fibroblasts. NIH 3T3 cells were cotransfected with a wild-type or a mutated GLI-luciferase reporter (point mutation that abolishes the binding of Gli) and expression plasmids, Gli1 and constitutively active PKCα. The constitutively active PKCα significantly increased the wild-type GLI-luciferase activity, but not the mutant, confirming that PKCα negatively regulates HH signaling. It has been demonstrated that PKCδ increased the activity of Gli1 in NIH 3T3 and 293T cells. Therefore, we cotransfected NIH 3T3 cells with Gli-luciferase reporter, Gli1, and either wild-type PKCδ, kinase-dead PKCδ K376R, or constitutively active PKCδΔNPS in which the N-terminal pseudosubstrate domain was deleted. Treatment with PMA increased Gli-luciferase activity only in cells cotransfected with control vector, which is consistent with the previous findings showing that the endogenous PKCδ positively regulates Gli activity [19]. In contrast, in cells transfected with wild-type PKCδ, Gli-luciferase activity was significantly decreased by PMA treatment; this effect was blocked by rottlerin. In the cells transfected with PKCδΔNPS, Gli-luciferase activity was further decreased either in the presence or absence of PMA, whereas Gli-luciferase activity was not altered in cells transfected with kinase-dead PKCδ K376R. In cells transfected with empty vector (pcDNA3) or kinase-dead PKCδ, PTCH1 mRNA expression was not altered either with or without PMA treatment. In contrast, PMA treatment decreased PTCH1 mRNA levels in cells transfected with wild-type PKCδ as well as PKCδΔNPS either in the presence or absence of PMA. Taken together, PKCδ appears to play a negative role in the regulation of Gli activity stimulated by PMA.

Crosstalk of HH and PKC in Development

The role of HH signaling in development is well known [21]. PKC isoforms have been implicated in a number of key steps during gametogenesis, fertilization, and early development [22]. However, the interaction of the two signaling pathways in the regulation of development has not been studied extensively. Lu et al. [23] tested the efficacy of the competitive inhibitors chelerythrine chloride and Gö6976 (specific inhibitors of PKC) and sphingosine (inhibits PKC and other kinases) in primary limb bud mesenchyme cultures. PKC inhibition resulted in smaller buds and truncated wings and caused complete loss of sonic hedgehog (Shh) expression in the buds, suggesting the possibility that PKC may control Shh expression. Indeed, the PKC inhibition-induced phenotype and lost Shh expression were rescued by providing ectopic Shh. These experiments demonstrated that, providing exogenous Shh to wing buds in which PKC signaling had been blocked, restored limb development and that Shh is one of the primary targets of PKC signaling.

Crosstalk of HH and PKC in Stem Cells

HH signaling has an essential role in the control of stem cell growth in embryonic tissues. Heo et al. [24] examined the effect of Shh on the self-renewal of mouse embryonic stem cells and its related mechanisms. They treated these cells with Shh and noted translocation of PKCα, δ, and ζ isoforms from the cytosol to the membrane, demonstrating the activation of these PKC isoforms by Shh stimulation. On the other hand, Shh-induced PKC activation was blocked by cyclopamine, a steroid alkaloid that blocks Shh signaling. Pretreatment with bisindolylmaleimide I (a PKC inhibitor) inhibited Shh-induced Gli1 gene expression and [^{3}H] thymidine incorporation, demonstrating that Shh stimulated mouse ES cell proliferation through Gli1 activation as well as PKC. Consistently, in mesenchymal stem cells transfected with Shh, the expression of angiogenic and pro-survival growth factors was increased and migration and tube formation were significantly improved in a PKC-dependent manner.

Crosstalk of HH and PKC in Human Cancer

The HH signaling pathway, when mutated or dysregulated, contributes to tumorigenesis. Recent studies provide evidence demonstrating the crosstalk of HH and certain PKC isoforms in human cancer cells.

Gli1 expression is associated with the development of BCC. Gli1 is expressed in the outer root sheath (ORS) of the hair follicle which is thought to be a potential source of BCC. PKCα was expressed in the epidermis and ORS of human hair follicles and PKCδ in the inner root sheath. Neill et al. [18] found that PKCα is downregulated in BCC, suggesting that loss of PKCα expression may be relevant to tumor formation. We screened PKCδ expression in a set of hepatocellular cancer (HCC) in which the activation status of HH signaling had previously been determined by in situ hybridization using probes against Gli1 and PTCH1 [20]. Interestingly, the expression of PKCδ was not detected in any of these specimens with activated HH signaling. These results suggest that decreased expression of PKCδ may account for activation of HH signaling in certain HCC, further demonstrating a negative role of PKCδ in the regulation of HH signaling in cancer cells. Additional evidence to support these findings was provided by in vitro studies using Hep3B cells, a human hepatoma cell line [20]. By a combination of overexpression of PKCδ and knockdown with PKCδ siRNA, we demonstrated that overexpression of wild-type or active PKCδ decreased Gli-luciferase activity, mRNA levels of PTCH, and Gli and endogenous Gli protein levels, whereas knockdown by PKCδ siRNA had opposite effects on these HH target proteins. Furthermore, PKCδ knockdown with siRNA enhanced the proliferation and significantly blocked the inhibitory effects of KAAD-cyclopamine (a potent derivative of cyclopamine). Taken together, the loss of PKCδ increased HH signaling and Gli1 protein expression and rescued the inhibitory effect of KAAD-cyclopamine on cellular proliferation, demonstrating that PKCδ negatively regulates HH signaling.

MEK/ERK Pathway in PKC-Mediated HH Signaling

The Raf/MEK/ERK signaling pathways regulate a variety of cellular activities including proliferation, differentiation, survival, and death. HH signaling exerts a positive feedback effect on these pathways; furthermore, PKC is well known as an activator of the ERK pathway. Therefore, it is very likely that the ERK pathway is involved in PKC-mediated HH signaling. Riobo et al. [19] investigated whether PKC activates Gli activity through the ERK pathway in LIGHT2 cells. They showed that PMA-induced GLI-luciferase reporter activity was blocked by the selective MEK-1 inhibitor PD98059 or the dual MEK-1/2 inhibitor U0126. These findings place MEK-1 downstream of PKC in the activation of GLI. Moreover, PKCα plays a positive role in the regulation of Gli1 activity; this effect was mediated by the MEK/ERK pathway.

Summary

We have highlighted data demonstrating evidence of crosstalk between HH and PKC. Consistently, PKCα has been shown to negatively regulate HH signaling. However, studies demonstrated that PKCδ plays either a negative or positive role in the regulation of HH signaling. We proposed that the balance between PKCα and PKCδ is important in the regulation of Gli activity. When PKCα is dominant, the negative effect of PKCδ is weak, and PMA increases Gli activity through the PKCα/MEK/ERK pathway. However, when PKCδ is dominant, PMA treatment decreases Gli activity through the activation of PKCδ. It is clear that the HH pathway plays an important role in tumor cell growth and survival. However, several issues regarding the precise role of the HH signaling pathway in human cancer remain unresolved, including the exact mechanisms of signal transduction. We anticipate that more mechanistic studies will further illuminate the conserved and divergent aspects of HH signaling. A better understanding of HH signaling and its crosstalk with other signaling pathways is of importance for developing a more precise understanding of HH-associated diseases and, furthermore, holds great promise for developing new therapies based upon this information.

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Chapter 7
Activation of Hedgehog Signaling in Human Cancer

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Keywords Hedgehog • Smoothened • PTCH1 • Cancer • Basal cell carcinomas • Signal transduction • Clinical trials and animal model

Introduction

Major advances in the studies of the hedgehog pathway have been made in the last 30 years. The hedgehog (Hh) gene was identified in 1980 through genetic analysis of fruit fly *Drosophila* segmentation [1]. In early 1990s, 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 hereditary developmental disorder holoprosencephaly, whereas hyperactivation of this pathway was linked to human cancer [7–11]. More recently, one Hh signaling inhibitor has been successfully used in clinical trials of human cancer, which further indicates the feasibility of Hh signaling inhibitors for treating human cancers.

The general signaling mechanisms of the Hh pathway is conserved from fly to the humans [12]. In the absence of Hh ligands, 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 homolog) binds to its receptor PTC and releases this inhibition, allowing SMO to signal downstream, eventually leading to activation of Gli transcription factors. As transcription factors, Gli molecules can associate with specific consensus sequences located in the promoter region of the target genes and regulate target gene expression [13, 14]. Figure 7.1 shows a simplified diagram of the Hh pathway.

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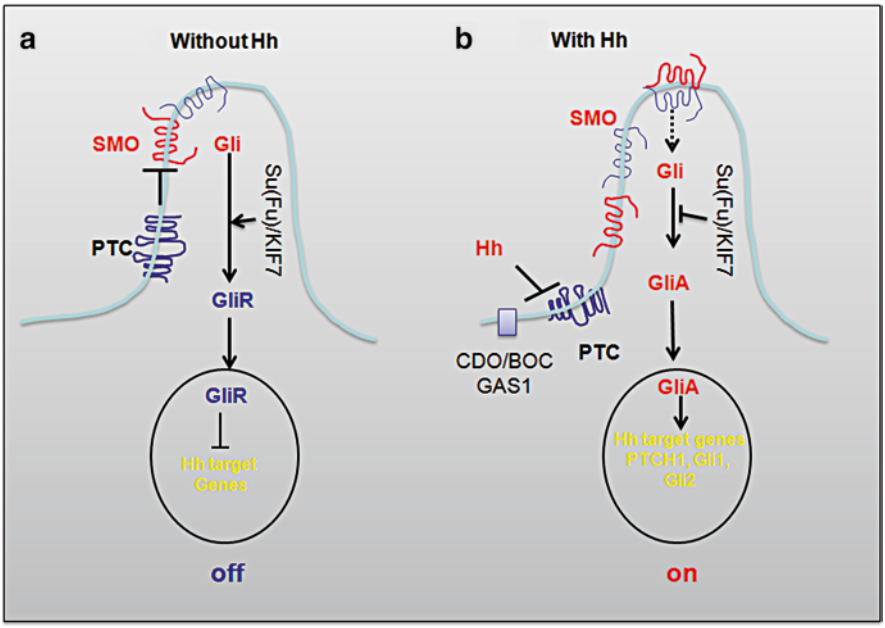

Fig. 7.1 A simplified model for Hh signaling in mammalian cells. SMO is the key signal transducer of the Hh pathway. (**a**) In the absence of the Hh ligands, Hh receptor PTC is thought to be localized in the cilium to inhibit SMO signaling via an unknown mechanism. Gli molecules are processed with the help of Su(Fu)/KIF7 molecules into repressor forms, which turn off 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 changes to form dimers. Gli molecules are now processed to active forms (GliA), which will activate the Hh target genes. 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 *yellow*. This figure was modified from Yang et al. *Oncogene* 29, 469–481 (2010)

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 molecules, functioning both at short range on nearby cells and at long range to distant cells during development [15–17]. After translation, Hh protein precursor undergoes autoprocessing 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 acyltransferase Skinny Hedgehog 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 reception of Hh ligands, with Patched (PTC, one PTC in fly and two PTCs in vertebrates – PTCH1 and PTCH2) as the major receptor [32]. Studies from cultured cells indicate that PTC inhibits SMO at a sub-stochiometrical concentration [33]. Hh-interacting protein (HIP) can compete with PTC on Hh binding, resulting in 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 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 pro-vitamin D3 [43–46]. It is not known how these molecules regulate SMO signaling.

Significant progress has been made in our understanding of SMO signaling. Several recent reports support SMO-G protein coupling [47–50]. In particular, a study in *Drosophila* provides direct evidence for SMO-coupling to Gαi in regulation of Hh pathway activation [48]. The physiological relevance of the G-protein coupling of SMO for Hh signaling in carcinogenesis is currently not known. In *Drosophila*, SMO function is promoted 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, indicative of a different mechanism for SMO signaling in higher organisms [52]. There are two important events during mammalian SMO signaling. First, SMO protein undergoes conformational change to favor SMO signaling [53] although the regulatory mechanism underlying this conformational change is not clear. Second, ciliary translocation of mammalian SMO protein is critical for Hh signaling (see below).

Accumulating evidence indicate that the primary cilia play an important role for the Hh pathway [54–59]. The function of primary cilium is regulated by protein complexes involved in intraflagellar transport (IFT), which functions in retrograde and anterograde movement of cargo within the primary cilia [60]. Mutations in IFT proteins 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]. Presence of several Hh components, including SMO and Gli molecules at the primary cilium upon Hh stimulation, further supports the relevance of cilium in Hh signaling [62–65]. It has been shown that a SMO mutant lacking ciliary translocation signal is unable to mediate Hh signaling [54]. However, translocation of SMO to cilium alone is not sufficient to activate Hh signaling [64, 65]. Using tissue-specific gene knockout, recent studies revealed dual roles of cilium (via knocking out cilium component Kif3a) for Hh signaling-mediated carcinogenesis

in mouse models [66, 67]. While Kif3a gene is required for activated SMO-mediated tumor formation, knocking out Kif3a accelerates Gli2-mediated carcinogenesis. We still do not understand how SMO is translocated to the cilium in response to Hh signaling and how SMO activates downstream effectors. It is known that Beta-arrestin 2 can regulate ciliary localization of SMO [68]. The role of cilium for Hh signaling downstream of SMO is less clear. Not all the signaling events occur in cilium. For example, cilium is not required for Su(Fu)-mediated regulation of Gli functions [69, 70].

Several molecules are identified to be genetically downstream of SMO signaling in *Drosophila*, including COS2 and Fused. Recent in vivo studies support that a COS2 homolog 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 from vertebrate Fused knockout is not similar to that observed as 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* homologs, mammalian cells have several novel cytoplasmic regulators of Hh signaling, including Rab23 [76] and tectonic [77]. Rab23 and tectonic are all negative regulators downstream of SMO, but the exact mechanisms of action remain to be established. Unlike many Rab proteins, we found that Rab23 is localized in the nucleus and in cytoplasm (Acta Histochem 2010, July 23 [Epub ahead of time]), 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 [78, 79], but their exact functions are not clear.

Several lines of evidence indicate that Suppressor of Fused [Su(Fu)] plays a major negative regulatory role in mammalian Hh signaling. Su(Fu) is originally identified genetically in *Drosophila* by its ability to suppress active *fused* mutations, but itself is not required for pathway activity. *Su(Fu)* null mouse mutants fail to repress the pathway [80] and have some phenotypes similar to *Ptch1* inactivation. While $Ptch1^{+/-}$ mice are predisposed to developing medulloblastoma, rhabdomyosarcoma, and basal cell carcinomas (BCCs) [81–83], $Su(Fu)^{+/-}$ mice mainly develop basaloid epidermal proliferation. Su(Fu) plays a central role in pathway repression as indicated from the data derived from *Su(Fu)* null MEFs and wild-type cells treated with Su(Fu) siRNAs [80] that loss of Su(Fu) results in Hh signaling activation. At the molecular level, Su(Fu) associates with and inhibits Gli molecule function, and is required for Gli3 processing [84, 85]. One potential molecular basis by which Hh signaling releases the suppressing activity of Su(Fu) is the enhanced Su(Fu) protein degradation upon Hh signaling activation [86].

The ultimate effect of Hh signaling activation is the activation of downstream Gli transcription factors, which can regulate target gene expression by direct binding of a consensus binding site (5′-tgggtggtc-3′) in the target gene's promoter [13, 14, 87, 88]. The activity of Gli transcription factors can be regulated at several levels. First, nuclear-cytoplasmic shuttling of Gli molecules is tightly regulated

[84, 89–91]. Protein kinase A can retain Gli1 protein in the cytoplasm via a PKA site in the nuclear localization signal domain [89], whereas activated Ras signaling induces Gli nuclear localization [91]. Second, ubiquitination, acetylation, and protein degradation of Gli molecules are regulated by several distinct mechanisms, including β-TRCP-, cul3/BTB-, and numb/Itch-mediated Gli ubiquitination [92–97]. In addition, Gli3 (Gli2 to a less extent) can be processed into transcriptional repressors, which may be mediated by the β-TRCP E3 ligase [94]. Defects in the retrograde motor for IFTs can affect Gli3 processing [98]. Furthermore, transcriptional activity of Gli molecules is tightly regulated. Su(Fu) not only prevents nuclear translocation of Gli molecules, but also inhibits Gli1-mediated transcriptional activity [99].

Several feedback regulatory loops exist in this pathway to maintain the level of Hh signaling in a given cell. PTC, HHIP, GAS1, and Gli1 are both components and the 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 loops would lead to abnormal signaling of this pathway, such as inactivation of PTCH1 in BCCs.

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 hereditary form of BCC – Basal Cell Nevus Syndrome (also Gorlin syndrome) [100–102]. Gorlin syndrome is a rare autosomal genetic disease with two distinct sets of phenotypes: an increased risk to developing cancers such as BCCs and medulloblastomas; developmental defects such as bifid ribs, ectopic calcification. The tumor suppressor role of *PTCH1* is demonstrated in mice with knockout of one Ptch1 allele that *Ptch1*$^{+/-}$ mice develop tumors in addition to other features observed in Gorlin syndrome patients, such as spina bifida occulta [82, 83, 103]. Figure 7.2 shows the association of Hh signaling with human cancer.

Activation of the Hedgehog Pathway in Human Cancer

BCCs and Medulloblastomas

BCCs consistently have abnormalities of the Hh pathway with *PTCH1* mutations in 50% and mutations of *SMO* in 10% of patients [104–108]. Unlike wild-type SMO, expression of SmoM2, an activated SMO mutant molecule identified in human BCCs, in mouse skin results in the formation of BCC-like tumors [104].

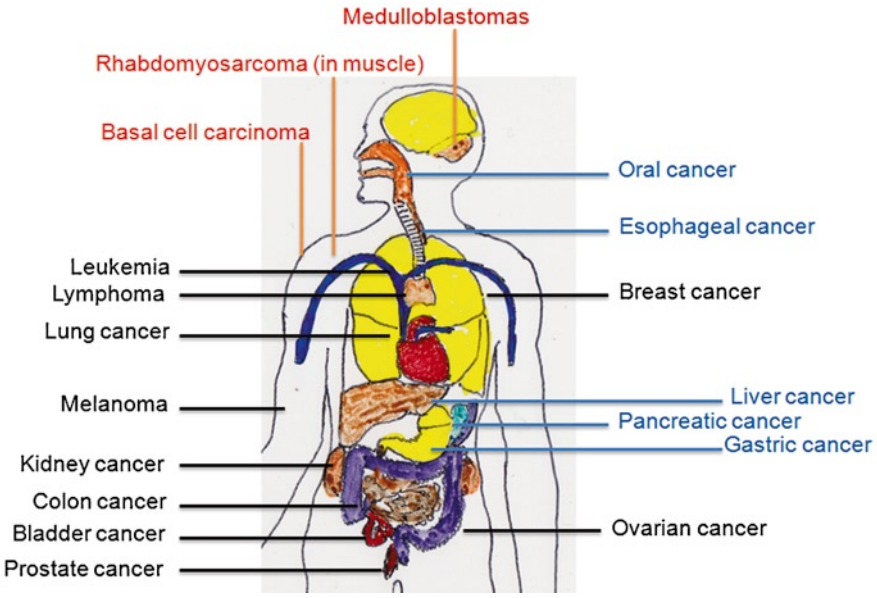

Fig. 7.2 Activation of Hh signaling in human cancer. Since the link of Hh signaling activation in Gorlin syndrome, increasing evidence indicate that Hh signaling is frequently activated in human cancer. Based on the current data, we group these cancers into three groups. Group one is associated with 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 reports or variable results from different groups (in *black*). Several common cancer types are in group three. Therefore, additional studies in group three cancers will provide insights as to the significance of Hh signaling in different types of cancer. This figure was from [177] with permission from the publisher

Su(Fu) is also mutated in a small number of BCCs [106]. Taken all the data together, the genetic alteration of the Hh pathway is detected in about 70% of BCCs. Since almost all BCCs have activated Hh signaling, we predict that alterations in other Hh signaling molecules or related molecules may be responsible for the Hh pathway activation in 30% of sporadic BCCs. At the molecular level, activated Hh signaling in BCCs leads to cell proliferation through elevated expression of PDGFRα [109], whereas targeted inhibition of the pathway causes apoptosis via Fas induction [110].

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 in a few cases. In addition, noncanonical activation of Gli2 via ATOH1 and Yap1 has been detected in medulloblastomas. Hh signaling is activated both in the desmoplastic form (more often) and in the classic form of medulloblastomas.

Activation of Hh Signaling in Cancers Not Associated with Gorlin Syndrome

Increasing data indicate that Hh signaling is activated in many types of human cancers, including those associated with Gorlin syndrome as well as those not associated with this syndrome. It is estimated that over 30% of human cancers exhibit activated Hh signaling to some extent. These cancers include brain tumors, melanomas, leukemia, 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) [111, 112]. Current understanding is that Hh signaling activation in type II cancers is caused by ligand-dependent mechanisms or noncanonical Hh signaling activation. The association of ligand-dependence (or ligand-independence) with a specific cancer type, tumor morphology, or tumor stage has not been established.

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

Increasing evidence indicate that Hh signaling is involved in different stages of carcinogenesis in different cancer types. 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 [113]. Sox9, as a target gene of BMP4, is highly expressed in the epithelial lesion [113]. These results indicate that Hh signaling plays an important role for the initiation of esophageal adenocarcinomas. In pancreatic cancer, activation of this pathway is found in PIN lesions as well in metastatic cancer [114–116], 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 are different from pancreatic ductal adenocarcinomas (PDAC) [116–118], suggesting that activation of Hh signaling alone is not sufficient to drive the development of PDAC. In other tumors, such as gastric and prostate cancers, Hh signaling activation is associated with cancer progression [119–122]. Consistent with these findings, inhibition of Hh signaling in prostate and gastric cancer cells reduces cell invasiveness [120, 122, 123] (our unpublished data). Reports also suggest that Hh signaling is required for development and progression of melanoma, gliomas, breast cancer, ovarian cancer, leukemia, and B-cell lymphomas [124–129]. However, the role of Hh signaling in each of the cancer types 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 indicate that Hh signaling is critical for cancer stem cell maintenance and function [130–132]. For example, leukemia stem cell maintenance and expansion is dependent on Hh signaling [130, 131]. 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 [131, 133–136]. Based on the cancer stem cell theory, it is anticipated that Hh signaling activation exerts resistance to cancer chemotherapy and radiotherapy [137]. Indeed, several studies have shown that Hh signaling activation is associated with chemotherapy resistance or radiotherapy resistance [138–140]. Hh signaling inhibitor IPI-926 enhances the delivery of chemotherapeutical drug Gemcitabine in a mouse model of pancreatic cancer [139]. Despite all these progress, more experiments are necessary to determine whether and how Hh signaling is involved in cancer stem cells in solid tumors.

In reviewing literatures on Hh signaling in human cancer, we notice that different results of Hh signaling activation are often reported from the same cancer type. This discrepancy may come from several 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 suggest that Hh signaling functions in maintaining cancer stem cell proliferation [130–132], not proliferation of all cancer cells. The percentage of cancer stem cells varies a lot from different tumor types. Second, heterogeneity in the tumor tissue often accounts for the difference in the analysis of Hh target gene expression by real-time PCR. For example, only a small portion of prostatectomy specimens (5–10%) is cancer tissue, whereas specimens of transurethral resection of the prostate (TURP) frequently have a large fraction of cancer tissue. Thus, the data from these two different types of specimens may result in different data, mainly due to the percentage of cancer cells in the tissue [122]. Even laser microscope captured tissues that will have significant amount of noncancerous cells, and the percentage may vary from operator to operator. Third, a standard is needed to define Hh signaling activation as different standards are being used. Some use increased expression of Gli1 as the read-out [91, 125], whereas others test the expression of several Hh target genes, such as Gli1, PTCH1, sFRP1, and HIP [116, 120, 141, 142]. Still others use only immunohistochemistry to detect Hh signaling activation [128, 143] while most studies use multiple approaches. Therefore, we need to read the literatures with cautions. Particular attention should be paid to the methodology used in the studies and reproducibility of the results. We believe that detection of Hh signaling pathway activation using immunohistochemistry of only one Hh target gene is not a reliable approach.

Animal Models for Hh-Mediated Carcinogenesis

It is known that correlation of Hh target gene expression with 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 the role

of Hh signaling in carcinogenesis. Currently, the mouse models for BCCs and medulloblastomas are well established, whereas mouse models for other Hh signaling-mediated carcinogenesis still need more work.

Mouse Models for BCCs

Wild-type mice never develop BCCs even after carcinogen treatment, UV, or ionizing radiation. Ptch1$^{+/-}$ mice are susceptible to BCC development following UV irradiation or ionizing radiation [103]. The frequency of BCC development under these conditions is around 50% with one or two tumors per mouse [110, 144]. Due to the embryonic lethality of Ptch1$^{-/-}$, tissue-specific knockout of Ptch1 has been generated [145]. By combing conditional gene knockout and the inducible activity of the keratin 6a promoter, Krt6a-cre:Ptch1$^{neo/neo}$ mice develop BCCs following the stimulation of retinoic acid [146]. In addition to Ptch1 k/o mouse model, transgenic mice expressing Smo using Krt5 or Krt14 promoters also develop BCC-like tumors [104, 147]. However, these transgenic mice eventually lose the expression of Smo via unknown molecular mechanisms. Using conditional knock-in technology, skin-specific knock-in of SmoM2^{YFP} (Krt14-creER:R26-SmoM2^{YFP}) mice develop multiple microscopic BCCs at very early age, which provide an easy genetic assay for putative Hh signaling downstream of Smo [148]. Su(Fu)$^{+/-}$ mice develop skin lesion resembling skin hyperplasia but not BCC-like tumors [80]. Several transgenic mice have been developed using downstream transcriptional factors Gli1 and Gli2 as well as Shh [149–151]. The inducible expression of Gli2 in the skin results in BCCs in a few weeks. These mouse models provide rich resources for further understanding of Hh signaling-mediated BCC development.

Mouse Models for Medulloblastomas

A small portion of Ptch1$^{+/-}$ mice (10–30%) develops medulloblastomas and Rhabdomyosarcomas [82, 83]. The synergy between p53 pathway and Hh signaling is clearly shown in the medulloblastoma model. While p53 null mice do not develop this type of tumors, Ptch1$^{+/-}$ p53 null mice all develop medulloblastomas [152]. On the other hand, Ptch2$^{+/-}$ mice do not develop medulloblastoma per se but increase the incidence of medulloblastomas in Ptch1$^{+/-}$ mice [153, 154]. Su(Fu)$^{+/-}$ mice develop skin phenotypes similar to Gorlin syndrome but are generally not tumor prone [80]. However, Su(Fu)$^{+/-}$ mice with p53 null background frequently develop medulloblastomas with the signature of Hh signaling alterations [155]. Although Ptch1$^{+/-}$:Su(Fu)$^{+/-}$ mice are more likely to develop medulloblastoma than Ptch1$^{+/-}$ mice, the difference was not statistically significant [156]. In addition to the loss of tumor suppressor genes, transgenic mice using activated Smo mutant molecule SmoM2 under the control of neuroD2 promoter results in medulloblastomas in

most mice [157]. Tissue-specific activation of Hh signaling via Ptch1 knockout or SmoM2 expression using granule neuron precursor lineage-specific promoters (Math1, GFAP, Oligo-2, and TLx3) but not the purkinje neuron-specific promoter leads to the formation of medulloblastoma [158, 159], indicating that granule neuron precursors are the source for medulloblastoma development. Further analysis shows that CD15 is the medulloblastoma stem cell marker [132, 160].

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 [148]. In this model, all mice exhibited rhabdomyosarcoma and BCC; and 40% also developed medulloblastoma. In addition, pancreatic lesions resembling low-grade mucinous cystic neoplasms in humans and diverticular harmartomatous lesions in both 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 drive tumor formation in prostate, lung, breast, and GI-tract.

Similar data are also observed in other studies. For example, it is shown in orthotopic mouse models that Hh signaling is necessary for pancreatic cancer tumor metastasis [161] (our unpublished data). Pancreas tissue-specific deletion of *Smo*, on the other hand, did not affect the formation of PDAC, whereas *GLI2* expression (CLEG2:Pdx1-cre mice) or Shh expression only lead to the formation of undifferentiated pancreatic tumors [116–117, 162]. These results indicate that activation of Hh signaling alone is not enough to drive PDAC formation, but it is essential for tumor progression and metastasis. In consistent with this theory, PDAC development of $Kras^{+/G12D}$:Pdx1-cre mice is not affected by the removal of Smo gene, and pdx-1 cre-driven expression of SmoM2 does not result in PIN lesions despite the fact that paracrine Hh signaling is observed in the pancreatic tissue [117, 162].

Recent study of Barrett's esophagus indicates that Shh expression in the epithelium of Barrett's esophagus can lead to stromal expression of Hh signaling target genes [113]. Using Shh transgenic mouse model, it is shown that epithelial expression of Shh can lead to stromal expression of Hh target gene BMP4, its target gene Sox9, and columnar phenotype of mouse esophageal epithelium, which resembles a feature in human Barrett's esophagus. These data suggest that Hh signaling activation can drive the formation of some features resembling Barrett's esophagus in mice.

For the role of Hh signaling in other cancer types, the major mouse models are based on xenograft in immunodeficient mice (nude or SCID mice) [163]. With potential implications of Hh signaling inhibitors for clinical cancer treatments, more established mouse models will be needed. Because modeling cancer

metastasis is quite a challenge, we anticipate an increase in the use of orthotopic mouse models for studying Hh signaling in cancer progression and metastasis in the next few years.

Small Molecule Modulators of Hedgehog Signaling

More than 50 compounds have been disclosed to have inhibitory effects on Hh signaling. Of these, four are being used in clinical trials. There are three major targeting sites for Hh signaling inhibitors identified so far: Hh molecules (Shh neutralizing antibodies and 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) [164]. We can divide Hh signaling inhibitors into three groups: natural products (cyclopamine, its derivatives, and other natural products); synthetic small molecules, and Hh signaling modulators. Table 7.1 lists the major Hh signaling inhibitors.

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 [165]. Identification of

Table 7.1 Hedgehog signaling inhibitors[a]

Inhibitor	50% Maximal inhibition (IC50)	In vitro/in vivo studies
Cyclopamine	300 nM	In vivo and in vitro
KAAD-cyclopamine	20 nM	In vitro cultured cells
Jervine	500 nM	In vitro and cultured embryos
Cyclopamine tartrate acid salt (Cyc-T)	20 nM	In vitro and in vivo studies
Cur-61414	200 nM	Phase I clinical trial
Sant-1,2,3,4	20–200 nM	In vitro studies
Gant-58,61	5 μM	In vitro and in vivo studies
IPI-926	<20 nM	Phase I clinical trial
GDC-0449	<20 nM	Phase I/II/III clinical trials
BMS-833923 (XL139)	<20 nM	Phase I clinical trial
LDE-225	<20 nM	Phase I clinical trial
Vitamin D3	100 μM	In vitro
Robotnikinin	>10 μM	In vitro
HPI-1,2,3,4	<10 μmol/L	In vitro
Itraconazole	<1.5 μM	In vitro and xenograft

[a] Modified from Yang et al. *Oncogene* 29, 469–481 (2010) and see text for references

specific small molecule antagonists of SMO has opened up exciting new prospects for targeted cancer therapy for human cancers associated with Hh signaling.

Specificity of cyclopamine varies depending on the concentration used. While cyclopamine at a low concentration (<10 μM) has specific inhibitory effects on Hh signaling, high doses of cyclopamine can result in cell death without effecting Hh target gene expression [166]. 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% [110]. The treatment in this mouse model also prevents the development of additional microscopic BCCs, implying a cancer prevention potential of cyclopamine. Similarly, cyclopamine is shown to be effective in reducing medulloblastoma development in *Ptch1*$^{+/-}$ mice [167] and tumor growth of cancer cell lines in nu/nu mice [91, 116, 120, 168]. Additional modifications on cyclopamine aiming at increasing acid stability and aqueous solubility are now available, such as IPI-926 and Cyc-T [169, 170]. IPI-926 is now in Phase I clinical trial.

Synthetic Hh Signaling Antagonists

Increasing number of synthetic Hh antagonists are reported in the literature, with most compounds targeting at SMO. Four of these compounds are now in clinical trials (Table 7.1), including GDC-0449. The successful clinical trials with GDC-0449 on human BCCs further encourage the translational studies in this area [171]. Clinical trial of the same compound in a medulloblastoma patient led to rapid tumor shrinkage but later developed drug resistance due to a SMO mutation, which disables the binding of GDC-0449 to SMO. This case report implies a need for novel alternative strategies for the treatment of Hh signaling-associated cancers. There are also a couple of small molecules targeting at Shh or Gli [171–173]. Because the wide spread existence of nonconical regulation of Gli transcriptional factors and potential resistance to Smo inhibitors, the antagonists targeting Hh downstream effectors constitute a valuable source for developing chemotherapeutical strategies for Hh pathway-related cancers.

Hh Signaling Modulators

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 a possibility to treat BCCs with nutrition 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 [174]. Curcumin has been shown to be able to block Hh signaling-mediated carcinogenesis. A commonly used antifungal agent Itraconazole is shown to affect Hh

signaling [175]. Several natural products, genistein, curcumin, EGCG, and resveratrol [176], are also shown to affect Hh signaling in a mouse model of prostate cancer. The detailed molecular mechanisms of action for these signaling modulators remain elusive.

Summary

In summary, link of Hh signaling activation to a variety of human cancer implies the relevance of studying Hh signaling to human health. Rapid advance in the discovery of novel Hh signaling inhibitors has provided many opportunities for developing novel cancer therapeutic strategies. Several major challenges exist for moving the Hh signaling inhibitors into clinic including the lack of basic understanding for the molecular mechanisms of Hh signaling-mediated carcinogenesis; identifying the right tumors for therapeutic application; reliable and reproducible mouse models for testing and optimization of drug dosages to minimize the side effects.

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Chapter 8
Hedgehog Signaling in Pediatric Brain Tumors

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Keywords Sonic hedgehog • Central nervous system • Cerebellum • Pediatric brain tumors • Medulloblastoma • Hedgehog inhibitors

Introduction

Sonic hedgehog (SHH) signaling plays roles in patterning and normal development of the mammalian central nervous system (CNS). Precise regulation of the pathway appears to be crucial in the CNS since dysregulation of SHH signaling has been associated with CNS birth defects and brain tumors. In this chapter, we focus on (1) the role of SHH signaling in mammalian CNS development, (2) the role of SHH signaling in pediatric brain tumors, and (3) potential clinical applications of Hedgehog (HH) pathway inhibitors in the treatment of pediatric brain tumors. We use the following conventions in this chapter: upper case = human protein (e.g. SHH, GLI1), lower case = mouse protein (e.g. shh, gli1), upper case italics = human gene (e.g. *SHH*, *GLI1*), and lower case italics = mouse gene (e.g. *shh*, *gli1*). When we are discussing a pathway in a general way without specific reference to gene, protein, or species, we use upper case without italics.

Role of SHH Signaling in CNS Development

During development, the CNS arises from the neural plate, which is composed of a single layer of cells derived from midline ectoderm. Neuroepithelial cells in the neural plate undergo rapid proliferation and morphologic changes to form the neural tube.

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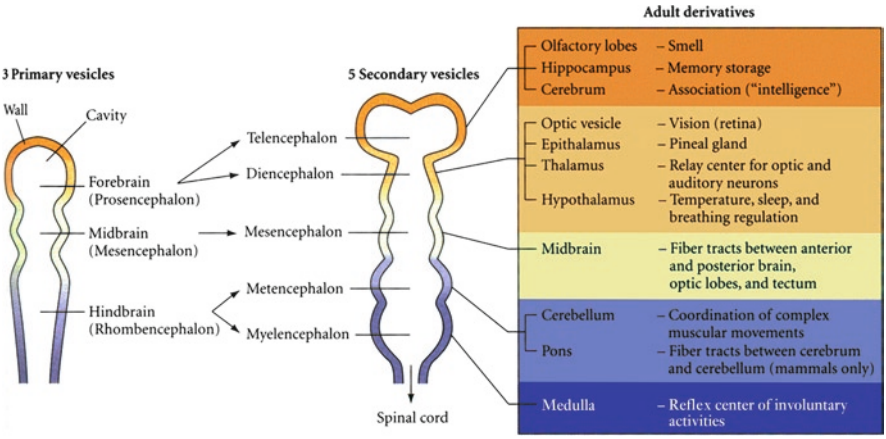

Fig. 8.1 Early human brain development. The three primary brain vesicles, five secondary brain vesicles, and their adult brain derivatives are shown. Reproduced and adapted from Developmental Biology, eighth edition with permission from Sinauer Associates, Inc.

Further proliferation of cells in the anterior region of the neural tube causes expansion and creates three primordial brain vesicles called the prosencephalic, mesencephalic, and rhombencephalic vesicles. These primary vesicles subsequently develop into secondary vesicles: the telencephalon and diencephalon (forebrain), mesencephalon (midbrain), and metencephalon and myelencephalon (hindbrain) (Fig. 8.1).

At a molecular genetic level, the events described above require complex interactions between key signaling pathways, including the SHH, Wingless (WNT), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) pathways and their target genes. Activation of these signaling pathways has been associated with fundamental events during CNS patterning. These events include (1) establishment of polarity within the developing nervous system, (2) rapid expansion of cells in the region of the developing brain, (3) establishment of inter-brain boundaries, and (4) establishment of regional specificity. Remarkably, SHH signal transduction appears to be critically involved in each of these developmental events in a spatial- and temporal-dependent manner.

Dorso-Ventral Polarity and SHH Signaling

During early mammalian development, CNS patterning requires the establishment of axes in the neural tube. shh signaling contributes to establishing the dorso-ventral axis as the neural tube fuses at embryonic (E) day 8.5 in the mouse. shh is secreted by the notochord which lies immediately ventral to the neural tube and is also expressed by the ventral floor plate in the developing neural tube [1, 2]. Expression of *shh* in the ventral region of the developing neural tube establishes a gradient of shh within the neural tube, highest ventrally and lowest dorsally (Fig. 8.2).

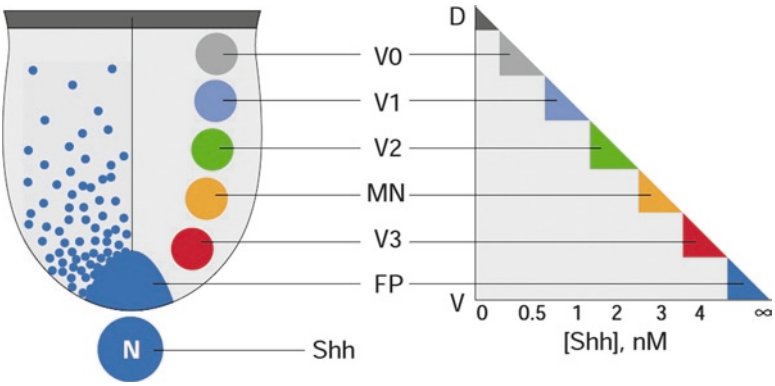

Fig. 8.2 A shh gradient regulates transcription factors that establish ventral specification in the developing CNS. The shh gradient (shown on the *right*) induces ventral floor plate and specifies five ventral cell types (shown on the *left*). *D* dorsal, *FP* floor plate, *MN* motor neuron, *N* notochord, *V* ventral, *V0–V3* ventral inter neurons 0–3. Reproduced and adapted from EMBO reports 4(8):761–765 (2003) with permission from the Nature Publishing Group

This gradient differentially regulates the expression of transcription factors that specify polarity and ultimately cell fates in the developing CNS, including *pax*6, the homeobox gene *nkx*2.2, and the floor plate marker *hnf3*-β [3, 4]. The importance of shh to early CNS development has been demonstrated in $shh^{-/-}$ mice, in which the notochord degenerates and the ventral floor plate and motor neurons fail to form. The ability of shh to induce differentiation of ventral cell types in the nervous system has been demonstrated by aberrantly expressing *shh* in the dorsal CNS. Aberrant expression of *shh* in the dorsal CNS activates dorsal expression of *hnf3-β* and causes aberrant dorso-ventral patterning [5–7].

Although shh induces differentiation of ventral neural precursor cells [6, 8, 9], further differentiation into motor neurons, interneurons, glial cells (oligodendrocytes), and other cell types in the CNS appears to require complex and incompletely understood interactions between shh signaling and the wnt, bmp, fgf, and tgf-β signaling pathways [7]. For example, shh regulates expression of *fgf8* receptors [10–13], and fgf8 together with shh induce dopaminergic neurons in the ventral region [10].

Rapid Expansion of Cells in the Region of the Developing Brain

Early mammalian brain development is characterized morphologically by rapid growth and expansion of the neural tube, which results in the formation of the brain vesicles (Fig. 8.1). The three primary vesicles have formed by week 4 of human development. This morphologic change results from both increased proliferation and reduced apoptosis of neuroepithelial cells. Several experimental approaches have been used to show roles for shh in regulating both proliferation and survival of cells that contribute to brain development. First, studies placing a transplanted

notochord, the source of shh, near the neural tube demonstrate increased proliferation, differentiation, and survival of nearby neural tube cells [14–16]. On the contrary, surgical removal of the notochord disrupts midbrain expansion by promoting cell death and inhibiting cell proliferation [17]. Second, *shh*$^{-/-}$ mouse embryos show multiple defects, including an overall reduction in the size of the brain, especially the forebrain [5]. Finally, ectopic expression of shh by electroporation into the developing midbrain region stimulates cell proliferation to regulate growth and morphology of the ventral region of the midbrain [17, 18].

Brain Boundaries

Studies in vertebrate embryos suggest that shh signaling specifically regulates genes at the midbrain–hindbrain boundary. Evidence suggests that shh functions to maintain this distinct boundary once it has formed rather than establishing the boundary [19]. Indeed, ectopic expression of *shh* by microinjection into one blastomere at the 2–4 cell stage expands the domain of expression of the shh target gene *sal* at the midbrain–hindbrain boundary [20, 21]. Disruption of shh signaling at the boundary by a mutant form of patched (ptc1) that cannot bind shh causes the midbrain–hindbrain boundary to become broader and indistinct, with midbrain and hindbrain cells inter-mixing across the expanded border [22]. Dorso-ventral cell fates are also affected in this model. As seen in other regions and periods during CNS development, blocking endogenous shh activity in the midbrain transforms cell fates from ventral to dorsal and correlates with the movement of dorsal cells into the ventral midbrain [19].

Regional Specification of the Developing Brain

shh is expressed along the entire anterior–posterior axis of the developing neural tube. It is believed that the establishment of regional specificity along the anterior–posterior axis of the developing CNS is achieved by differential expression of *shh* together with other key signaling pathways, such as the *wnt*, *bmp*, *fgf*, and *tgf-β* pathways in a regional specific manner. Roles for shh in regional specification is reviewed in the following sections.

Forebrain

shh signaling appears to regulate the size, ventral cell fate specification, and ventral patterning of the developing telencephalon. Targeted loss of shh in *shh*$^{-/-}$ mice results in multiple morphologic defects in the forebrain, including a reduction in size, fused telencephalic vesicles, and fused optic vesicles [5]. On the contrary, ectopic expression of *shh* by retroviral injection in early mouse embryos (E9.0) enhances proliferation and causes a substantial expansion in the size of the telencephalon [23].

If neural explants from the telencephalic region are incubated with *shh*-expressing cells, the neural plate in the prospective forebrain region differentiates into motor neurons which are normally observed in the ventral CNS [8]. shh treatment at E10.5 also represses expression of dorsal telencephalic markers such as *emx1* and *tbr-1* [24].

Ventralization of the telencephalon is also tightly regulated at the level of the gli family transcription factors. For example, shh inhibits expression of the repressor form of *gli3* (*gli3-R*) in the ventral telencephalon, presumably to promote active shh signaling and ventralization as well as to inhibit dorsalization [25]. Indeed, "extra-toes" mice carry a mutation in *gli3*, and E12.5 mutant embryos lack expression of dorsal marker *bmp* genes in the telencephalon, even though shh expression is unaltered [25]. Also, ventral marker genes, such as *dlx*2 and *gsh*2 are expressed in the dorsal telencephalon in "extra-toes" mice [23].

Midbrain and Hindbrain

shh signaling appears to regulate cell proliferation, apoptosis, and cell fate specification in the developing midbrain and hindbrain. Loss of *shh* expression causes decreased cell proliferation and increased apoptosis in the midbrain region. Similar results are obtained when the shh signal is reduced by surgical separation of the notochord from the floor plate, injection of cyclopamine into the lumen of the midbrain, or in the setting of $shh^{-/-}$ mice [17, 26]. These cellular changes collectively alter early expansion of the brain, causing a reduction in the size of the midbrain and ultimate collapse of the brain vesicles. Of interest, while the growth of the developing midbrain in E8.5 $shh^{-/-}$ mice is significantly reduced, the sizes of the diencephalon and hindbrain are unaffected [26].

shh signaling also specifies dopaminergic neuron cell fate in the developing midbrain [27]. Recent evidence using cultured midbrain suggests that higher level shh signaling inhibits cell proliferation and dopaminergic neuron specification, pointing out that shh signaling functions in a concentration-dependent manner to establish cellular and morphologic phenotypes [28]. In addition, in the hindbrain, shh signaling defines the ventral region and promotes hindbrain growth.

shh signaling in the midbrain and hindbrain regions is mediated through gli family transcription factors [29, 30]. Ectopic expression of *gli1* in the midbrain and hindbrain regions activates the ventral markers *ptc1* and *hnf3-β* [31]. Conditional *gli2* knockout mouse embryos at E9.0 and E11.5 demonstrate that the activator form of gli2 (gli2-A) promotes growth of the ventral midbrain and hindbrain regions, whereas the repressor form of gli3 (gli3-R) is continuously required for the overall growth of the dorsal midbrain and hindbrain, presumably by inhibiting shh signaling in these regions [27–29]. Detailed analysis in the developing hindbrain reveals that *gli*2 **–/–** mouse embryos show a more severe ventral defect in the hindbrain than in the spinal cord, since gli3 can compensate for the loss of gli2 in the spinal cord but not in the hindbrain [32].

Interactions between the shh signaling pathway and the bmp pathway appear to specify the ventral region of the hindbrain. Aberrant expression of *bmp-7* in the

floor plate region inhibits *shh* expression and interrupts dorso-ventral patterning of the hindbrain, suggesting that bmp-7 regulates shh signaling in this domain [33].

Cerebellum

The cerebellum originates from the metencephalon. It is the largest part of the hindbrain and is connected to the other parts of the brain through projection fibers. Through these fibers, the cerebellum receives input from sensory systems and integrates the signals to coordinate and accurately time movement. The cerebellum is composed of many different types of neurons, including Purkinje neurons, granule neurons, Bergmann glia, astrocytes, interneurons, and neurons of the deep nuclei. Cerebellar development has been reviewed previously in detail [34–37].

shh signaling plays an integral role in the developmental biology of the Purkinje neurons, Bergmann glia, and granule neurons in the cerebellum (Fig. 8.3).

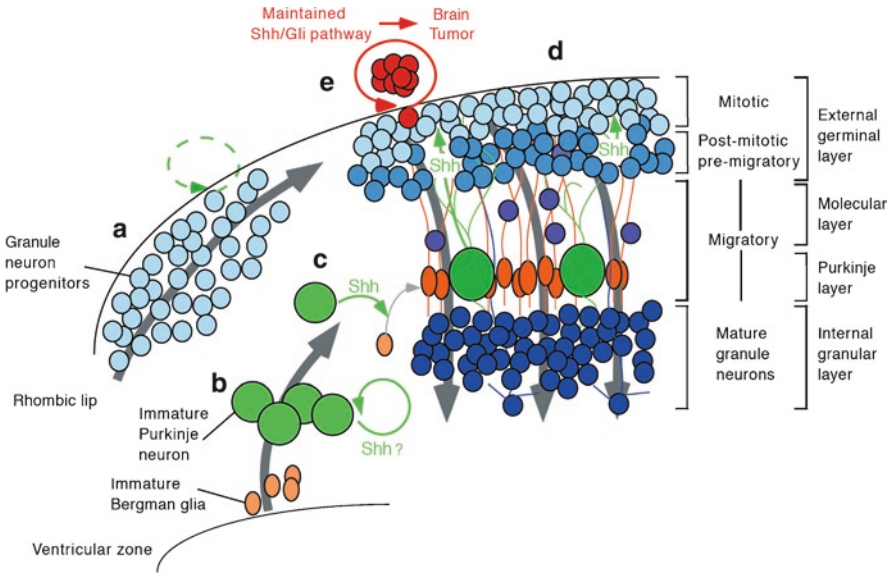

Fig. 8.3 The role of shh in cerebellar development. (**a**) Granule neuronal precursors (CGPs in text; *light blue*) migrate tangentially from the rhombic lip toward the EGL. During migration, the shh pathway may be transiently active in an autocrine manner (*dashed green arrow*). (**b**) Purkinje neurons (*green*) and Bergmann glia (*pale orange*) migrate from the ventricular zone toward the Purkinje layer. Purkinje neurons may initially use the shh pathway in an autocrine manner (*green arrow*). (**c**) shh from Purkinje neurons (*green arrows*) induces Bergmann glia maturation (*bold orange*). (**d**) In the later stage EGL, granule neuronal precursors (CGPs; *light blue*) proliferate in the outer zone and mature glia send extensions (*orange lines*) toward the inner EGL. Post-mitotic granule cells (*bold blue*) then migrate (*purple cells*) on glial fibers to form the internal granular layer (*dark blue cells*). (**e**) Constitutive shh signaling in EGL cells or failure to induce their differentiation may contribute to the development of medulloblastoma (*red arrows and cells*). Reproduced and adapted from Development 126, 3089–3100 (1999) with permission from the Company of Biologists, Ltd

During early embryonic development, Purkinje cells are derived from progenitor cells located in the ventricular zone of the neural tube, and granule neurons arise from the thickened alar plate of the embryonic rhombencephalon called the rhombic lip. Both cell types migrate into the region of the developing cerebellum, where cerebellar granule precursors (CGPs), also called granule cell precursors, granule neuron progenitors, or granule cell neuron precursors, form the external granular (or germinal) layer (EGL). Once the cells arrive in the EGL, dramatic growth of the neonatal mouse cerebellum ensues, increasing over 1,000-fold in volume. This period of growth is driven by the rapid proliferation of CGPs [38]. As a result of this rapid proliferation, granule neurons become the most abundant neurons in the cerebellum. In fact, more than 50% of the neurons in the entire mouse brain are comprised of granule neurons [39]. Amplified CGPs located in the EGL eventually exit mitosis, differentiate, and migrate internally to form the internal granular layer [39]. Bergmann glia interact with post-mitotic CGPs during their migration.

shh signaling regulates the proliferation and differentiation of CGPs and induces maturation of Bergmann glial cells. In situ hybridization studies for *shh*, *ptc1*, and *gli1* demonstrate that the shh ligand is produced by the Purkinje cells, and the *ptc1* receptor and *gli1* transcription factor are expressed in CGPs in the EGL. This expression pattern suggests paracrine signaling from Purkinje cells to CGPs in the developing cerebellum. Indeed, blocking shh activity with neutralizing anti-shh antibody disrupts CGP proliferation in the EGL [39–41]. Also, CGPs treated with shh in vitro remain undifferentiated while untreated CGPs undergo spontaneous differentiation, suggesting a role for shh in preventing their differentiation [39].

shh drives CGP proliferation by activating G_1-cyclins and N-myc [42, 43]. *atoh1* (also called *math1*) is a basic helix-loop-helix transcription factor, which is highly expressed in CGPs [44] that directly activates expression of the shh mediator *gli2*, thereby significantly promoting shh signaling [45]. Conditional knockout of *atoh1* in the post-natal mouse cerebellum reduces the size of the EGL, since CGPs cannot respond to the shh signal. In addition, *gli2* expression is significantly inhibited even in the presence of constitutively activated shh signaling in *atoh1* null conditional mutants, supporting the concept that atoh1 is a critical regulator of *gli2* and therefore shh signaling in the cerebellum [45].

Interactions of shh with several other proteins and signaling pathways are required for CGPs to exit from the proliferative cycles and begin differentiation. The extracellular matrix protein vitronectin is continuously expressed in the developing cerebellum very close to the *shh*-expressing cell population. Physical interaction of vitronectin with shh inhibits shh-mediated proliferation of CGPs and promotes their differentiation [46]. bmp-2 antagonizes shh-mediated CGP proliferation through smad5 [47] and through tieg-1, which inhibits N-myc [48]. The BTB/POZ domain-containing protein REN also antagonizes shh by negatively regulating gli1 and gli2 activity in CGPs, thereby promoting growth arrest, enhancing differentiation, and activating apoptosis of CGPs [49]. Finally, the fgf signaling pathway suppresses shh-induced proliferation of CGPs by down-regulating expression of *gli1*, *N-myc*, and *cyclin D1* [39, 50] and promotes differentiation of CGPs in the presence shh, suggesting an inhibitory role on shh during CGP differentiation [50].

It remains uncertain whether shh plays a direct role in the migration of CGPs. shh increases migration of granule cell explants [40], whereas blocking shh activity with neutralizing anti-shh antibody inhibits the migration of the cells. However, two complementary models, which prevent shh signaling by Purkinje cells show significantly compromised expansion of the CGPs and post-mitotic granule cells, but migration is not affected [38].

Aberrant Activation of SHH Signaling in Pediatric Brain Tumors

Collectively, CNS tumors represent the most common solid tumors among children and are a leading cause of pediatric cancer-related morbidity and mortality [51–53]. Pediatric brain tumors are a heterogeneous group of malignancies that differ in scope, behavior, and biology compared to adult CNS tumors. The majority of adult brain tumors are high-grade gliomas, meningiomas, and metastases from extra-CNS solid tumors [54–56]. Metastases to the brain and meningiomas are rarely seen in pediatrics, and high-grade gliomas only represent 10–15% of all childhood brain tumors [53]. On the contrary, astrocytomas and medulloblastomas are the most common CNS tumors in children, accounting for approximately 60 and 20% of pediatric brain tumors, respectively [54–56].

Despite multimodal therapies for childhood brain tumors, including surgical resection, cytotoxic chemotherapy, and radiation therapy, there remains a significant group of patients who succumb to their disease. In addition, many children who survive sustain significant late effects related to their original tumor and therapies, including neurocognitive deficits, endocrine dysfunction, ototoxicity, and the development of secondary malignancies [57–60]. A significant amount of research is underway evaluating the molecular, biologic, and cytogenetic characteristics of pediatric CNS tumors. The hope is that future targeted therapies tailored to the specific aberrant molecular pathways within a tumor will not only improve survival, but also may help to minimize some of the late effects. The childhood brain tumor that has advanced the furthest along this research trajectory is medulloblastoma, in large part based on research directed at the SHH signal transduction pathway.

Genetic Alteration of Components of the SHH Pathway in Pediatric Brain Tumors

Based on the fundamental roles that the SHH pathway plays in cell proliferation and cell fate specification during CNS development, it is not surprising that constitutive activation of the pathway is associated with brain tumors. Constitutive pathway activation has been described in association with continuous somatic expression of the SHH ligand in a variety of cancers outside the CNS. In pediatric brain tumors,

Table 8.1 Dysregulation of SHH signaling in childhood brain tumors

Tumor	Gene	Type of abnormality	References
Medulloblastoma	*PTCH1*	Loss of function mutation	[68, 96, 112, 142]
	SMO	Activating mutation	[141]
	SuFu	Loss of function mutation	[101, 143]
	GLI2	Amplification	[102]
Ependymoma	*IHH*	Overexpression	[121]
	GLI2	Overexpression	[120, 121]
	GLI1	Overexpression	[120]
Pilocytic astrocytoma	*PTCH1*	Overexpression	[124]
	GLI1	Overexpression	[124]
Craniopharyngioma	*PTCH1*	Loss of function mutation	[119]

constitutive pathway activation is more typically independent of HH ligands and is the result of mutations in downstream components of the HH pathway. These genetic alterations may be inherited constitutional mutations associated with cancer predisposition syndromes, such as basal cell nevus syndrome, or may be somatic. The most common genetic alterations in HH pathway components in childhood brain tumors are summarized in Table 8.1.

Medulloblastoma: Clinical Aspects

Medulloblastoma is the most common malignant brain tumor in childhood [54, 61]. It is a highly malignant embryonal tumor that is believed to arise from CGPs in the cerebellum. It is considered a central primitive neuroectodermal tumor (PNET) based on the histologic appearance of the cells mimicking embryonic neuroectoderm. The name "medulloblastoma" implies that the primary tumor is located within the cerebellum. Central PNET can occur in other locations within the CNS, including the supratentorium, brainstem, and spine; however, in these locations the tumor is not referred to as medulloblastoma and only represents approximately 2–3% of all pediatric brain tumors [62, 63]. These non-cerebellar central PNETs are thought to be biologically distinct from medulloblastoma based upon genetic and biologic studies as well as worse clinical outcomes compared to medulloblastoma [62, 64]. The cell of origin for these CNS non-cerebellar PNETs is not yet known.

Medulloblastoma is more commonly seen in males than females. The peak age at diagnosis is typically between 5 and 7 years old, however, it can be seen from birth to young adulthood [54, 65]. In fact, age at the time of diagnosis is one of the few important clinical prognostic factors known in medulloblastoma. The etiology of the majority of medulloblastomas is unknown, however, there are a few rare genetic disorders that predispose some patients to medulloblastoma, including basal cell nevus syndrome, Li–Fraumeni syndrome, ataxia telangiectasia, and Turcot's syndrome [66–69].

Children with medulloblastoma typically present with a relative short history of symptoms related to obstructive hydrocephalus, including early morning headaches,

emesis, and papilledema. They can also present with signs of cerebellar dysfunction, such as truncal ataxia and unsteady gait [54]. Finally, some patients may present with symptoms related to metastatic foci of disease in other parts of the brain and spine, such as seizures and signs of spinal cord compression.

Medulloblastoma has an inherent tendency to spread throughout the CNS. Therefore, staging at diagnosis is essential and includes a complete brain and spine MRI as well as evaluation of lumbar cerebro-spinal fluid (CSF) cytology to evaluate for metastasis. Typically, the spine MRI and lumbar CSF collection is performed 10–14 days postoperatively in an effort to avoid false positives secondary to surgical debris. The modified Chang staging system is used to stage these patients at diagnosis, where M0 patients have no signs of metastasis, M1 patients only have tumor cells on lumbar cytology, M2 patients have macroscopic spread of tumor to distant parts of the brain, M3 patients have macroscopic metastases to the spine, and M4 patients have spread outside the CNS, which is exceedingly rare in the modern era [70].

Despite a significant understanding of medulloblastoma biology, biologic characteristics have not yet been incorporated into up-front treatment strategies and prognostication. Currently, the major prognostic factors utilized to stratify patients with medulloblastoma include age at diagnosis, extent of tumor resection, absence or presence of CNS dissemination/metastases, and tumor histology. Disease characteristics that render a patient at high risk include residual disease greater than 1.5 cm^2 after primary surgery, metastasis to distant parts of the brain or spine, and anaplastic histology [61]. Patients with one or more of these characteristics are typically treated with an intensified regimen that includes both craniospinal irradiation and chemotherapy. In addition, patients less than 3 years old at the time of diagnosis are also considered at high risk due to their worse clinical outcomes and are treated with unique therapy approaches. These strategies often delay or avoid irradiation, since this group of patients is highly susceptible to the deleterious effects of radiation therapy.

Patients who are greater than 3 years of age with high risk disease are currently treated with a combination of full dose craniospinal irradiation (36 Gy to the neuraxis and boost to the posterior fossa up to 54 Gy) and chemotherapy. These patients have a 5-year progression-free survival ranging from 40 to 70% [54, 61, 71, 72]. Those patients who are less than 3 years old at diagnosis are often treated with a combination of high-dose chemotherapy followed by autologous hematopoietic cell rescue with or without adjuvant radiation therapy. These patients have 5-year progression-free survival ranging from 30 to 60% in published prospective series, and many of these patients have avoided radiation therapy completely [73–75]. Superior survival has been published using the German HIT protocol for this group of young patients. This protocol uses a chemotherapy alone regimen that includes intrathecal methotrexate. However, over half of the patients reported in this series had desmoplastic histology which is believed to confer a better prognosis in younger patients [75]. Also, there is concern that the intrathecal methotrexate contributed to neurocognitive sequelae seen in these patients. For these reasons, this approach has not been universally adopted.

The current approaches to patients with standard risk of medulloblastoma, that is, age greater than 3 years at diagnosis, less than 1.5 cm² residual disease postoperatively, no signs of metastasis (as seen on MRI of the brain and spine and lumbar fluid cytology), and a classic histology, include a dose reduction of craniospinal irradiation (23.4 Gy to the neuraxis and a boost to the posterior fossa up to 54 Gy) and adjuvant chemotherapy. This strategy maintains good outcomes, and preliminary data suggest it may reduce neurocognitive sequelae [76]. These patients have a 5-year progression-free survival of approximately 85% [71].

Desmoplastic medulloblastoma is a less common histologic variant of medulloblastoma, most commonly seen in patients with basal cell nevus syndrome. The association between desmoplastic histology and basal cell nevus syndrome suggests that abnormalities in SHH signaling contribute to the development of this form of medulloblastoma. This histologic variant accounts for approximately 10–20% of sporadic medulloblastomas as well [77]. Histologically, desmoplastic medulloblastomas are characterized by pale nodular areas surrounded by densely packed cells and a significant reticulin network between these areas. The nodular areas are made up of more mature tumor cells with fewer mitoses and more abundant cytoplasm. The densely packed cells, surrounding the nodules appear more typical of classic medulloblastoma [54, 75, 78]. Interestingly, this subtype shows superior survival compared to other subtypes in patients with or without basal cell nevus syndrome [75, 77, 79].

Despite all of the aforementioned strategies, approximately 30% of patients with medulloblastoma will relapse, and unfortunately most of these patients will succumb to their disease [80, 81]. If they have not yet received radiation therapy, as is the case in some very young children, a small percentage may be salvaged using radiation therapy. Unfortunately, based on the international experience, most patients who have already received craniospinal irradiation do not appear to be curable once they recur; however, there are some data to suggest that the use of high-dose chemotherapy with autologous hematopoietic cell rescue may be of value in a select group of patients [81, 82]. Ongoing phase I and phase II trials are attempting to utilize molecularly targeted agents in an effort to improve survival at the time of recurrence.

Medulloblastoma: Biologic Aspects

Studies continue to more completely understand the molecular biology and cytogenetics of medulloblastoma. The most common cytogenetic abnormality in medulloblastoma is isochromosome 17q, which is present in up to 40% of cases [83, 84]. Numerous reports have also identified gains of chromosomes 4, 6, 7, 8, and 18 as well as losses of chromosomes 1, 2, 6, 8, 9, 10, 11, and 16 [84–86]. Approximately 5% of medulloblastomas also harbor a high level of amplification of the *N-MYC* oncogene [84].

Many groups have suggested using these biologic and molecular aberrations as a means of risk stratification. Pfister et al. developed a five-tier system based on screening 80 medulloblastoma samples by array-based comparative genomic hybridization and an independent validation of 260 samples via fluorescence in situ hybridization. This hierarchical medulloblastoma molecular staging system from worst to best outcome includes (1) *c-MYC/N-MYC* amplification, (2) 6q gain, (3) 17q gain, (4) 6q and 17q balanced translocation, and (5) 6q deletion [87]. These data show quite convincingly that molecular and cytogenetic abnormalities are powerful tools for prognostication, and one day may be more useful than the traditional risk categorization based solely on clinical characteristics.

Another group evaluated gene-expression profiles of 46 human medulloblastoma samples. Unsupervised analysis divided the samples into five distinct groups (A–E) enriched for specific genetic alterations that were later confirmed by gene sequence analyses and fluorescence in situ hybridization [88]. Some of the specific abnormalities include WNT pathway mutations and chromosome 6 deletions in subgroup B and SHH pathway mutations in subgroup D [88]. This type of analysis and separation of tumors by genetic alterations may help stratify patients for the most appropriate targeted therapies.

To date, three main molecular signaling pathways have been implicated in medulloblastoma development, including the WNT pathway, the Notch pathway, and the SHH pathway. Better understanding of the WNT pathway and medulloblastoma development has come from a rare disorder called Turcot's syndrome. Turcot's syndrome, also known as glioma-colonic polyposis syndrome, is a genetic disorder characterized by colonic polyposis and an increased risk of developing colon cancer and malignant neuroepithelial CNS tumors. Most commonly, these patients develop glioblastoma multiforme and medulloblastoma [69, 89, 90]. One of the main mutations identified in this syndrome is a defect in the adenomatous polyposis coli (APC) gene, a tumor suppressor, which is a component of the WNT pathway and helps to coordinate the proliferation and ultimate fate of neural progenitor cells. Differing phenotypes may result from mutations at unique regions within the APC gene. Activation of the WNT pathway inhibits phosphorylation of beta-catenin, allowing its translocation into the nucleus [91]. This in turn increases the expression of a variety of genes that ultimately lead to cell proliferation, inhibition of apoptosis, and differentiation within the CNS. In addition, mutations of beta-catenin and other WNT pathway genes have been described in approximately 10–20% of sporadic medulloblastomas [84, 91]. The accumulation of beta-catenin within the nucleus, suggesting WNT pathway activation, appears to predict a favorable outcome in medulloblastoma [84, 92].

The Notch signaling pathway is vital to a variety of developmental processes, including hematopoiesis, somitogenesis, vasculogenesis, and neurogenesis [93]. This pathway has been implicated in the development of a variety of malignancies, including medulloblastoma. Notch signaling is activated by four transmembrane receptors, including Notch 1–4. Notch 1 is thought to be essential to the normal development of the cerebellum, whereas Notch 2 is implicated in medulloblastoma development [54, 93]. Once the receptors bind their extracellular ligands, proteolytic cleavage

leads to the release of the intracellular domain of the receptors into the intracellular compartment and eventual translocation into the nucleus. A variety of downstream targets are then activated, such as *cyclin D1* and apoptosis associated genes [54, 94]. If unregulated, this activation is thought to drive a variety of processes, including neural "stem" cell maintenance, gliogenesis, and oncogenesis [93].

Medulloblastoma: Dysregulation of SHH Signaling

Evidence linking the SHH signaling pathway and medulloblastoma originated from recognizing that patients with basal cell nevus syndrome are at increased risk for medulloblastoma. Basal cell nevus syndrome is a rare autosomal dominant genetic disorder affecting 1 in 60,000 individuals [77]. It is characterized by skeletal anomalies (frontoparietal bossing, rib and vertebrae abnormalities, and dural calcifications), large body habitus, soft tissue fibromas, radiation sensitivity (increased risk of developing radiation-induced tumors such as meningioma, ependymoma, fibrosarcoma, and basal cell carcinoma), and a high incidence of basal cell carcinoma, rhabdomyosarcoma, and medulloblastoma [67, 77, 95]. Approximately 3–5% of patients with basal cell nevus syndrome will develop medulloblastoma, typically the desmoplastic variant.

Basal cell nevus syndrome is caused by inherited inactivating germ-line mutations in one patched (*PTCH1*) allele [91, 95]. During normal development of CGPs in the cerebellum, PTCH1 maintains the SHH pathway in an inactive state except when it binds SHH. On the contrary, mutant forms of PTCH1 maintain the pathway in a constitutively activated state, even in the absence of ligand. Constitutive SHH pathway activation appears to account for the birth defects and cancer predisposition in patients with basal cell nevus syndrome. Cancers presumably develop in the setting of basal cell nevus syndrome if a somatic inactivating mutation occurs in the remaining wild type *PTCH1* allele in limited cell types, including CGPs.

Loss-of-function somatic mutations in *PTCH1* have been subsequently described in approximately 10–15% of sporadic medulloblastomas [68, 96]. It is now believed that SHH pathway activation occurs in 20–30% of all medulloblastomas, largely accounted for by inactivating *PTCH1* mutations. A variety of loss of function mutations in *PTCH1* have been described in the setting of medulloblastoma, including frame shift mutations, small deletions, duplication insertions, and splice site mutations. Interestingly, the sporadic medulloblastomas that exhibit abnormal signaling via the SHH are not all desmoplastic. Constitutive activation of SHH signaling in cerebellar CGPs is believed to contribute directly to the genesis of human medulloblastoma, based on the fact that *ptc1*$^{+/-}$ mice develop medulloblastoma [97].

Mutations in components of the SHH signaling pathway that are downstream of *PTCH1* have been more rarely described. Although activating mutations in *SMO* have been widely described in basal cell carcinomas, they appear to be rare in medulloblastoma and only have been reported in recent years [98, 99]. Suppressor of Fused (SuFu) normally binds to GLI1 and inhibits GLI1-mediated transcriptional

activation by exporting GLI1 from the nucleus to the cytoplasm [100]. Several somatic mutations in *SuFu* have been described in medulloblastomas, including frame shift and exon skipping mutations [101]. The mutant forms of the SuFu protein lack the carboxy terminal domain and are unable to bind GLI1. Therefore, the SHH pathway remains in an active state since mutant SuFu cannot sequester GLI1 in the cytoplasm. A single case of medulloblastoma with *GLI2* gene amplification has been reported in a patient with the Li–Fraumeni familial cancer syndrome [102]. The significance and the biological role of *GLI2* amplification have not been studied in medulloblastoma.

Other Pathways Affecting SHH Signaling in Medulloblastoma

Although mutations in the SHH signaling genes *PTCH1*, *SMO*, and *SuFu* are believed to directly contribute to the genesis of medulloblastoma, such mutations are observed in only a subset of the tumors, suggesting that there are other mechanisms and gene pathways that can cause or play an important role in the biology of medulloblastoma either independent of SHH signaling or by dysregulating SHH signaling. Indeed, noncanonical activation of GLI family transcription factors, which mediate HH signaling, has been described in the setting of cancer. For example, TGF-β activates the expression of *GLI1* and *GLI2* through SMAD3, independent of SHH signaling in human pancreatic cancer cells [103]. *GLI1* also appears to be activated independent of the canonical HH pathway by the EWS–FLI11 oncoprotein in Ewing sarcoma [104]. A number of studies using mouse models and human specimens demonstrate interactions between SHH signaling and other pathways and genes, both in CGPs and in medulloblastoma. Recent progress in this field is summarized in Table 8.2.

Table 8.2 Interactions between SHH signaling and other pathways in medulloblastoma

Gene	Effect on pathway	References
Genes that enhance SHH signaling in medulloblastoma		
atoh1	Increases *gli2* expression	[45]
c-myc	Enhances shh tumorigenicity	[107]
yap1	Increases gli2 expression	[108]
Genes that inhibit SHH signaling in medulloblastoma		
bmp-2,4	Degrades atoh1	[105]
bFGF	Decreases *gli1*, *N-myc*, and *cyclin D1* expression	[50]
pacap	Inhibits gli1 activity	[109]
p53	Inhibits gli1 activity	[114]
REN	Decreases *gli1* expression, nuclear localization of gli1, and gli2 activity	[49]
Targets of SHH signaling in medulloblastoma		
igf2	Increases expression	[116]
insm1	Increases expression	[118]
irs1	Stabilizes protein	[117]
nhih	Increases expression	[118]

Some proteins and pathways appear to enhance HH pathway activity in CGPs and medulloblastoma. *ATOH1* is highly expressed in CGPs and in a subset of human medulloblastomas. Conditional deletion of *atoh1* in mice downregulates *gli2* expression. In fact, atoh1 directly activates *gli2* by binding a *gli2* intron, therefore, significantly promoting the activity of the shh signaling pathway [45]. The role of atoh1 in the genesis of medulloblastoma has been tested using a mouse medulloblastoma model, carrying an activating mutation in *smo*. In this background, loss of expression of *atoh1*, using an *atoh1* null conditional mutant, significantly inhibits tumor formation, suggesting that atoh1 and activation of shh signaling are required for medulloblastoma formation [45]. bmp-2 and bmp-4 down-regulate expression of *gli1* in medulloblastoma by degrading the atoh1 protein [105].

MYC family genes are amplified and overexpressed in 5–10% of medulloblastomas [106]. Overexpression of *c-myc* alone does not appear to cause medulloblastomas in mice. However, *c-myc* greatly enhances the tumorigenicity of shh signaling in CGPs, suggesting cooperation of c-myc with hh signaling in shh-mediated medulloblastoma formation [107]. The molecular mechanism of this cooperation is unknown.

Finally, the transcriptional coactivator yes-associated protein (*yap1*) that is a key factor in Hippo signaling pathway is amplified in some medulloblastomas [108]. It activates transcription of target genes by interacting with a tea domain family transcription factor (tead). The yap1–tead1 complex drives *gli2* transcription by directly interacting with the CATTC consensus sequence in the *gli2* promoter and thus promotes constitutive shh pathway activation in medulloblastoma.

On the contrary, other proteins and pathways appear to inhibit SHH signaling in CGPs and in medulloblastoma. bFGF dramatically downregulates the expression of *gli1*, *N-myc*, and *cyclin D1*, and thereby suppresses shh-induced proliferation of CGPs [39, 50]. The fgf pathway also inhibits *gli11* expression and proliferation of medulloblastoma cells derived from $ptc1^{+/-}$ mice, suggesting an inhibitory role in the genesis of shh-induced medulloblastoma [50].

Pituitary adenylyl cyclase-activating peptide (pacap) activates PKA and functions as a powerful inhibitor of medulloblastoma formation. In fact, double heterozygote $ptc1^{+/-}$ $pacap^{+/-}$ mice demonstrate a 2.5-fold increase in medulloblastoma incidence [109]. pacap inhibits gli1 in medulloblastoma cells by activating PKA. The tumor suppressor *REN* is deleted in 39% of sporadic human medulloblastomas and inhibits the growth and the tumorigenicity of medulloblastomas [49, 110]. REN inhibits *gli1* expression and gli1 activity in medulloblastoma by blocking Dyrk1-mediated nuclear localization of gli1. REN also impairs gli2-dependent gene transcription.

Finally, inherited germ-line *p53* mutations are associated with the development of medulloblastoma in some patients with Li–Fraumeni syndrome [111]. Normally, only a small subset of $ptc1^{+/-}$ mice develop medulloblastoma [112]. However, $ptc1^{+/-}$ $p53^{-/-}$ mice develop medulloblastoma significantly more frequently (>95%) and at an earlier age [113]. This finding strongly suggests that loss of p53 and constitutive activation of the shh signaling pathway interact functionally. The mechanism of

this interaction in medulloblastoma is not known. However, recent studies suggest a feedback loop between p53 and gli1. p53 inhibits the transcriptional activity, nuclear localization, and level of expression of gli1, while gli1 inhibits the activity p53 [114]. Thus, loss of p53 may enhance GLI1 activity and thereby medulloblastoma formation. In addition, increased expression of *p53* has been observed following the transfection of *GLI1* into rat kidney epithelial cells (RK3E cells) and in the subset of medulloblastomas with HH pathway activation, suggesting that GLI1 may regulate *p53* expression [115].

shh signaling appears to regulate several genes in the "insulin regulatory pathway" in both CGPs and medulloblastoma. Insulin-like growth factor 2 (igf2) promotes cell proliferation in developing embryos. Normally, *igf2* is expressed in the meninges and at lower level in CGPs. However, *igf2* is highly expressed in medulloblastomas that develop in *ptc1*$^{+/-}$ mice. In addition, *igf2* expression in CGPs is directly activated by shh in vitro and is inhibited by cyclopamine treatment in medulloblastoma cell lines [116]. Loss of *igf2* expression decreases medulloblastoma formation in *ptc1*$^{+/-}$ mice, suggesting a role as a vital downstream target of the shh signaling pathway in medulloblastoma. Another insulin-related gene that is necessary for proliferation of CGPs and aberrantly activated in medulloblastoma is the insulin receptor substrate 1 (*irs1*) [117]. shh signaling stabilizes the irs1 protein by inhibiting the mTOR pathway that directs the degradation of irs1. Neural basic helix-loop-helix 1 (*nhlh1*) and insulinoma-associated 1/IA1 (*insm1*) are also highly expressed in rapidly expanding CGPs and medulloblastomas [118]. Both *nhih1* and *insm1* are activated transcriptionally by shh signaling in cultured CGPs, and activation of *nhih1* is directly mediated by gli1. Understanding the interactions between genes/proteins in the HH signaling pathway with those of other pathways that modulate HH signaling, as well as identifying vital effects of HH signaling in CGPs and medulloblastomas, will be important when developing targeted therapy and making informed decisions about which agents to test in combination.

Dysregulation of SHH Components in Other Pediatric Brain Tumors

Limited information is available about genetic alterations or aberrant activation of the SHH signaling pathway in other pediatric brain tumors. Craniopharyngiomas, which arise from the embryonic remnants of Rathke's pouch and account for 5.6–6.2% of all pediatric brain tumors [52], have been reported in members of a family with basal cell nevus syndrome [119]. Analysis of the *PTCH1* gene in this family shows an insertion mutation, causing a frame shift. The craniopharyngiomas from these patients have loss of heterozygosity in the *PTCH1* region, suggesting potential involvement of SHH signaling in this tumor [119]. It is of interest that the shh, bmp, and fgf pathways appear to be involved in establishing dorso-ventral polarity in Rathke's pouch during development.

A study conducted to identify an ependymoma-specific gene signature identified overexpression of *GLI1* and *GLI2*, suggesting a potential role for HH signaling in this type of tumor [120]. More recently, gene-expression analysis using 34 ependymoma samples demonstrated that a subset of HH pathway components were highly expressed, including *GLI2* and Indian Hedgehog (*IHH*) [121]. During development, ependymal cells are derived from *nkx6.1*-expressing ventral neuroepithelial cells, which are regulated by shh [122]. A mechanism for HH pathway dysregulation in ependymomas has not been reported.

Pilocytic astrocytoma is a very heterogeneous tumor that is the most frequently occurring brain tumor during childhood [52]. It typically arises in the cerebellum (40–70%) and generally is benign with an excellent survival rate [123]. The level of *PTCH1* mRNA is elevated in approximately 45% of pilocytic astrocytomas and its level is inversely correlated with the age of patient [124]. Higher expression of *PTCH1* and *GLI1* is associated with younger age at diagnosis and more rapid tumor growth, suggesting a role for the pathway in regulating proliferation. It will be important to expand the analyses of HH signaling in these tumors and to see if any genetic alterations are associated with the activation of the SHH pathway in any of these non-medulloblastoma tumors.

Potential Clinical Applications of HH Pathway Inhibitors in the Treatment of Patients with Pediatric Brain Tumors

Specifically targeting the SHH pathway as cancer therapy becomes possible as our understanding of the pathway improves. An observation reported in 1962 provided the first evidence that blocking the SHH pathway is feasible. It was noted that when pregnant ewes ingested the *Veratrum californicum* plant during their first trimester, they bore lambs with congenital cyclopean-type malformations [125]. Later, a steroidal alkaloid, called cyclopamine, was isolated from this same plant, which induced midline deformities in lamb fetuses [126]. It is now known that cyclopamine directly binds to the heptahelical bundle of SMO, likely changing the protein's conformation and thereby inactivating the SMO protein. In vitro, cyclopamine has been shown to inhibit SHH-dependent expression of *GLI1*, *GLI2*, and *PTCH1* and cause medulloblastoma cell cycle arrest [127]. In murine medulloblastoma tumor allograft and xenograft models, cyclopamine induces rapid tumor cell death [128, 129]. Unfortunately, due to its pharmacokinetic and side effect profile cyclopamine is not ideal for clinical use in humans [130]. Cyclopamine has poor solubility, acid sensitivity, weak potency and is a known teratogen [131]. More recently, a variety of naturally occurring and synthetic small molecule antagonists have been discovered [127, 128, 132].

Most of the small molecule antagonists to the SHH pathway also target SMO [132]. Romer and Curran have evaluated a small molecule inhibitor that binds and inhibits SMO called HhAntag-691 (Genentech). This compound is a benzimidazole derivative, which readily enters the brain of mice [128]. When $ptc1^{+/-}$ $p53^{-/-}$ mice

with medulloblastomas are treated with HhAntag-691, there is dose-dependent down-regulation of several genes overexpressed in medulloblastomas, including *gli1*, *ptc2*, and *atoh1* [128]. Importantly, treatment of these mice improves tumor-free survival with minimal noted toxicities [128, 133].

Rubin and de Sauvage conducted cell-based screens for novel compounds that block SHH-activated gene transcription [134]. A variety of agents were discovered that target the pathway. One in particular, HhAntag, was initially very promising as it had oral bioavailability and showed potent antitumor activity in both murine *ptc1*-mutated medulloblastoma and human xenograft models [133]. Unfortunately, when evaluated in humans, it was determined that this drug had low potency and unpredictable pharmacokinetics. Therefore, newer drugs, such as GDC-0449 (Genentech) have been developed to improve the pharmacokinetics and potency of HhAntag. GDC-0449 also blocks the SHH pathway by binding and inhibiting SMO. Preclinical studies evaluating absorption, distribution, metabolism, and tumor responses have been promising. The compound's characteristics include the following: low plasma clearance, a volume of distribution estimated to be approximately equal to total body water, high protein binding, and oral bioavailability ranging from 13 to 53% in different species [135]. It is currently undergoing evaluation in phase I and phase II studies in adults with a variety of cancers, including basal cell carcinoma, stomach cancer, pancreatic cancer, breast cancer, lung cancer, ovarian cancer, glioblastoma multiforme, and medulloblastoma. There is also an ongoing pediatric brain tumor consortium (PBTC) phase I trial evaluating the use of GDC-0449 in children and adults with recurrent medulloblastoma [136].

There will be many challenges and questions to address while introducing inhibitors of HH signaling into medulloblastoma therapy. Activation of HH signaling is believed vital in tumor initiation and maintenance for some medulloblastomas, often but not exclusively of the desmoplastic histologic subtype. We would expect HH inhibitors to be most effective in this subset of tumors. Therefore, a reproducible and clinically useful method to identify active HH signaling must be established in medulloblastoma. Immunohistochemistry for components of the HH signal transduction pathway, such as PTCH1 and GLI1 may be promising. On the contrary, using gene-expression profiles to identify subsets of patients is still difficult in real time across centers.

Patients with desmoplastic histology tend to be young and have a more favorable prognosis. Moving newer agents into therapy for this subset of patients may be challenging; but may ultimately be of considerable value in improving outcomes and especially limiting toxicity. Typically, phase I and phase II studies are conducted in the setting of recurrence, as is the case with the ongoing Phase I PBTC HH inhibitor study. The youngest patients have been initially excluded based on the concern of a role for HH signaling in post-natal bone growth and development. Indeed, chondrocyte and osteoblast development require IHH, and there have been significant defects noted in long bone development in IHH knockout mouse models [137]. Mutant mice have reduced chondrocyte proliferation, failure of osteoblast development in endochondral bones, and premature closure of growth plates [137]. Interestingly, in humans, IHH mutations have recently been associated with a

disorder known as acrocapitofemoraldysplasia [138]. This is a rare autosomal recessive growth disorder characterized by short stature, short limbs, brachydactyly, large head, narrow thorax, and pectus deformities [138, 139]. Osteopontin has also been identified as a target of GLI1 [140].

Efficacy of HH inhibitors may differ at the time of diagnosis versus at the time recurrence. Using a HH inhibitor at the time of diagnosis may optimize chances of observing a therapeutic benefit, since cells have not yet been exposed to agents that may select for resistant clones, however, treating at the time of recurrence may more effectively allow identification of agents that treat drug-resistant clones and more effectively build upon current therapeutic approaches. The optimal approach for testing HH inhibitors remains uncertain.

As with other agents, HH inhibitors may be most beneficial when either paired with traditional chemotherapy and radiotherapy or with other biologically active agents. Identifying other pathways that enhance or inhibit SHH signaling in medulloblastoma may help to inform decisions concerning drug combinations. For example, HH signaling in medulloblastoma activates the "insulin regulatory pathway." Inhibitors of this pathway are currently undergoing testing in a wide range of tumors. Targeting the upstream HH pathway and downstream "insulin regulatory pathway" may enhance efficacy. Identification of critical downstream targets will be essential [115].

A growing body of literature suggests a potential pitfall to the use of a HH inhibitor that targets SMO. There is now evidence that SMO's targets, GLI1, GLI2, and/or GLI3, may be activated in ways other than through the canonical HH pathway. Activation of GLI family transcription factors may then bypass the effect of the SMO inhibitor. A more complete understanding of ways to activate GLI family transcription factors in medulloblastoma and in other cancers is needed. In addition, an amino acid substitution in SMO was recently reported in human medulloblastoma, conferring resistance to GDC-0449, rather than disrupting the pathway which suggests another possible mechanism of resistance to this agent [141].

Summary

Childhood brain tumors are significantly different from their adult counterparts, since the latent period is very short, growth is fast, and the cell populations causing the tumors arise from the embryonic cells. The causes of childhood brain tumors remain incompletely understood. However, significant progress in the genetics and biology of childhood brain tumors has been made in the past 15 years. In particular, important genes and signaling pathways involved in the development of childhood brain tumors have been identified [106]. We describe recent important discoveries of the role of SHH signaling pathway in brain development and tumorigenesis. SHH signaling appears to play fundamental roles in regulating proliferation and differentiation during development of a variety of cell types in the CNS. A role for SHH signaling in the normal development of CGPs in the cerebellum appears to

reflect a role that the pathway plays in the genesis of some medulloblastomas. For this reason, drugs targeting major components of the SHH signaling pathway and interacting genes may prove to be a valuable alternative or adjunctive approach for the treatment of some children with medulloblastoma and potentially children with other brain tumors.

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Chapter 9
Hedgehog Signaling in Mammary Gland Development and Breast Cancer

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Introduction

The *Hedgehog* (*Hh*) gene was discovered in *Drosophila* as a gene specifying segment polarity [1]. Since that initial discovery, the Hedgehog signaling network has been shown to regulate development of most structures and organs in both invertebrate and vertebrate species [2]. For example, in mammals, the network is known to pattern the limb and the dorso-ventral axis of the neural tube to stimulate proliferation of neural precursor cells and to regulate hair follicle regeneration [3]. Like Wnt and Notch signaling, Hedgehog signaling has also been implicated in the maintenance and self-renewal of several adult stem/progenitor cell types, including those of the mammary gland [4–6].

Mammalian Hedgehog Signaling

Although autocrine signaling can occur, mammalian Hedgehog signaling generally takes place between two cells, a signaling cell and a receiving cell [7–9].

In the absence of a Hedgehog ligand, the PTCH receptor catalytically inhibits Smoothened (SMO), the primary signal transducer. Inhibition of SMO allows the phosphorylation and cleavage of two members of the GLI-family of transcription factors (GLI2 and GLI3). This cleavage is promoted by a complex of kinases, and the scaffold protein Suppressor of fused, which phosphorylate the GLI proteins and

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target them for processing. The cleaved, repressor forms of GLI can then translocate into the nucleus and block transcription of target genes.

Whereas in *Drosophila* there is only a single gene encoding one Hedgehog ligand, mammals have three genes encoding three distinct proteins: *Sonic Hedgehog* (*Shh*), *Desert Hedgehog* (*Dhh*), and *Indian Hedgehog* (*Ihh*). Hedgehog proteins are translated and autocatalytically cleaved to an N-terminal signaling fragment in the signaling cell, and are secreted as palmitoylated and cholesterol-modified ligands via function of the Dispatched protein. Once the ligand is secreted by the signaling cell, it binds the Patched (PTCH) family of 12-pass trans-membrane receptors (PTCH1 or PTCH2) on the receiving cell. Ligand binding to PTCH can be facilitated by Commodo, Brother of Commodo, and Growth Arrest Specific-1, or it can be inhibited by Hedgehog Interacting Protein by sequestration of the ligand [10–13].

Upon ligand binding to a PTCH receptor, inhibition of SMO by PTCH is released. Once SMO is allowed to function, it inhibits the phosphorylation and cleavage of the GLI family transcription factors to yield full-length transcriptional activators. Of the three GLI proteins (GLI1, GLI2, and GLI3), GLI1 acts exclusively as a transcriptional activator, as it lacks the proteolytic cleavage site. GLI2 and GLI3 can function either as activators or repressors, depending on whether or not they are cleaved. In vivo data suggests that GLI2 functions primarily as a transcriptional activator, while GLI3 is found primarily in its repressor form [14–16].

The ability to activate Hedgehog signaling in mammalian cells has been associated with the presence of a primary cilium on the receiving cell. As such, proteins involved in cilium formation and intraflagellar transport (IFT) play vital roles in Hedgehog signaling. Mouse IFT proteins have been shown to be required for GLI activator and GLI repressor functions, and mice lacking these proteins exhibit Hedgehog loss-of-function phenotypes [17, 18]. *Drosophila* has homologs to IFT components as well, yet they do not appear to be required for Hedgehog signaling since flies lacking these components do not exhibit Hedgehog loss-of-function phenotypes [19–21]. Localization of PTCH1 and SMO to cilia is mutually exclusive. Ligand-bound PTCH1 is internalized upon binding, and only then can SMO translocate to the primary cilium to activate GLI proteins [17, 22, 23]. The role of primary cilia in Hedgehog signaling is reviewed in detail by Wong et al. [24].

Non-canonical Hedgehog Network Functions

In addition to canonical GLI-mediated transcriptional responses induced by Hedgehog ligands, several non-canonical functions of Hedgehog network genes have been identified.

Among the most versatile Hedgehog network components is the PTCH1 protein. In addition to preventing signaling in the absence of ligand, and transducing the Hedgehog signal in their presence, PTCH1 has a number of additional functions. First, the PTCH1 receptor can act to sequester the Hedgehog ligand and restrict its range of diffusion such that otherwise signaling-competent cells may not come into contact with ligand [25, 26]. Second, the PTCH1 receptor has been demonstrated to

act as a dependence receptor in cell types that require the presence of ligand-bound PTCH1 in order to survive. In these cells, unbound PTCH1 receptor initiates apoptotic cell death [27–29]. Third, the PTCH1 was shown to act as a "gatekeeper" of the cell cycle by controlling the transition from G2 to M phase. PTCH1 performs this function by binding phosphorylated cyclin B1 and sequestering it in the cytoplasm. Upon SHH ligand addition, this interaction is disrupted allowing cyclin B1 to localize to the nucleus followed by cell cycle progression [30]. Functional significance of this interaction was supported in a skin-specific PTCH1 loss-of-function model, in which PTCH1 loss led to basal cell carcinoma (BCC) lesions accompanied by nuclear accumulation of cyclin D1 and cyclin B1 [31]. Fourth and finally, recent data using mammary epithelial cells suggest that PTCH1 can activate ERK1/2 independent of SMO-mediated signaling in mammary epithelium via one or more SH3-domain-mediated protein–protein interactions, including potential interactions with Grb2, c-src, and p85β (PIK3R2) [32].

SMO may also have multiple functions. SMO is a seven-pass trans-membrane protein which is a nonredundant component and serves as an essential, rate-limiting mediator of canonical signal transduction. SMO shares structural and evolutionary homology with G protein-coupled receptors (GPCRs) such as rhodopsin and β-adrenergic receptor, and is most closely related to the Frizzled family of proteins that mediate Wnt signaling. While the Wnt-Frizzled signaling network is known to transduce signal via heterotrimeric G proteins [33], evidence to support a role for heterotrimeric G-proteins in activated Hedgehog signaling has only recently emerged [34–36].

The first solid evidence that SMO-coupled heterotrimeric G protein activation can occur was an in vitro study by Riobo et al. which demonstrated that mammalian SMO can couple with selected G_{α} subunits primarily of the $G_{\alpha i}$ family upon co-transfection [37]. This study indicated that mammalian SMO can couple with G_{i1}, G_{i2}, G_{i3}, G_0, G_z, and G_{15} in Sf9 (insect) cells. On the contrary, SMO could not couple with G_s, G_q, G_{qzIc}, G_{qG66D}, $G_{qG66Dx5}$, G_{12}, G_{13}, or G_{16}, although a different study found that G_{12} and G_{13} mediated the SHH–SMO response in neuroblastoma cells [38]. While G protein activation could be partially or completely blocked by cyclopamine, a relatively specific inhibitor of SMO, activation of G_z was largely insensitive. Four of the G_{α} subunits shown to couple with SMO (G_{i1}, G_{i2}, G_{i3}, and G_0) are irreversibly inhibited by pertussis toxin (PTX)-mediated ADP-ribosylation.

Of potential importance for the interpretation of Hedgehog network function, G protein coupling by SMO could be genetically separated from GLI activation using a truncated SMO protein [39]. Thus, it may be possible that these two functions can be uncoupled in vivo under the right conditions. Consistent with the possibility of uncoupled function, SHH was found to mediate axon guidance in a SMO-dependent, but GLI-independent, manner via activation of SRC family kinases [40]. Additionally, Hedgehog ligands were found to activate a pro-angiogenic response in endothelial cells in a GLI-independent manner. These GLI-independent effects could be suppressed by PTX treatment, suggesting an independent GPCR function for SMO is critical for at least some aspects of SMO activity [41].

There have been other indications that SMO might function as a GPCR. For example, SMO function can be enhanced by catalytically active G protein-coupled receptor kinase 2 (GRK2), but not catalytically inactive GRK2. GRK2 activity promoted association of SMO with β-arrestin-2 [42, 43]. GPRK2 was also found to participate in Hedgehog signaling in *Drosophila*, further suggesting that SMO function as a GPCR might be evolutionarily conserved [44]. Subsequently, *Drosophila* SMO was found to interact with $G\alpha_i$ in vivo, and this interaction is apparently essential for Hedgehog signal transduction [45].

With respect to the GLI transcription factors, the GLI proteins were long believed to act solely as the transcriptional activators of the Hedgehog network. However, ligand- and SMO-independent function for the GLI family of transcription factors have also been suggested, with emerging evidence indicating that these transcription factors are regulated by other signaling networks including that of TGF-β, as well as by activation of AKT- and RAS-mediated signaling [46–49].

Hedgehog Network Control of Mammary Gland Development and Breast Cancer

Mammary Gland Development

In both mouse and human, mammary gland development begins during embryonic life with growth of a rudimentary ductal tree. After this initial hormone-independent growth, the rudimentary ducts remain relatively growth-quiescent from birth until puberty. With the onset of ovarian hormone secretion during puberty, the rudimentary ducts begin to grow and elongate as secondary and tertiary ducts, led by rapid and

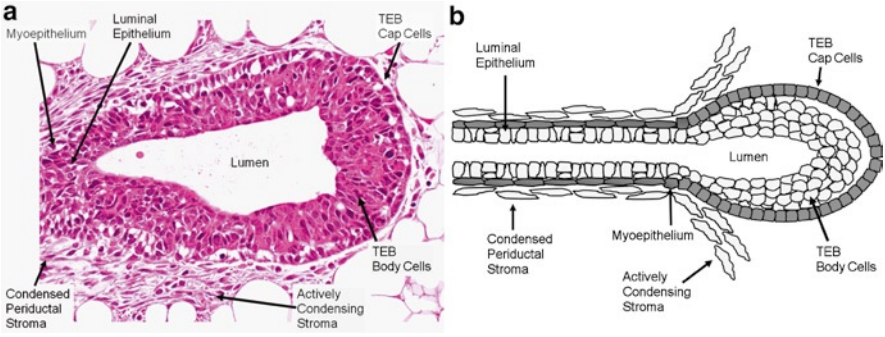

Fig. 9.1 The terminal end bud (TEB). (**a**) Histological preparation showing the histoarchitecture of a typical TEB. Distinct cap cell and body cell layers are identified, as well as areas of actively condensing and condensed periductal stroma. (**b**) Schematic diagram of a TEB and mature subtending duct showing a layer of heterogeneous luminal epithelial cells surrounded by a layer of myoepithelial cells and a condensed mammary stroma

invasive growth of the terminal end bud (TEB) (Fig. 9.1a, b). The TEB is a bulb-shaped structure consisting of multiple layers of rapidly dividing, immature epithelial cells. The outer cell layer is composed of cap cells, which differentiate into the duct-associated myoepithelial cells as the duct elongates. The inner layers are composed of body cells that give rise to and/or differentiate into ductal and alveolar progenitors as well as the more differentiated luminal ductal epithelial cell types. Upon reaching the edges of the available mammary fat pad, TEB structures regress, leaving an arborized tree of differentiated ducts that are open to the surface of the skin via the nipple.

While strikingly similar, mouse and human mammary gland development in the virgin are not identical. In humans, a relatively high level of alveolar development also occurs due to the presence of a luteal phase of the menstrual cycle. On the contrary, most commonly used mouse strains lack a luteal phase to their estrus cycle, and thus show a relative lack of alveolar development in virgin animals. As a consequence, the relative proportion of ductal vs. alveolar cell types in the mouse vs. human mammary gland is quite different in the nonpregnant female.

Regardless of the degree of alveolar development in virgins, neither the human nor mouse mammary gland can be considered fully differentiated until pregnancy and lactation. With pregnancy, alveolar development culminates, followed by the production and secretion of milk during lactation [50]. Upon weaning of young, the mammary gland involutes. Involution is characterized by extensive apoptosis and epithelial remodeling that yields a mammary ductal tree that is similar to, but not identical, with that of the adult virgin [51, 52].

Hedgehog Network Gene Expression and Function in Mammary Gland Development

Most major insights into Hedgehog network function in breast development and breast cancer come from gene expression analyses in situ, and the study of genetically engineered mice.

In mice, the Hedgehog ligands are expressed in mammary epithelium at several stages of development (Fig. 9.2a, b). *Ihh* and *Dhh* are detectable in the pubertal gland by in situ hybridization and *Ihh* mRNA levels are upregulated during pregnancy and lactation, suggesting a role in alveolar differentiation or function [53, 54]. Expression of *Ihh* mRNA becomes undetectable in early involution, but returns to roughly pre-pregnant levels after gland remodeling [55]. Despite the developmentally regulated changes in gene expression as a function of pregnancy, embryonic tissue transplantation of individual knockouts of the *Ihh* and *Shh* genes showed no overt developmental phenotype [56, 57]. The *Dhh* knockout has not been analyzed for a mammary phenotype, but homozygous females are able to feed their pups successfully, suggesting no major impairments in mammary gland function [58]. Hedgehog ligands can compensate for one another functionally, thus, the lack of phenotype in single gene knockouts might be explained by functional redundancy.

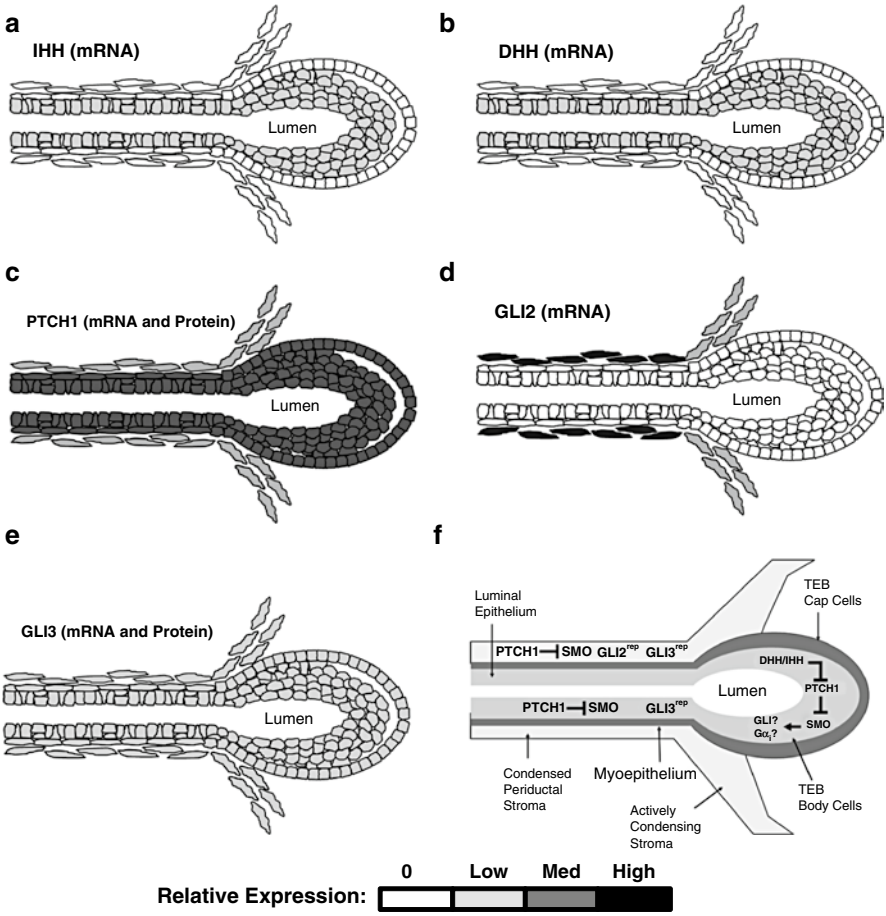

Fig. 9.2 Schematic representation of Hedgehog network gene expression patterns and associated working model for Hedgehog network function in ductal development. A scale of relative expression is shown below the figure. (**a**) Low-level expression of *Ihh* mRNA by in situ hybridization. (**b**) Low-level expression of *Dhh* mRNA by in situ hybridization. (**c**) Robust expression of *Ptch1* mRNA and protein in both epithelium and stroma. (**d**) Stroma-limited expression of *Gli2* mRNA. (**e**) Widespread low-level expression of *Gli3* mRNA and protein in both epithelium and stroma. (**f**) Schematic diagram of a TEB and subtending duct overlayed with a working model for Hedgehog network gene function during ductal development based on gene expression and phenotypic analysis of genetically engineered mice. Expression and function of SMO is postulated, but not yet supported by in vivo experimentation

Ptch1 mRNA and protein are expressed in both epithelial and stromal compartments of the virgin mouse mammary gland (Fig. 9.2c). Coordinate with the increase in *Ihh* mRNA levels during pregnancy and lactation, expression of *Ptch1* mRNA also appears elevated during pregnancy and lactation [53, 55]. Further, coordinate with *Ihh* expression in involution, *Ptch1* message becomes undetectable in early involution but becomes detectable again after gland remodeling.

Loss-of-function studies using several different *Ptch1* mutants have demonstrated a role for *Ptch1* for normal patterning and elongation of the mammary ductal tree [53, 59, 60]. Heterozygous loss of *Ptch1* (*Ptch1*^{Tm1Mps} allele) led to mild TEB defects, and abnormal proliferation and accumulation of luminal epithelial cells within the ducts. The accumulated cells were lost during pregnancy, but returned during involution, thus demonstrating conditional haploinsufficiency for *Ptch1* in the nonpregnant animal [53]. Homozygous *Ptch1*^{mes}/*Ptch1*^{mes} (a hypomorphic allele) mice showed failure of ductal elongation in a high percentage of glands examined, as well as ductal dysplasia in "escape" ducts that did form. Finally, complete loss of Ptch1 in the epithelium using a *Cre*-dependent conditional null allele led to dilated and dysmorphic ducts, but did not compromise ductal elongation or cause accumulation of cells within the ducts as did *Ptch1* heterozygosity. Subsequent transplantation studies demonstrated that *Ptch1* was required in both mammary epithelium and stroma for appropriate mammary gland development [53, 60]. Additionally, *Ptch1* appears to be required in the pituitary since failure of ductal elongation in homozygous *Ptch1*^{mes}/*Ptch1*^{mes} mice could be rescued by a pituitary isograft [60].

Loss of Ptch1 function in heterozygous *Ptch1*$^{Tm1Mps/+}$ mice has been associated with forfeiture of quiescence, increased proliferation, and expansion of a progenitor cell pool via differential regulation of the *TP63* promoter [59]. However, because these studies were conducted in intact mice rather than via epithelial transplantation, it is still somewhat unclear whether this effect is due to local or systemic functions, or some combination thereof. Nevertheless, the *Ptch1*$^{Tm1Mps/+}$ studies complement work using a constitutively activated Smo transgene driven by the Mouse Mammary Tumor Virus promoter (*MMTV-SmoM2*) selectively in mammary epithelium. Glands in virgin *MMTV-SmoM2* mice showed increased proliferation and ductal dysplasias, but did not show accumulation of cells within the duct, thereby demonstrating that *Ptch1* loss was not functionally equivalent to activation of SMO strictly in the epithelium [61]. However, consistent with the *Ptch1*$^{Tm1Mps/+}$ data, *MMTV-SmoM2* activation led to an approximately twofold *decrease* in regenerative stem cell frequency, but an approximately twofold *increase* in a progenitor cell pool capable of anchorage-independent growth as mammospheres.

These data remain to be reconciled with data using human mammary cells in which activated Hedgehog signaling was associated with self-renewal of normal mammary epithelial stem cells [62, 63]. Treatment of normal cells with SHH ligand increased the mammosphere-forming potential and self-renewal of multipotent epithelial cells capable of giving rise to mixed luminal and myoepithelial colonies. Treatment with cyclopamine, as expected, decreased the frequency of these cells. In addition, genetic knockdown of *Gli1* and *Gli2* resulted in reduced anchorage-independent growth in mammosphere cultures, while overexpression of *Gli2* in human cells led to hyperplasia upon transplantation into immunocompromised hosts. One plausible explanation for the apparent discrepancy between the mouse and human data is the observation that alveolar progenitor cells are capable of giving rise to both luminal and a distinct population of alveolus-associated myoepithelial cells [64, 65]. Given that the relative frequency of alveolar cell types is far greater in human

vs. mouse (as mentioned above), and that activated Hedgehog signaling may play a role in alveolar differentiation [55], it is possible that ligand treatment acts to promote self-renewal of a bipotent alveolar progenitor cell capable of anchorage-independent growth, rather than the most primitive regenerative stem cell.

Ptch2, although detectable by gene expression analysis in mature virgin mice, has not been studied in mammary gland development. *Ptch2* knockout mice are viable, and no defects in lactation were reported. As with Hedgehog ligands, functional compensation by *Ptch1* is possible [66].

SMO protein is not detectable by immunolocalization using currently available antibodies in either the mouse or human mammary gland, but mRNA can be detected in the mouse [61]. Whether *Smo* is required in mammary gland development, and thus whether canonical (or non-canonical) signaling is required during mammary gland development is still an open question. The requirement for *Smo* is currently being tested in our laboratory using a conditional, *Cre*-dependent disruption allele.

Expression and functional analyses of the *Gli* family of transcription factor genes in mammary epithelium have yielded somewhat ambiguous results. Using a *Gli1-lacZ* knock-in reporter line (*Gli1*^{lzki}), lacZ activity was only detected in lymphatic vessels in both the embryonic and adult mammary gland, but not in mammary ducts or alveoli [67]. Consistent with lack of expression in mammary epithelium, homozygous *Gli1*^{lzki} loss-of-function had no phenotype as a single gene mutation. On the contrary, targeted overexpression of Gli1 in mammary epithelium via the MMTV promoter (MMTV-Gli1) led to impaired lobuloalveolar development and lactation defects in transgenic female mice [68].

Gli2 mRNA was detected by in situ hybridization and localized exclusively to the periductal stroma during virgin development (Fig. 9.2d) [55]. However, expression was both epithelial and stromal during pregnancy and lactation, and levels appeared to increase coordinately with those of *Ihh* and *Ptch1* mRNAs consistent with activation of the canonical signaling network during these phases of development [55]. Transplantation analysis of whole embryonic mammary glands derived from a targeted disruption mutant for *Gli2* revealed that *Gli2* function is required for normal mammary duct development. However, transplantation of epithelial fragments from homozygous mutant embryos into cleared fat pads of immune compromised mice failed to recapitulate the dysplastic ductal phenotypes observed in whole gland transplantation, indicating that *Gli2* functions primarily in the mammary stroma to affect mammary epithelial cell behavior [55]. Because ligand levels appear to be low during virgin phases of development, GLI2 protein is predicted to be in its repressor state (Fig. 9.2d, f).

In a different study [67], both *Gli2* and *Gli3* were found to be expressed in stromal cells in the virgin (Fig. 9.2d, e), and in myoepithelial cells after pregnancy. *Gli3* was also expressed in luminal epithelial cells, and was shown to be essential in the somites for proper formation of the embryonic mammary buds from surface ectoderm [67, 69]. There was no requirement for either *Gli1* or *Gli2* in embryonic mammary gland development. Together, these data suggested that the Hedgehog

network must remain inactive for appropriate embryonic and pubertal ductal development of the mammary gland [67]. However, as stated above, the phenotypic effect of the complete loss of Hedgehog signaling by disruption of Smo has not yet been evaluated fully.

Role of Primary Cilia in Mammary Gland Hedgehog Signaling

The existence of cilia in mammary epithelium had not been carefully examined until recent work by McDermott et al. [70]. This study examined the distribution of cilia in the mouse mammary gland and found that epithelial, myoepithelial, and stromal cells all contained cilia. Cilia distribution in luminal epithelial cells was highest during pubertal development (~17%) and less abundant as the animal matured, leveling out at ~4% in both the virgin and pregnant adult animal. Disruption of cilia led to a reduction in branching morphogenesis in the mammary ductal tree. Decreased branching was accompanied by an increase in Wnt signaling, but a decrease in canonical Hedgehog signaling as defined by reduced *Gli1* mRNA expression [70].

The Hedgehog Network in Breast Cancer

The first evidence for a role of the Hedgehog network in cancer came from the study of patients with Gorlin's syndrome. Individuals with this disease carry inherited loss-of-function mutations in the *Ptch1* gene, and are strongly predisposed to BCC [71, 72]. Since then, activated Hedgehog signaling has been implicated in medulloblastoma, glioblastoma, rhabdomyosarcoma, and melanoma as well as in cancers of the breast, pancreas, lung, prostate, gastrointestinal system, and hematopoietic system, among others. The role of the Hedgehog network in these malignancies is reviewed in other chapters of this book (see also [73]).

A potential role for activated Hedgehog signaling in breast cancer was postulated almost immediately upon identification of mutations in *Ptch1* associated with Gorlin's syndrome and BCC. However, Gorlin's syndrome patients do not show increased risk of breast cancer. Thus, significant evidence supporting such a role has been lacking (reviewed in [74, 75]).

Analysis of mutations in breast cancers has thus far shown little evidence that mutation of Hedgehog signaling genes are common [76], with most studies failing to identify mutations [77, 78]. More recent genomic sequencing efforts identified three missense mutations in the *Gli1* gene in 11 breast cancer samples examined. The functional significance of these mutations is not known [79]. At the genomic level, array comparative genomic hybridization analyses indicate that genomic loss at the *Ptch1* locus was the fourth most commonly detected change among the tumor suppressor genes identified in the study, occurring in 19% of human breast cancers

and 33% of breast cancer cell lines [80]. Amplification of the *Gli1* gene has also been demonstrated [81].

Data related to expression of Hedgehog network genes in human breast cancer are currently ambiguous, most likely due to use of a variety of unvalidated, or poorly validated, commercially available immunoreagents. The discrepancies among the various expression studies recounted below remain to be resolved.

Initial immunohistochemical staining studies suggested that Hedgehog signaling is activated in a majority of human invasive breast cancers (IBC) based on ectopic expression of PTCH1 and GLI1 [82], which were not detected in normal tissue. Another small study using patient-matched samples showed SHH, PTCH1, and GLI1 expression [83] in both normal and cancer tissues, with about half of the samples showing increased expression of at least one protein in cancerous epithelium relative to normal. A recent publication [84] analyzed 21 normal breast samples and 121 invasive ductal carcinomas by immunohistochemistry for expression of IHH, PTCH1, SMO, GLI1, GLI2, and GLI3. For all six proteins, expression was higher in invasive ductal carcinoma relative to normal breast epithelia, with several correlations between expression of individual genes with clinical biomarkers and behaviors. Expression of GLI1 protein has been associated with the emergence of estrogen receptor negative (ER−) breast cancer, as well as with increased survival of ER− breast cancer cells. Further, high GLI1 expression has been associated with poor clinical outcome [85, 86].

Some aspects of these studies conflict with other published analyses. Using a relatively large panel of normal, ductal carcinomas in situ (DCIS), and IBC samples [61], SMO protein expression was undetectable in normal breast, but was ectopically expressed in ~70% of DCIS and ~30% of IBC [61]. Specificity of the SMO antibody was controlled by detection of MMTV-SmoM2 transgene expression in the mouse mammary gland, whereas wild type animals showed no detectable expression. Increased SMO protein expression in human breast cancer was consistent with Q-PCR results of Mukherjee et al. [83], which showed increased mRNA expression in ~40% of their samples.

Contrary to SMO, PTCH1 protein was detectable at moderate levels throughout the epithelium, and in isolated stromal cells of the normal breast. This pattern was observed with two independent antibodies, and was consistent with expression patterns in the mouse mammary gland assayed by both immunolocalization and in situ hybridization [53, 61]. Consistent with the observation of genomic loss at the *Ptch1* locus, PTCH1 protein expression was decreased or absent in ~50% of DCIS and IBC. Reduced PTCH1 expression in this study was in remarkable agreement with an independent study by Wolf et al. [87] in which epigenetic silencing of the *Ptch1* gene, as well as reduced protein expression in clinical specimens, was demonstrated in ~50% of the samples examined.

If, in fact, Hedgehog signaling is ectopically activated in human breast cancer, activated signaling may influence breast cancer development in a number of ways in addition to those mentioned above. Perhaps most important from both a biological and clinical perspective, Hedgehog signaling has been implicated in

the regulation of self-renewal of the $CD44^{+};CD24^{-}$ population of breast cancer stem cells (tumor-initiating cells) [62, 63]. One recent study suggests that activated signaling (via expression of activated SMO) can compromise the tumor suppressor function of TP53 (p53) by promoting MDM2-mediated TP53 degradation [88]. With respect to metastasis, osteolytic behavior of MDA-MB-231 cells was dependent on *Gli2* [89].

Despite provocative indications of an importance of activated Hedgehog signaling in human breast cancer, and the development of ductal hyperplasia and dysplasia in several genetically engineered mouse models [53, 55, 60, 61], long-term tumor formation studies in either *Ptch1* heterozygotes or MMTV:*SmoM2* transgenic mice did not indicate increased frequency of mammary tumors [61, 90]. As the sole exception, conditional overexpression of *Gli1* was recently shown to result in tumor development [91] with multiple histopathologies and expression of basal cell type markers. Together, these data suggest that inappropriate activation, or de-repression, of Hedgehog network target genes may have important consequences for tumor growth and behavior, but that activated signaling may not be the primary driver of tumorigenesis as it is in cancers such as BCC and medulloblastoma.

Table 9.1 Mammary gland phenotypes in Hedgehog pathway mouse models

	Mouse model	Effect on pathway	Mammary gland phenotype	Epithelial/ stromal role	References
Ligands	*Shh*$^{-/-}$ (*Rescue Transplants*)	Decreased activity	No overt phenotype	–	[56, 57]
	Ihh$^{-/-}$ (*Rescue Transplants*)	Decreased activity	No overt phenotype	–	[56]
Receptors	*Ptch1*$^{Tm1Mps/+}$	Increased activity	Aberrant TEBs, ductal dysplasia. No embryonic phenotype	Both	[53, 59]
	Ptch1^{Mes}	?	Ductal dysplasia	Both	[60]
	MMTV-Cre; *Ptch1*$^{fl/fl}$	Increased activity	Dilated ducts	Epithelial	[60]
Transducer	*MMTV-SmoM2*	Increased activity	Increased branching and budding	Epithelial	[61]
Effectors	*MMTV-Gli1*	Increased activity	Impaired lobuloalveolar development	Epithelial	[91]
	Gli1$^{lzk1/lzk1}$	Decreased activity	No overt phenotype	–	[67]
	Gli3$^{ext/+}$	Increased activity	Aberrant mammary placode formation	Both	[67]
	Gli2$^{lzki/lzki}$	Decreased activity	No overt phenotype	–	[67]
	Gli2$^{-/-}$ (*Rescue Transplants*)	Decreased activity	Aberrant ductal morphogenesis	Stromal	[55]

Hedgehog Signaling as a Therapeutic Target in Breast Cancer

A number of Hedgehog signaling antagonists have been identified and characterized (Table 9.2) (exhaustively reviewed in [92] and references therein). Antagonists include a group of plant-derived steroidal alkaloids (e.g. cyclopamine and jervine) first identified as potent teratogens in sheep, rodents, and other vertebrates [93–95]. Compounds in this chemical class directly bind SMO to inhibit downstream signaling [96–98]. In addition to these naturally occurring antagonists, several other Hedgehog signaling agonists and antagonists have been identified or synthesized that target either the SHH ligand, SMO, or the downstream GLI transcription factors (Table 9.2). Most recently, a screen of FDA-approved compounds identified the antifungal agent itraconazole as a potent inhibitor of SMO, but in a manner distinct from that of cyclopamine and related compounds [99].

In preclinical studies, cyclopamine and CUR0199691 have been used in vitro to treat a limited panel of breast cancer cell lines [82, 83, 100]. Results are generally consistent across studies, with cyclopamine doses of 10 μM or higher leading to significant inhibition of cell growth via both reduction of proliferation and induction of apoptosis. However, the specificity of these compounds at the doses required for inhibition is in question due to the observation that these two compounds showed activity against two different subsets of cell lines. Further, activity did not correlate with detectable expression of *Smo* mRNA, nor did activity correlate with the ability of cell lines to respond to treatment with recombinant dual lipid-modified SHH ligand [100].

While in vivo tests of Hedgehog signaling inhibitors have yet to be reported for the treatment of breast cancers, preclinical studies in mice using SMO inhibitors have shown promising effects in prevention of metastases from pancreatic cancer [101], as well as inhibition of tumor growth in medulloblastoma [102].

Several of the Hedgehog signaling modulators listed in Table 9.2 are entering clinical trials (http://www.clinicaltrials.gov). GCD-0449 has completed a phase I clinical trial in patients with locally advanced or metastatic BCC with measurable responses in 29 of 33 patients (including two complete responses), with no dose-limiting toxicities [103, 104]. This compound is now in phase II trials in patients with a variety of cancers including advanced or metastatic BCC, pancreatic cancer, gastric cancers, colorectal, and ovarian cancers. In advanced breast cancer, GCD-0449 is being investigated in combination with a gamma secretase inhibitor (RO4929097) to block Notch signaling based on data indicating that both Hedgehog and Notch networks regulate mammary stem cell self-renewal.

The Pfizer SMO inhibitor PF-04449913 has entered a phase I clinical trial in patients with hematological malignancies including CML for use either alone or in combination with Dasatinib (a c-src inhibitor). The BMS compound BMS-833923 (XL139) has entered a phase I clinical trial for patients with BCC. The infinity compound IPI-926 has entered a phase 1 study in patients with advanced and/or

Table 9.2 Selected small molecule modulators of Hedgehog signaling

Activity	Name	Target	Company	Usage	A.K.A
Agonists	*Purmorphamine*	SMO		Basic/preclinical	
	SAG	SMO		Basic/preclinical	
Antagonists	*Cyclopamine*	SMO	Natural product	Basic/preclinical	
	Jervine	SMO	Natural product	Basic/preclinical	
	SANT1	SMO		Basic/preclinical	
	SANT2	SMO		Basic/preclinical	
	SANT19	SMO		Basic/preclinical	
	SANT74	SMO		Basic/preclinical	
	SANT75	SMO		Basic/preclinical	
	Cur61414	SMO	Curis	Basic/preclinical	
	Cur0199691	SMO	Curis	Basic/preclinical	HhAntag 691
	GDC-0449	SMO	Curis/Genentech/Roche	Clinical	RG3616
	IPI926	SMO	Infinity Pharmaceuticals	Clinical	
	LDE225	SMO	Novartis	Clinical (planned)	
	XL-139	SMO	Bristol-Meyers Squibb	Clinical	BMS-833923
	PF-04449913	SMO	Pfizer	Clinical (planned)	
	Itraconazole	SMO	Janssen Pharmaceutica Products	Antifungal/preclinical	Sporanox
	Unknown	SMO	Amgen		
	Unknown	SMO	Eli Lilly		
	Unknown	SMO	Takeda		
	GANT58	GLI		Basic/preclinical	
	GANT61	GLI		Basic/preclinical	
	Physalin F	GLI		Basic/preclinical	
	Physalin B	GLI		Basic/preclinical	

(continued)

Table 9.2 (continued)

Activity	Name	Target	Company	Usage	A.K.A
	NMDA298-1	GLI		Basic/preclinical	
	JK-184	GLI		Basic/preclinical	
	Robotnikinin	SHH		Basic/preclinical	
	HPI-1	Unknown			
	HPI-2	Unknown			
	HPI-3	Unknown			
	HPI-4	Unknown			

metastatic solid tumor malignancies. Similarly, the Novartis compound LDE225 is also in phase I trials in patients with advanced solid tumors as well as BCC and medulloblastoma. Since advanced solid tumors will include breast cancers, results from these phase I and phase II trials should be informative with respect to potential efficacy against advanced breast cancers.

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Chapter 10
Hedgehog Signaling and Cancer Treatment Resistance

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Introduction

The hedgehog (HH) signaling is critical for growth and differentiation during embryonic development and is required for the maintenance of somatic stem cells [1]. In adult cells, HH signaling has been implicated in the maintenance of homeostasis of stem or progenitor cells in a number of epithelial tissues, including intestinal epithelia [2]. HH signaling also contributes to physiologic processes of epithelial repair and regeneration after injury [3]. However, aberrant activation of HH signaling in tumors from a wide range of tissues may allow escape from regulatory mechanisms that cause the return to quiescence that normally follows regeneration [4, 5]. Activation of HH signaling by binding of secreted HH ligands (Sonic, Indian, and Desert) to the membrane receptor Patched (PTCH) results in the nuclear translocation of the Gli family and initiation of HH-related gene expression [1, 6], including genes controlling the cell cycle, cell adhesion, signal transduction, angiogenesis, and apoptosis [7]. Several studies have shown that unregulated progenitor cell proliferation induced by abnormal Sonic hedgehog (SHH) signaling has a role in carcinogenesis [5, 8, 9]. For example, small cell lung carcinoma (SCLC), one of the identified malignancies with HH overexpression, could block the growth by the treatment of smoothened (Smo) inhibitor cyclopamine or a monoclonal antibody blocking SHH [10].

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Cancer Treatment Resistance

Cancer treatment, including radiotherapy (RT) and chemotherapy are considered effective for many types of cancers for clinical benefit and improvement in survival. Chemoradiotherapy (CRT) has been recommended as a standard treatment strategy for solid cancers, such as those arising from head and neck [11], cervix uteri [12], lung [13], esophagus [14], pancreas [15], stomach [16], and rectum [17]. Although genotoxic agents are strongly linked to tumorigenesis, the cytotoxic effect of DNA damage is also a critical facet of cancer therapy. In fact, the majority of human tumors treated with genotoxic agents possibly induced secondary primary malignancies and facilitated therapy-resistant forms [18, 19]. The self-renewal of cancer stem cells (CSCs), DNA repair, drug trafficking system, and other factors expressed in cancer cells are considered to prevent the injury by the therapy. For example, radiation therapy enhanced HSP90 chaperone function, causing radio-resistant lung cancer cells [20]. CRT, concurrent combination of RT and chemotherapy, in cervical cancer has been evaluated that improved treatment outcomes and/or maximize efficiency in comparison with RT alone [21]. Articles reported that the causes for chemotherapy failure in cancer treatment reside in multiple levels, such as poor vascularization, hypoxia, intratumoral high interstitial fluid pressure, and phenotypic resistance to drug-induced toxicity through upregulated xenobiotic metabolism or DNA repair mechanisms and silencing of apoptotic pathways [22, 23]. Factors that have been demonstrated with cancer treatment resistance are listed as follows:

1. *Growth factors* (*GFs*). In the cervical cancer patients, tumors with higher GF were more sensitive to radiation than those with low GF [24]. For instance, radiation-activated epidermal growth factor receptor (EGFR) signaling in cancers, such as non-small cell lung cancer (NSCLC), leading to radioresistance by inducing cell proliferation and enhanced DNA repair [20].
2. *Protumorigenic signaling cascade*. Some tumors cause rapid proliferation phenomenon, accelerated proliferation, by stimulation of irradiation during the course of RT. Accelerated proliferation increases dose required to control tumor cells and is an important cause of acquiring tumor radioresistance [21]. The phosphatidylinositol-3-kinase (PI3K)–Akt pathway involved in several human cancers is frequently upregulated [25], which may cause a tumorigenic phenotype with increased cell proliferation, metastasis, and angiogenesis. Akt inhibitors may significantly reduce viability of certain CSCs [26] and sensitize them to chemotherapeutic agents [27]. Inhibition of the Akt pathway further causes delayed repair of ionizing radiation (IR)-induced DNA double-strand breaks (DSB) formation and radiosensitization [28], indicating that the activation of Akt signaling may underlie at least some cases of radiation resistance [29].
3. *Hypoxia*. Research on an in vivo solid tumor demonstrated that contained a certain proportion of hypoxic fractions [30]. The existence of hypoxic cells is well recognized as one of the major factors causing radiation resistance which possibly results in local failure after RT [31].

4. *P53 and the factors relating apoptosis*. Apoptosis is an active mode of cell death which occurs in response to DNA damage by ionizing radiation, ultraviolet irradiation, and certain chemotherapeutic agents [32]. Mutations in proto-oncogenes or tumor suppressors, like Ras and p53, alters apoptosis signaling and changes the tumor microenvironment which traits of tumor cell resistance to therapy [33, 34] and subsequent tumor recurrence [35]. A large number of experimental studies have shown that apoptosis induced by irradiation is a determining factor of radiosensitivity [36].
5. *Molecular targeting agents* (*EGFR*, *COX2*, *and Mn-SOD*). Molecular targeting agents, such as EGFR, COX2, and Mn-SOD, may be possible to efficiently increase radiosensitivity of cancer cells when given with RT and also eradicate subclinical metastases by themselves [21].

The Role of Hedgehog Signaling in Cancer Treatment Resistance

HH ligands (Sonic, Desert, and Indian) bind to and antagonize the cell surface receptor PTCH, relieving the PTCH-mediated suppression of the transmembrane protein smoothened (Smo). Smo then initiates an intracellular signaling cascade that leads to the activation and nuclear translocation of the Gli family (Gli-1, 2, and 3). Gli family mediates transcription of genes controlling proliferation, differentiation, and survival of cells [1, 6]. Aberrations in hedgehog signaling have been found in cancers [37], resulting in overexpression of HH signaling pathway and an increase in endogenous production of HH ligands [4, 5]. Therefore, suppression of HH signaling might be a valid therapeutic option for overcoming drug resistance and for increasing the success of chemotherapy. Cui et al. analyzed 60 glioma samples, indicating that overexpression of Gli-1 is correlated with glioma recurrence after chemotherapy including VCR, VP16, CDDP, and ACNU [8]. Blockade of HH pathway enhanced cytotoxicity of chemotherapeutic agents in glioma cells through downregulating the expressions of MDR1, MRP1, MVP, MGMT, Bcl-2, and Survivin genes [38].

A growing body of evidence indicates that HH signaling plays an important role in regulating cancer treatment resistance. For example, IR-induced DSBs activate the PI3K-related kinases ATM and ATR, which regulate apoptosis, cell cycle progression, and DNA repair [39]. Research on basal cell nevus syndrome (BCNS; also known as Gorlin syndrome) patients and *Ptc1*$^{+/-}$ mice have shown a defect in the IR-induced activation of the ATR-Chk1 checkpoint signaling pathway (a pathway that serves as a barrier to the development of tumors), resulting in dramatically increases the incidence of tumors in *Ptcl*$^{+/-}$ mice [40]. Likewise, transient expression of Gli-1 disrupts Chk1 activation in human cells, suggesting that SHH signaling attenuates the activation of a genotoxin-triggered ATR–Chk1 checkpoint signal transduction pathway, and inappropriate SHH pathway activation promotes tumorigenesis by disabling a key signaling pathway that helps maintain genomic stability and inhibits tumorigenesis [40].

Induction of Tumor Regrowth and Cancer Stem Cells

Stem cells and CSCs share some features, including signaling pathways to regulate self-renewal and differentiation [41]. Similar to normal stem cells, CSC are thought to be relatively quiescent, to be resistant to drugs and toxins, and to possess the DNA repair capacity [42]. For radiation sensitivity in cancer cells, the vast majority of experimental and clinical studies support four determinant phenomena in radiobiology: repair of DNA damage, redistribution of cells in the cell cycle, repopulation, and reoxygenation of hypoxic tumor areas [43]. The effectiveness of each radiation fraction decreases with increasing repopulation of tumor cells, suggesting repopulation by an RT-resistant progeny [44]. The mechanisms that underlie accelerated repopulation are poorly understood, but may involve the proliferation of previously quiescent treatment-resistant clonogenic cells or, CSC [45].

Signaling pathways, such as the Bmi-1, Notch, Wnt, and SHH pathways [46, 47] that support the dysregulated self-renewal and proliferation of CSC may be targets for regulating tumor regrowth and improving treatment outcomes [48, 49]. The investigations of CSC signaling activation during tumor repopulation suggest that the SHH pathway is an important target to regulate proliferation of surviving clonogens after concurrent chemoradiotherapy (CCRT) [50]. A significant upregulation of SHH and Gli-1 expression was observed in the majority of residual tumors after chemoradiotherapy, suggesting that HH signaling may contribute to cancer resistance [4]. Treatment of cancer cell lines with the HH-inhibitory compound cyclopamine results in downregulation of the proliferation marker Ki67 and reduced proliferation rates [5, 50], indicating that HH pathway activation may be essential for tumor growth and maintenance. Smo knockout studies in chronic myeloid leukemia (CML) CSCs (Bcr-Abl-driven $Lin^{-}/Sca1^{+}/c\text{-}Kit^{+}$) cells reduced their ability to form tumors in irradiated mice [51]. Smo antagonists inhibit the growth of these CML CSCs in vitro and prolong survival in vivo, importantly also in cells with resistance to the currently used Bcr-Abl inhibitors imatinib or nilotinib, suggesting that combination therapy might help to prevent relapses in this chronic disease [51]. Sims-Mourtada et al. indicated that the SHH signaling pathway was extensively activated in both chemoradiotherapy-resistant residual esophageal carcinoma specimens and animal tumor xenografts, causing the promotion of tumor repopulation and contribute to chemoradiation resistance through upregulation of the G1-cyclin-Rb axis [4].

Anti-apoptosis and Cell Cycle Regulation

Research on SHH pathway has demonstrated that SHH contributes to the survival of cells by opposing the execution of intrinsic and extrinsic apoptotic cascades [52, 53]. Research in lymphocytes demonstrated that this effect of SHH signaling may go through the prevention of Fas-induced apoptosis [54]. Many malignant

cells remain resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis, and blockade of HH using pharmacological and genetic tools sensitized the cells to TRAIL cytotoxicity [55]. Small interfering RNA (siRNA)-targeted knockdown of Gli-3, but not Gli-1 or Gli-2, restored receptors death receptor 4 (DR4) expression and TRAIL sensitivity, suggesting that modulation of HH–Gli pathway might be a new therapeutic approach for TRAIL-resistant neoplasms [55]. In B-cell chronic lymphocytic leukemia (B-CLL), selective downregulation of Gli-1 by antisense oligodeoxynucleotides results in decreased Bcl-2 expression and increased apoptosis, suggesting that Gli-1 may regulate Bcl-2 and, thereby, modulate cell survival [56]. For tumor cells, inhibition of SHH signaling has been shown to induce apoptosis in tumors through the activation of both intrinsic and extrinsic apoptosis cascades [57], such as SHH signaling increased Bcl-2 expression in BCCs [58].

Additionally, the sensitivity of cells to the cytotoxic effects of radiation is cell cycle dependent, with the S-phase being more resistant and the G1-S boundary and G2/M phase being more sensitive [4]. Sims-Mourtada et al. demonstrated that the treatment of SEG-1 cells with ionizing radiation alone led to a slight but not significant reduction in the radiation-resistant S-phase fraction. Treatment with HH inhibitors alone led to a significant reduction in the S-phase fraction, and the combination of radiation and HH inhibitors caused a greater reduction in the S-phase fraction compared with untreated cells [4]. In a mouse xerograft model, SHH–Gli-1 signaling pathway was shown a high association with the increase in proliferation and repopulation of esophageal cancer observed after CCRT [4].

Repair of DNA Damage

DNA damage includes endogenous (such as oxidative metabolites) and external exposures (such as environmental pollution), causing single-strand breaks (SSB) and DSBs that may limit survival and the regenerative potential of cells [29]. Repair of DNA DSB can occur via nonhomologous end joining (NHEJ) or homologous recombination (HR). HR is required for a sister chromatid present in the S/G2 phase of replicating cells to provide an error-free template for DNA repair [59] while NHEJ is an error-prone repair mechanism that enzymatically modifies the two ends of a DNA break so that they are compatible for direct ligation [60]. UV light is known to induce DNA repair in irradiated cells through the upregulation of damaged DNA-binding (DDB) proteins, DDB1 (127 kDa) and DDB2 (48 kDa), which mediate a key process in nucleotide excision repair after UV damage [61, 62]. EGFR is also involved in DNA synthesis and DNA repair through its interactions with DNA proliferating cell nuclear antigen (PCNA) [63] and DNA-dependent protein kinase (DNA-PK), which is required for DNA repair [64]. Other factors, such as ionizing radiation, heat, H_2O_2, and cisplatin treatment, induce Ku70/80 and phosphatase I translocation to the nucleus and increase DNA-PK activity for initiation of DNA repair [64–66].

Drug resistance can, in some cases, be attributed to increased DNA repair response but may also result from a variety of other alterations, including decreased apoptotic signaling in response to this form of DNA damage [67, 68]. Articles reported that p53- and DNA mismatch repair (MMR) deficiency are two key genetic changes that have been associated with resistance to cisplatin [68, 69]. Frappart et al. have demonstrated that inactivation of the DNA repair factors, together with p53 loss, led to rapid medulloblastoma formation [60]. Genomic analysis of the tumors showed recurring chromosome 13 alterations via chromosomal loss or translocations involving regions containing *Ptch1*. Sequence analysis of the remaining *Ptch1* allele showed a variety of inactivating mutations in all tumors analyzed, highlighting the critical tumor suppressor function of this hedgehog-signaling regulator and Ptch1 tumor suppressor activity [60]. Moreover, mutations of multiple genes involved in the SHH pathway (including *PTCH1*, *SUFU*, *Smo*) or the Wingless (*WNT*) pathway (such as *AXIN1* or β-*CATENIN*) have also been found in sporadic human medulloblastomas, the most common malignant pediatric brain tumor, highlighting the importance of these pathways for preventing cancer [70]. Couvé-Privat et al. (2002) demonstrated that the presence of relatively high levels of ultraviolet-specific mutations in the *Smo* proto-oncogene in BCC from DNA repair-deficient xeroderma pigmentosum patients has confirmed its importance in BCC development [71]. Research on engineered loss of Pten or expression of a constitutively active Akt can synergize with engineered dysregulation of SHH signaling in mouse models to generate medulloblastoma [72]. Both pathways were targeted by somatic changes arising in medulloblastoma with defective HR, which showed abnormalities in Pten and PI3K signaling in combination with biallelic inactivation of *Ptch1* [60]. Shafaee et al. reported that cyclopamine increased the cytotoxic effects of paclitaxel and ionizing radiation in HH expressing pancreatic carcinoma cells [73]. Although potential interactions between DNA repair mechanisms and the HH pathway is suspected, the radiosensitizing mechanism of cyclopamine is still not fully understood [74].

Stimulation of Multiple Drug Resistant Transporter System

Multidrug resistance (MDR) is a common problem in cancer chemotherapy, resulting from enhanced drug efflux from cancer cells mediated by members of the ATP-binding cassette (ABC) transporter family [75]. Permeability-glycoprotein (P-gp), a product of the multidrug resistance gene 1 (*mdr1*), is one of the best characterized MDR molecules, which highly expressed in solid tumors and, moreover, in CSCs [75]. Recently, research demonstrated that imatinib mesylat (IM), a specific tyrosine kinase inhibitor commonly used in CML, was a substrate of P-gp so that *mdr1* gene overexpression can confer resistance to it [76, 77].

Constitutive activation of the HH pathway has been shown to contribute to the growth and maintenance of various cancers [78]. Previous studies have

shown that the HH pathway regulates cell cycle progression and apoptotic resistance; this likely contributes to HH-induced chemoresistance [4, 5]. Sims-Mourtada et al. show that HH signaling regulates the expression of the ABC transporter protein P-gp and breast cancer resistance protein (BCRP), and blockade of SHH activation by cyclopamine or a Gli-1 specific siRNA resulted in decreased expression of these transporters [74, 78]. In addition, simultaneous treatment of SHH ligand and cyclosporine A, a broad inhibitor of ABC transporter function, blocked this decrease of drug uptake in SEG-1 esophageal adenocarcinoma cells for [3] H-labeled Taxol, MTX, and VP-16 [78]. These findings suggest that SHH signaling may promote MDR via increasing drug efflux by ABC transporters [75].

Development of HH Regulating Therapeutics

It has been shown that topical application of cyclopamine inhibited the growth of human BCC [79]; however, concerns of neurological disturbances may limit the systemic application of this drug. Cyclopamine and other compounds, such as cyclopamine derivatives IPI-926 and Cyc-T, and synthetic compounds GDC-0449, XL-139, LDE-225, SANT1, and Cur-61414, action in binding to and antagonizing Smo. SHH-neutralizing antibodies and Robotnikinin were reported that block the SHH pathway by directly targeting SHH, while arsenic, HPI-1, HPI-2, GANT-56, and GANT-61 were potent Gli inhibitors [80–82]. Several small molecule compounds that prevent HH signaling by binding to and inhibiting Smo are currently under development, including Cur-61414 [83], which has shown promising results in the inhibition of BCC and pancreatic cancer in preclinical models. Vismodegib (GDC-0449, discovered by Genentech Inc. under collaboration with Curis Inc.) [84] is a small, orally administrable molecule with suppression effect on HH signaling by binding to and interfering with Smo. Preclinical studies of vismodegib in mouse models of medulloblastoma and in xenograft models of colorectal and pancreatic cancer, and phase I clinical trials in patients with advanced BCC and MB highlighted an objective response to vismodegib [84, 85]. Because of its low toxicity (with only one grade 4 adverse side effects) and specificity for the HH pathway, vismodegib is currently undergoing phase II clinical trials for the treatment of more solid tumors, and may also be used in combination treatments with conventional chemotherapy [84]. Although systemic inhibitors of HH signaling have been undergoing clinical trials, the discovery may provide potentially a novel therapeutic strategy in tumors because HH signaling blockade may not only impair tumor proliferation, but may increase chemotherapeutic efficacy, and result in improved treatment responses. The therapeutic effects of HH pathway blockade in combination with current CRT regimens are perspective to be investigated in the future. The differential regulation and timing of HH activity in normal and tumor tissue after CRT should also be investigated to optimize the most beneficial therapeutic index.

Concluding Remark

HH signaling increases the resistance of cancer cells to radiotherapy, chemotherapy, and CRT. Research results demonstrate that HH signaling confer treatment resistance of cancer cells through four aspects, including the induction of tumor regrowth and CSCs, anti-apoptosis and cell cycle regulation, modulation of DNA damage repair, and stimulation of MDR transporter system. Inhibition of HH activity may sensitize tumor cells to radiation and chemotherapeutic drugs to improve the treatment outcome [4].

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Chapter 11
Small-Molecule Inhibitors of the Hedgehog Pathway

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Introduction

The Hedgehog (Hh) pathway is one of several intercellular communication mechanisms used to establish and maintain complex tissues in multicellular organisms [33]. Initiated by a family of secreted, lipid-modified polypeptides – Sonic (Shh), Indian (Ihh), and Desert (Dhh) Hhs in mammals – this signaling pathway regulates tissue patterning during embryogenesis, sustains tissue function in postnatal life, and enables tissue repair and regeneration in response to injury. For example, Hh signaling and the resulting transcription of Hh target genes contributes to dorsal–ventral polarity in the neural tube [65], anterior–posterior digit identity in the limb bud [46], bulge stem cell renewal in the cycling hair follicle [45], and post-hepatectomy regeneration of the liver [56]. Dysregulated Hh pathway activity consequently can result in developmental defects such as holoprosencephaly [6, 66] and polydactyly [87], as well as abnormal hair growth [72] and impaired wound healing [44] in postnatal life.

Consistent with its function in controlling cell proliferation and differentiation, the Hh pathway can also promote the onset and/or progression of several human cancers when it is inappropriately upregulated in children and adults [77]. Autocrine, paracrine, and ligand-independent Hh pathway activation have been linked to cancers of the skin [34], brain [82], lung [84], pancreas [8], prostate [37], and blood [93], to name but a few examples, correlating with the role of Hh target gene expression in the development of these tissues and organs. This mechanistic connection between ontogeny and oncogenesis has generated significant interest in small molecules that can block Hh signaling, including natural products previously eschewed as potent teratogens and synthetic antagonists discovered in high-throughput screens. In the past decade, the development of Hh pathway inhibitors has advanced from cell-based models to preclinical animal studies to human clinical trials, resulting in tumor responses that inspire new hope for cancer patients.

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We discuss, in this chapter, current models of Hh signaling, genetic lesions that can lead to uncontrolled pathway activation and tumorigenesis, and the small-molecule blockade of these oncogenic transformations. In particular, we consider the mechanisms by which known Hh pathway inhibitors act, their efficacy in pre-clinical or clinical studies, and potential limitations in their therapeutic use. We conclude with an examination of the challenges associated with therapeutically targeting the Hh pathway and possible opportunities for future research.

Hedgehog Signaling Mechanisms and Cancer

As with any communication system, Hh signaling can be deconstructed into three basic steps: (1) signal generation, (2) a signal transport, and (3) a signal reception (Fig. 11.1) [33]. Dysregulation of any of these processes can cause aberrant inhibition

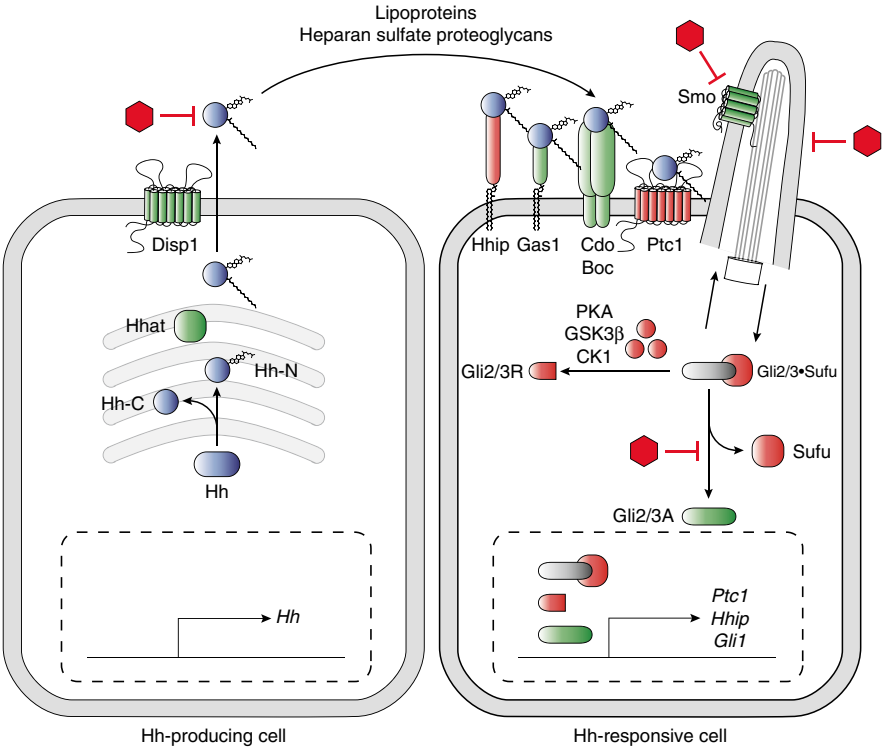


Fig. 11.1 Pharmacological modulation of Hh signaling mechanisms. Schematic representation of Hh protein biogenesis, secretion, transport, and reception. Hh ligands are depicted in *blue*, positive regulators of Hh signaling in *green*, and negative regulators in *red*. Signaling proteins or processes that can currently be inhibited by small molecules are indicated by the *red hexagons*

or activation of Hh target gene expression, resulting in human disorders and disease. The biochemical reactions and interactions that underlie Hh signal generation and transport appear to be largely conserved across metazoan organisms; Hh reception mechanisms in mammals, however, have evolutionarily diverged from those in their invertebrate counterparts, and we limit our brief discussion here to the former. First, production of the Hh ligand itself is a surprisingly complex process, involving the autoproteolytic cleavage of a polypeptide precursor to generate the signaling protein functionalized with a C-terminal cholesteryl ester [60]. The N -terminus of the Hh ligand is subsequently palmitoylated by Hh acetyltranferase (Hhat) to yield the fully active morphogen [13]. Secretion of the lipid-modified ligand is mediated at least in part by a 12-pass transmembrane protein Dispatched1 (Disp1) [49].

Perhaps because of these dual lipid modifications, movement of the secreted Hh morphogen through tissues relies on active transport mechanisms. Genetic and biochemical studies in *Drosophila* have suggested roles for lipoproteins and heparan sulfate proteoglycans in intercellular Hh transport [59, 79], and similar mechanisms appear to be operative in other organisms. Reception of the Hh signal is then mediated in mammals by the 12-pass transmembrane protein Patched1 (Ptc1) [50], which is structurally related to Disp1 but functionally disparate. Ptc1 binds directly to the Hh morphogens and this interaction can be either facilitated by other Hh-binding proteins at the cell surface, such as growth arrest-specific 1 (Gas1) and the Ig/fibronectin superfamily members Cdo and Boc [1, 78, 89], or competitively inhibited, as is the case with the membrane protein Hh-interacting protein (Hhip) [19]. When unbound by Hh ligands, Ptc1 localizes to the primary cilium, a microtubule-based sensory organelle that protrudes from the plasma membrane [68]. Members of the Gli transcription factor family (Gli2 and Gli3) traffic through the primary cilium [28], and in the absence of Hh signaling, these factors are sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) and then either proteolytically processed into N-terminal transcriptional repressors (Gli2R and Gli3R) or degraded by the proteosome [57, 83]. These enzymatic reactions may occur within the primary cilium or the centriole/basal body from which it extends, since genetic perturbations that block ciliogenesis can also prevent Gli repressor formation [47].

The binding of Ptc1 by Hh ligands results in the inhibition of this transmembrane protein, its trafficking out of the cilium, and activation of a G protein-coupled receptor (GPCR)-like protein called Smoothened (Smo) [67]. Active Smo accumulates within the primary cilium [20, 67], and through a poorly understood process it abrogates Gli2 and Gli3 proteolysis and converts the full-length factors into transcriptional activators (Gli2A and Gli3A). These events also release the Gli proteins from the repressive effects of Suppressor of Fused (Sufu) [30], a negative regulator of the Hh pathway that binds directly to full-length Gli proteins and restrains their activity through several mechanisms [17, 25, 41, 74]. Gli2A is the primary activator of Hh target gene expression under normal physiological conditions, and several regulators of cell proliferation and differentiation are transcribed in response to Hh ligands. Hh target genes also include pathway components, thereby creating negative (Ptc1 and Hhip) and positive (Gli1, a constitutively active Gli homolog) feedback loops.

Consistent with the mechanistic complexity of Hh signaling, there are several ways by which genetic lesions can promote uncontrolled Hh pathway activation and tumorigenesis. The role for Hh target gene expression and cancer was first recognized when it was discovered that mutations in the *Ptc1* locus are the primary cause of nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin's syndrome [34]. As implied by its name, individuals afflicted with NBCCS are markedly predisposed to basal cell carcinoma (BCC), as well as medulloblastoma and rhabdomyosarcoma. Subsequent studies have demonstrated that nearly all cases of sporadic BCCs are caused by ligand-independent, cell-autonomous Hh pathway activation, typically by loss of Ptc1 function [27] and less frequently by genetic lesions in Smo that render it constitutively active (e.g., the SmoM2 allele which has a W535L mutation) [42, 86]. Activating mutations in Smo can also be found in a subset of medulloblastomas [61], and inactive, truncated alleles of Sufu have been discovered in other cases of this pediatric brain cancer [76].

It is now widely appreciated that Hh pathway activation contributes to the etiology of several cancers, including those of the lung, stomach, pancreas, prostate, and blood [77]. Tumor cells derived from these tissues typically express at least one member of the Hh protein family, and it appears that ligand-dependent pathway activation is required for the progression rather than the onset of these diseases. Although autocrine Hh signaling was initially believed to be the predominant oncogenic mechanism in these cases, subsequent animal model studies suggest that paracrine signaling between the cancer cells and their surrounding stroma maintains a microenvironment favorable for tumor growth [90]. Tumor responses to Hh ligand-expressing stroma could, in principle, represent an alternative mode of oncogenic, paracrine Hh signaling.

Cancer more commonly associated with other oncogenic pathways may also involve Gli-dependent Hh target gene expression, albeit through noncanonical signaling mechanisms. For example, recent findings from several laboratories indicate that tumorigenic Ras signaling can activate Hh transcriptional programs. This crosstalk between the Ras and Hh pathways appears to be Smo-independent and may be mediated through SCL/TAL interrupting locus (SIL), a cytoplasmic protein that uncouples Gli proteins from Sufu-mediated repression [32, 55]. Transforming growth factor-β (TGFβ) signaling can also induce Gli2 expression, leading to Smo-independent Hh target gene expression [22], and phosphoinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) signaling have been reported to modulate Gli activity downstream of Smo [62, 63]. Noncanonical Hh pathway activation can even result from anomalous factors generated by chromosomal translocations; several reports demonstrate that the chimeric transcription factor EWS-FLI1 associated with Ewing's Sarcoma can directly induce *Gli1* transcription and consequently promote Hh target gene expression [5, 94]. In fact, Gli1 may be more critical for tumorigenesis than Gli2 in at least some instances, since genetic loss of Gli1 function significantly reduces the incidence of medulloblastoma in mouse models of NBCCS (39).

Given the variety of mechanisms that can lead to Hh target gene expression and cancer, developing a single pharmacological agent that effectively targets all Hh

pathway-related tumors will be a major challenge. Rather, gaining comprehensive small-molecule control of Gli-dependent transcription likely will require several classes of drugs that target multiple activities within the pathway. In the next sections, we summarize current attempts to develop Hh pathway antagonists, a growing pharmacopoeia of diverse structure and function.

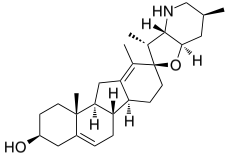
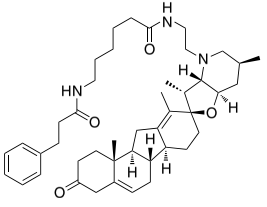
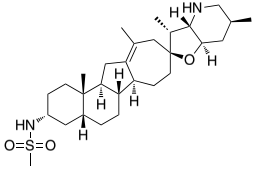
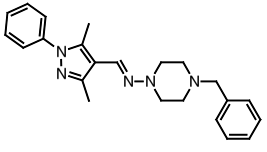
Small-Molecule Inhibitors of Smoothened

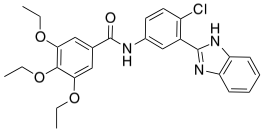
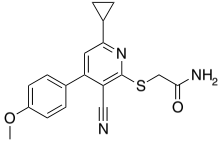
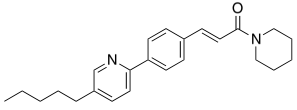
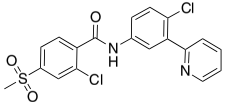
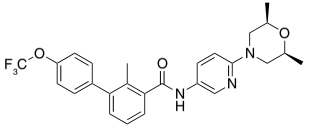
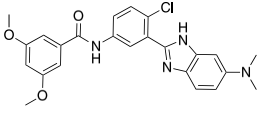
The first example of pharmacological blockade of Hh signaling actually pre-dates the discovery of genetic mutations that disrupt this pathway. During the 1950s, lambs exhibiting cyclopia, limb defects, and other congenital abnormalities were observed in ranches located in parts of the Boise, Sawtooth, and Challis National Forests of Idaho [9]. Scientists affiliated with the United States Department of Agriculture (USDA) determined that these birth defects were caused by ingestion of the corn lily *Veratrum californicum* by pregnant ewes [10], and further studies by the USDA Poisonous Plant Research Laboratory isolated the steroid alkaloid cyclopamine (1; Table 11.1) and structurally related compounds as the causative agents [38]. For over 30 years, these one-eyed lambs were featured in developmental biology textbooks as examples of naturally occurring teratogenesis, yet the mechanism of cyclopamine action remained a mystery. Studies of human genetics and knockout mice in the 1990s, however, revealed a role for impaired Shh signaling in holopresencephaly [6, 18, 66], a congenital disorder characterized by cyclopia and other axial defects. These findings suggested that cyclopamine might act as a Hh pathway inhibitor, and subsequent investigations demonstrated that this natural product binds directly to Smo and inhibits its activity [14, 75].

The discovery of cyclopamine as a specific Hh pathway antagonist has led to its extensive use as a basic research tool, allowing the blockade of Hh target gene expression in cultured cells, tissue explants, and whole organisms with temporal precision. Cyclopamine and its derivatives also provided the first pharmacological evidence that Hh pathway activation is required for the proliferation of certain cancers. For example, cyclopamine can inhibit abnormal cell growth associated with the loss of Ptc1 function, and the more potent analog KAAD-cyclopamine (2) can overcome oncogenic Smo mutations in cultured cells [75]. It was further shown that cyclopamine induces tumor regression in murine allograft models of medulloblastoma [7], demonstrating for the first time that sustained Hh pathway activity can be required for cancer cell growth.

Despite these successes, cyclopamine is not without its limitations. While its *in vivo* activity suggests that it inhibits Smo with good selectivity, the natural product can have off-target effects at doses similar to that required for Hh pathway blockade [51, 92]. In fact, it appears that at least some reports of cyclopamine-sensitive, autocrine Hh signaling in cultured tumor cells are confounded by Smo-independent cytotoxicity of the compound [90]. In addition, cyclopamine itself has limited potential as an orally bioavailable therapeutic agent, since its spiroketal functionality readily

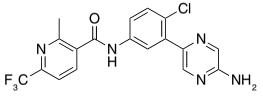
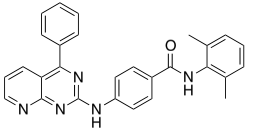
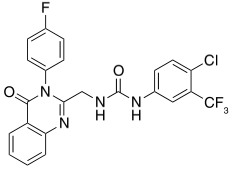
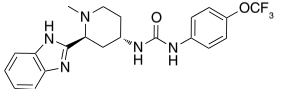
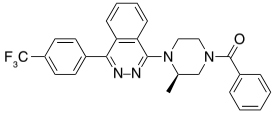
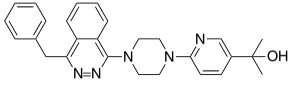
Table 11.1 Hp pathway inhibitors that target Smo

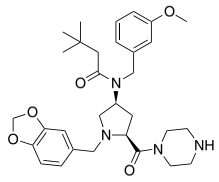
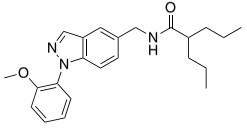
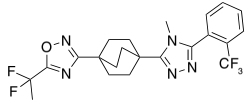
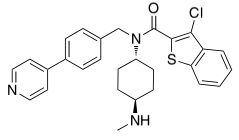
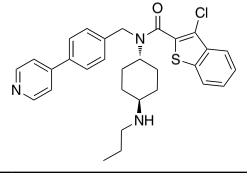
	Compound		IC50 (*in vitro*)	Preclinical anti-cancer activity	Clinical trials
1		Cyclopamine	300 nM	Inhibits growth of several tumor allografts and xenografts in mice	
2		KAAD-cyclopamine	20 nM		
3		IPI-926	7 nM	Inhibits growth of several tumor xenografts in mice	Currently in Phase I
4		SANT-1	20 nM		

5		SANT-2	30 nM		
6		SANT-3	100 nM		
7		SANT-4	200 nM		
8		GDC-0449	10 nM	Inhibits growth of medulloblastoma allografts in mice	Currently in Phase II
9		LDE225	0.6 nM	Inhibits growth of medulloblastoma allografts in mice	Currently in Phase I
10		HhAntag	30 nM	Inhibits growth of medulloblastomas that arise in $Ptc1^{+/-}$; $p53^{-/-}$ mice and several tumor xenografts	

(continued)

Table 11.1 (continued)

	Compound		IC50 (*in vitro*)	Preclinical anti-cancer activity	Clinical trials
11		AstraZenica inhibitor	10 nM		
12		Exelixis/BMS inhibitor	4 nM		
13		Compound Z	70 nM		
14		Pfizer inhibitor	Not reported		
15		Amgen inhibitor	3 nM	Inhibits growth of medulloblastoma allografts in mice	
16		Novartis inhibitor	3 nM	Inhibits growth of medulloblastoma allografts in mice	

17		Cur61414	100 nM	Inhibits growth of BCC-like lesions in *Ptc1*$^{+/-}$ mice	Unsuccessful Phase I trial as a topical agent
18		Merck inhibitor	5 nM		
19		Merck inhibitor	300 nM	Inhibits growth of medulloblastoma allografts in mice	
20		SAG	30 nM		
21		SANT-75	20 nM		

undergoes acid-catalyzed ring-opening to yield a functionally inactive derivative [11]. These pharmacological issues combined with the promise of Smo as an anti-cancer target have prompted several research groups in academia and industry to identify new chemical scaffolds capable of inhibiting Smo with high potency and specificity.

One strategy pursued by infinity pharmaceuticals has been the semisynthetic preparation of cyclopamine analogs with improved pharmacokinetic properties and potency [80]. In particular, they modified three structural features in cyclopamine to generate IPI-926 (3): expansion of the acid-sensitive D ring to a seven-membered system, saturation of the B ring, and replacement of the A ring hydroxyl group with a sulfonamide. IPI-926 is orally available and is now being evaluated in phase I clinical trials. Most Smo antagonists, however, have been discovered through high-throughput screens of synthetic libraries using Hh-responsive cell lines and enzymatic reporters of Hh pathway activation. The first synthetic Smo inhibitors to be reported were the SANTs (*S*mo *ant*agonists; 4–7), four compounds with distinct pharmacophores that abrogate Hh signaling with nanomolar potencies and bind to Smo in a manner that is competitive with cyclopamine [15]. The *N*-phenylbenzamide pharmacophore in SANT-2 (5) can be found in the largest class of Smo antagonists known to date, exemplified by GDC-0994 (8), a Genentech compound that was the first Smo inhibitor to progress to Phase II clinical trials [64], and LDE225 (9), a Novartis drug candidate currently in Phase I trials [58]. Additional Smo antagonists containing a *N*-phenylbenzamide core structure include HhAntag (10) [69] and a series of patented compounds from AstraZeneca (11) [26] and Exelixis (12) [3], the latter of which may be related to BMS-833923/XL139, a Phase I drug candidate of undisclosed structure developed through an Exelixis/Bristol Myers Squibb partnership.

Small molecules based upon a phenyl urea scaffold constitute a second class of Smo inhibitors, represented by compound Z (13) developed by Curis and Evotec [12] and a chemical series disclosed by Pfizer (14) [35] that may define structure elements of PF-04449913, a drug candidate currently in Phase I trials. According to the patent literature, these molecules can completely suppress Hh pathway activation in cultured cells at 2-μM doses, although detailed preclinical data is not yet available. A third class of synthetic Smo antagonists is characterized by a pharmacophore composed of piperazine and phthalazine elements that is somewhat reminiscent of SANT-1 (4). Representatives of this inhibitor class developed by Amgen (15) [48] and Novartis (16) [53] can inhibit Hh signaling with single-digit nanomolar potency. Novartis recently initiated a Phase I trial for a second Smo antagonist, LEQ506, although it has not yet been reported whether its structure is related to LDE225, contains the piperazine/phthalazine pharmacophore, or represents yet another class of Smo modulators. In fact, the structural diversity of synthetic Smo ligands is striking, perhaps reflecting the conformational flexibility of this GPCR-like protein and the myriad of inactive states that can be stabilized by small-molecule binding. Smo inhibitors that are structurally distinct from the three classes described above include Cur61414 (17) [85], a drug candidate developed by Curis and one of the first synthetic Smo antagonists tested in animal models, an aryl indazole (18) with nanomolar potency [23], and a family of bicyclooctyltriazoles (19) [4], the latter two being molecular leads reported by Merck. It has even been found that a chemical agonist of Smo called SAG (20) can be converted into Smo

antagonists by simple structural modifications, mirroring the structure–activity relationships of certain GPCR ligands [88]. For instance, replacing the methyl group in SAG with a propyl substituent yields SANT75 (21), a potent Hh pathway inhibitor *in vitro* and *in vivo*.

In principle, these structurally distinct inhibitors could selectively stabilize different inactive conformations of Smo, thereby targeting specific aspects of Smo function. Critical steps in Smo homeostasis include protein processing in the endoplasmic reticulum (ER) and Golgi, trafficking to and within the primary cilium, and changes in protein conformation and aggregation state upon Hh ligand-dependent activation [33]. Recent studies are consistent with this model, as there appear to be functional differences between SANT-1, SANT-2, and cyclopamine. For example, the Shh-dependent ciliary accumulation of Smo can be blocked by either SANT-1 or SANT-2, whereas cyclopamine treatment actually increases Smo levels in the primary cilium in the absence of Hh ligand [68]. The SANTs also appear to bind to a different site in Smo than cyclopamine, as the synthetic antagonists do not directly compete with SAG for Smo binding, while the steroid alkaloid does [70]. Furthermore, SANT-1 and SANT-2 but not cyclopamine preferentially reduce cellular levels of post-ER glycosylated Smo [68]. These observations indicate that SANT-1 and SANT-2 perturb a Smo activation step that is upstream of and necessary for its ciliary accumulation, while cyclopamine traps an inactive, ciliary form of Smo to block Hh pathway activation. They also suggest that small-molecule antagonists might differentially counteract oncogenic mutations in Smo, and Hh pathway activation induced by SmoM2 overexpression is in fact several-fold more resistant to cyclopamine than the SANTs [15].

To date, Smo inhibitors have been the primary focus of chemotherapies that target Hh pathway-dependent cancers, and several of the compounds described above have demonstrated efficacy in tumor models. Preclinical studies have typically involved cancers that require Hh target gene expression for their onset and progression, such as BCC and medulloblastoma. For example, cyclopamine, IPI-926, GDC-0449, LDE225, HhAntag, and other lead compounds from Amgen, Novartis, and Merck have been shown to induce the regression of subcutaneous medulloblastoma allografts derived from $Ptc1^{+/-}$, $Ptc1^{+/-}$;$p53^{-/-}$, or $Ptc1^{+/-}$;$Hic1^{+/-}$ mice [4, 7, 48, 53, 58, 64, 69, 80]. LDE225 has been similarly successful in orthotopic tumor models in which medulloblastoma cells are transplanted intracranially, demonstrating that this molecule is capable of passing the blood–brain barrier and inhibiting medulloblastoma growth in an environment similar to that of the native cancer.

More recent investigations have begun to explore the ability of Smo antagonists uncouple Hh pathway-dependent tumor–stroma interactions that promote cancer survival. HhAntag has been found to inhibit the proliferation of xenografted human colon cancer and pancreatic adenocarcinomas in mouse models, tumors that secrete Hh ligands but do not require Smo-dependent Hh signaling for their survival. In these cases, it is believed that Hh target gene expression in the surrounding stroma creates a physiological niche that fosters tumor growth, a process that is blocked by the Smo inhibitor. Hh ligand production by stromal compartments may also contribute to tumorigenesis, as illustrated by the Hh pathway-dependent expansion of Eμ-*Myc*-driven B-cell lymphomas in syngeneic mouse models [24]. Cyclopamine

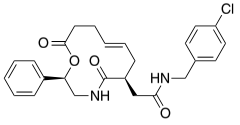
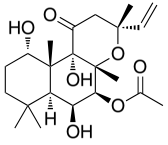
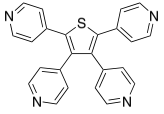
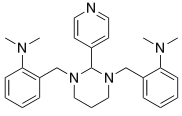
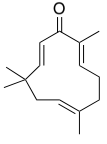
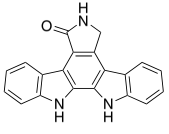
treatment of these mice disrupts paracrine Hh signaling presumably initiated by bone marrow, nodal, and splenic stroma, leading to tumor cell apoptosis and increased survival of the host animals.

Altogether, these preclinical results strongly suggest that Smo inhibitors may be effective chemotherapies for several types of cancers, many of which currently have poor clinical prognoses. The success of these animal model studies has generated considerable enthusiasm for human clinical investigations of Smo antagonists, and nearly 30 clinical trials have been initiated since 2007. A wide variety of therapeutic applications have been proposed, including the use of Smo-targeting compounds as single agents or in combination with other drugs in patients with medulloblastoma, glioblastoma, pancreatic adenocarcinoma, stomach cancer, colorectal carcinoma, breast cancer, hematological malignancies, and generally advanced or refractory solid tumors. Due to the recent inception of these studies, relatively little clinical data is yet available, but results from the first completed Phase I trial underscore the therapeutic potential of Hh pathway-targeting drugs. In this study, patients with metastatic BCC were treated with GDC-0449, with the majority exhibiting objective clinical responses and a few achieving complete regression of the disease [81]. A single patient with advanced, systemic medulloblastoma also responded dramatically to GDC-0449 within 2 months of therapy [91]. At least in this latter case, however, drug-resistant tumors subsequently emerged, resulting in withdrawal of the patient from the trial and eventual death. Genetic analyses of tumor biopsies from the medulloblastoma patient revealed the presence of a mutant Smo allele (D473H) that was not observed in pretreatment tumor samples. Cell-based studies of this mutant protein showed that it is phenotypically normal with respect to Hh response but resistant to binding and inhibition by GDC-0449. Smo D473H is also insensitive to KAAD-cyclopamine, and whether other Smo inhibitors will be similarly ineffective against this or other drug-resistant alleles that arise remains to be determined. Remarkably, a structurally equivalent Smo mutation was observed in one GDC-0449-resistant tumor after prolonged treatment of a mouse medulloblastoma model [91]. Two other GDC-0449-insensitive tumors examined in this preclinical study did not exhibit any Smo mutations, indicating that resistance to Smo inhibitors can arise through multiple mechanisms.

Small-Molecule Inhibitors that Act Upstream of Smoothened

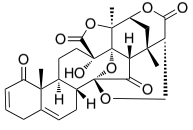
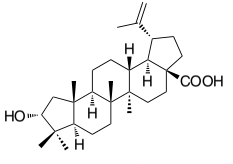
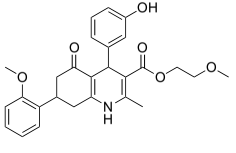
While Smo is perhaps the most druggable target within the Hh pathway, small molecules that block Hh signaling through other mechanisms could also be useful therapeutic agents (Table 11.2), especially given the emergence of Hh pathway-dependent tumors that are resistant to Smo inhibitors. The most upstream targets within the pathway would be those involved in the biogenesis and secretion of Hh ligands. To date, there have been no reports of compounds that block these steps in Hh signaling, although the feasibility of this approach is strongly suggested by the

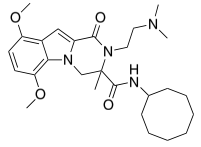
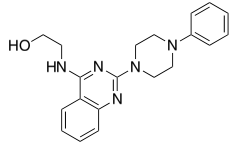
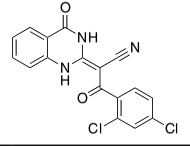
Table 11.2 Hh pathway inhibitors that do not target Smo

	Compound		IC50 (*in vitro*)	Preclinical anti-cancer activity	Clinical trials
22		Robotnikinin	Binds to Shh	10 μM	
23		Forskolin	Indirectly activates PKA and promotes Gli processing	10 μM	
24		GANT-58	Inhibits overexpressed Gli1 and Gli2	5–10 μM	Inhibits growth of prostate tumor xenografts in mice
25		GANT-61	Inhibits overexpressed Gli1 and Gli2	5–10 μM	Inhibits growth of prostate tumor xenografts in mice
26		Zerumbone	Inhibits overxpressed Gli1 and Gli2	7 μM for Gli1 1 μM for Gli2	
27		Staurosporinone	Inhibits overxpressed Gli1 and Gli2	2 μM for Gli1 3 μM for Gli2	Inhibits proliferation of a pancreatic cancer cell line

(continued)

Table 11.2 (continued)

	Compound		IC50 (*in vitro*)	Preclinical anti-cancer activity	Clinical trials
28		Physalin B	Inhibits overxpressed Gli1 and Gli2	0.6 μM for Gli1 2 μM for Gli2	Inhibits proliferation of a pancreatic cancer cell line
29		Betulinic acid	Inhibits overexpressed Gli1	30 μM	Inhibits proliferation of several cancer cell lines and tumor xenografts in mice
30		HPI-1	Inhibits overexpressed Gli1 and Gli2	2 μM	Inhibits proliferation of SmoM2-expressing CGNPs in primary cultures

31		HPI-2	Inhibits overexpressed Gli2	2 μM	
32		HPI-3	Inhibits Hh signaling downstream of Su(fu)	3 μM	
33		HPI-4	Inhibits Hh signaling downstream of Su(fu)	7 μM	Inhibits proliferation of SmoM2-expressing CGNPs in primary cultures

recent discovery of synthetic Wnt signaling inhibitors that target Porcupine, an *O*-acyltransferase that palmitoylates members of the Wnt morphogen family [16].

In contrast, small molecules that can bind directly to lipid-modified, secreted Hh ligands and block their signaling capabilities have been identified, challenging the conventional wisdom that ligand–receptor interactions cannot be pharmacologically targeted. In this study, a collection of compounds prepared by diversity-oriented synthesis was immobilized on glass slides and screened for molecules capable of binding fluorescently labeled recombinant Shh. Subsequent lead optimization efforts led to the macrocycle robotnikinin (22), which can inhibit Hh pathway activation induced by Hh ligands but not that caused by the loss of Ptc1 function or activation of Smo by a synthetic agonist [73]. These activities support a model in which robotnikinin prevents Shh from engaging the ligand reception machinery in Hh-responsive cells. However, it is not yet known which portion of the Shh morphogen binds to robotnikinin or which Hh ligand-binding proteins are unable to recognize the Shh/robotnikinin complex. Although the specificity of robitnikinin for Shh versus Ihh and Dhh has not been established, in principle it should be possible to develop robotnikinin-like compounds that discriminate between Hh ligand homologs. Such ligand specificity could be therapeutically advantageous, as it would allow the pharmacological intervention of diseases associated with a particular Hh ligand, while minimizing perturbations of other Hh pathway-dependent physiology. For example, Smo inhibitors used to treat Shh-dependent tumors in children would adversely and permanently disrupt Ihh-regulated bone development, resulting in dwarfism [40]; a Shh-specific inhibitor, however, might be able to avoid these deleterious side effects.

Inhibition of the Hh pathway upstream of Ptc1 may also be clinically desirable, as recent evidence suggests that Ptc1 can directly promote programmed cell death through the recruitment of a complex-containing DRAL (downregulated in rhabdomyosarcoma LIM-domain protein) and caspase 9 and that this process is inhibited by Hh ligands [52]. Small molecules that reestablish this Ptc1-facilitated apoptosis pathway in tumors, therefore, might be more efficacious than Smo inhibitors against certain cancers, such as those that rely on autocrine Hh signaling.

Small-Molecule Inhibitors that Act Downstream of Smoothened

Hh pathway inhibitors that act downstream of Smo could be particularly efficacious anti-cancer agents since they can counteract Hh pathway activation induced by drug-resistant Smo mutants and possibly even oncogenic lesions that affect downstream components (Table 11.2). Prior to the advent of high-throughput screening campaigns for Hh pathway inhibitors, the most common pharmacological method for disrupting cytoplasmic Hh signaling was treatment with forskolin (23), a plant-derived agonist of adenylate cyclase that increases cellular levels of cyclic adenosine monophosphate (cAMP) and consequently promotes PKA-dependent Gli repressor formation. While forskolin has been a valuable tool for studying Hh signaling mechanisms, its indirect mode of action limits its potential as a therapeutic agent.

Identifying new small molecules that alter Gli function more directly is an area of increasing biomedical interest and clinical importance, albeit one complicated by the exceptional sensitivity of Smo to pharmacological modulation and the regulation of Gli activity by signaling proteins with pleiotropic functions. Screens for compounds that block Hh ligand-mediated pathway activation have nearly exclusively yielded new Smo antagonists, reflecting the difficulty in discovering inhibitors with novel mechanisms amidst the large number of Smo-targeting pharmacophores. Efforts to find downstream-acting molecules have consequently relied on other means of Hh pathway activation. The first of these studies surveyed a collection of 1,990 compounds from the National Cancer Institute for their ability to block the expression of a Gli-dependent reporter in HEK293 cells transiently transfected with exogenous *Gli1* [43]. Two *G*li *ant*agonists, GANT-58 (24) and GANT-61 (25), were uncovered through this screen, and the compounds were also able to block exogenous Gli2 activity in HEK293 cells and Hh pathway activity in embryonic fibroblasts derived from *Sufu*$^{-/-}$ mice. GANT-58 and GANT-61 further demonstrated anti-proliferative activities toward cultured human pancreatic adenocarcinoma PANC1 and prostate cancer 22Rv1 cells, even inhibiting the growth of 22Rv1 xenografts in athymic nude mice.

GANT-58 and GANT-61 have subsequently been found to block the proliferation of Ewing's sarcoma cell lines and Gli-dependent transcription in several cellular contexts [36]. The precise mechanism by which either compound acts remains unclear, but exogenous Gli1 expressed in GANT-61-treated HEK293 cells exhibits diminished affinity for its DNA recognition sequence [43]. Since this effect is not observed *in vitro*, the action of GANT-61 on Gli1 is likely through an indirect mechanism, possibly involving a posttranslational modification that impedes DNA binding. It is also uncertain whether the GANT-58 or GANT-61 pharmacophores will be appropriate scaffolds for drug development; their simple, symmetrical structures are atypical for pharmacological agents, and the compounds may have target specificities and pharmacokinetic properties that are limiting.

Similar Gli overexpression screens have led to the discovery of several natural products that can inhibit Gli-dependent transcription. A survey of 286 natural substances yielded members of the zerumbone (26), staurosporinone (27), and physalin (28) families as potential Gli antagonists [29], and a subsequent study of *Zizyphus cambodiana* extracts identified three structurally related pentacyclic triterpenes as pathway inhibitors, exemplified by betulinic acid (29) [2]. How these compounds block Gli function has not yet been established either, but activities ascribed previously to molecules in these structural families may provide some clues. For example, molecules containing the staurosporinone pharmacophore are well known to promiscuously inhibit kinases by competitively blocking adenosine triphosphate binding [71]. Physalins have been reported to abrogate phorbol ester-induced activation of the nuclear factor-κB pathway and therefore may perturb Gli function through the inhibition of one or more PKC isoforms [21]. Betulinic acid is most commonly known for its pro-apoptotic activity in multiple cancer cell lines and murine tumor models [54], although how these mechanisms might relate to Gli function is unclear.

In spite of these unanswered questions, the screens described above demonstrate the feasibility of pharmacologically modulating Gli activity. The mechanisms by

which the Gli antagonists act are likely to be divergent, and it is almost certain that several cellular targets remain untapped. In addition, Hh target gene expression induced by exogenous Gli1 or Gli2 escapes at least some regulatory processes that would otherwise control the endogenous transcription factors. To search for Hh pathway inhibitors in manner that surveys downstream signaling more comprehensively, our laboratory established an NIH-3T3 cell-based assay in which a stably transfected Gli-dependent reporter is activated by high doses of SAG. Under these conditions, Smo antagonists such as cyclopamine are largely ineffectual while forksolin and other downstream-acting compounds retain their inhibitory activities. A collection of 122,755 synthetic compounds was screened for molecules capable of blocking Gli-dependent transcription in SAG-treated cells. Four *H*h pathway inhibitors (HPI-1 through HPI-4; 30–33) were discovered in this study, each based upon a unique molecular scaffold.

Consistent with their differing structures, the four Gli antagonists appear to act through distinct mechanisms. HPI-1 (30) and HPI-2 (31) can overcome Hh pathway activation induced by Gli1 or Gli2 overexpression, with the latter exhibiting selectivity for the Gli2 isoform [31]. HPI-3 (32) and HPI-4 (33) are less effective against exogenous Gli transcription factors, but their potent activities in *Sufu*$^{-/-}$ fibroblasts indicate that they can block endogenous Gli function. As with the other Gli antagonists, the cellular targets of the HPIs await further study, but their effects on Gli processing provides some mechanistic insights. HPI-1 and HPI-4 uncouple Shh signaling from Gli2 repressor formation, whereas Shh-induced Gli2 stabilization is maintained in cells treated with either HPI-2 or HPI-3. Most strikingly, HPI-4 perturbs primary cilia structure, and it is possible that this compound disrupts Gli activator formation by targeting a ciliary transport protein. Some of the HPIs also exhibit activities against murine SmoM2-expressing cerebellar granule neuron precursors (CGNPs), which give rise to medulloblastoma-like tumors. In contrast to cyclopamine, HPI-1 and HPI-4 can effectively inhibit SmoM2 CGNP proliferation *in vitro*, coincident with their ability to block the expression *Gli1*, *N-Myc*, and other Hh target genes in these cells. Surprisingly, HPI-2 and HPI-3 exhibit neither activity, yet both are able to prevent Hh pathway activation in SmoM2-expressing NIH-3T3 cells. This unexpected result indicates that Hh pathway inhibitors can have tissue-specific activities, a finding that could have important clinical implications.

Conclusions and Future Directions

Since the connection between Hh pathway activation and oncogenesis was first recognized nearly 15 years ago, there have been significant advances toward the development of chemotherapies that specifically target this signaling mechanism. In this relatively brief period, Smo has been identified as a druggable target within the Hh pathway, potent and specific inhibitors of Smo have been developed, and the anti-cancer activities of these compounds have been validated in preclinical models and now human subjects. The dramatic responses of some clinical trial participants

to Smo antagonists may herald new targeted therapies for cancer patients, including those battling metastatic BCCs, medulloblastoma, and other Hh pathway-dependent tumors that currently lack effective treatments.

This new-found optimism is tempered somewhat, however, by the appearance of drug-insensitive tumors in these initial clinical trials, which focused on cancers caused by ligand-independent pathway activation. The subset of these cancers that acquire drug-resistant Smo mutations could be ameliorated by new classes of Smo inhibitors, but murine models suggest that the majority of tumors that gain resistance to Smo antagonists will have genetic lesions in downstream effectors or parallel oncogenic pathways. Whether drug resistance will emerge in Hh ligand-dependent tumors and the mechanisms by which it occurs remains to be seen. Cancers that proliferate in response to Hh ligands, either by autocrine or stroma-to-tumor paracrine signaling, could acquire resistance as these genomically unstable cells are subjected to pharmacological pressure. Cancers that require tumor-to-stroma paracrine Hh signaling to maintain a tumorigenic microenvironment, however, might be more likely to maintain their drug sensitivity since the Hh-responsive stroma cells presumably will be less subject to drug-mediated selection pressure and have normal genomic mutation rates. In any case, choosing the most appropriate chemotherapy for each cancer patient will depend on the mode of Hh pathway activation, the susceptibility of the tumor cells to drug-resistance mechanisms, and the patient's need for either systemic or tissue-selective Hh pathway inhibition.

Realizing this level of personalized medicine will require a structurally and mechanistically diverse ensemble of Hh pathway-targeting drugs. While our growing collection of Smo antagonists has been a logical and promising starting point, new Hh pathway inhibitors need to be developed. Small molecules that block the biogenesis or trafficking of Hh ligands, as well as robotnikinin-like compounds that directly target these secreted morphogens, would constitute valuable additions to our anti-cancer arsenal. Discovering drugs that more directly perturb Gli function is a particularly important area of future research, since in principle, Gli antagonists would be more broadly applicable to cancers that require Hh target gene expression, including those in which Gli activation is achieved through noncanonical signaling mechanisms. Achieving these goals will undoubtedly be challenging, involving new high-throughput small-molecule screens, extensive medicinal chemistry, and the identification of cellular targets for the resulting chemical leads. Yet if these efforts are successful, Hh pathway inhibitors will be known not only as the "monster-generating" compounds in our developmental textbooks, but also as life-saving drugs in our fight against cancer.

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Chapter 12
Cyclopamine and Its Derivatives for Cancer Therapeutics

Martin Tremblay and Karen McGovern

Introduction

In the 1950s, researchers from the United States Department of Agriculture (USDA) investigated cases of congenital cyclopia in sheep grazing in high mountain ranges in central Idaho. After nearly a decade of research, steroidal alkaloids present in the corn lily plant (*Veratrum californicum*) were found responsible for the induction of cyclopic-type craniofacial birth defects that occurred when *Veratrum* was ingested by pregnant sheep on day 14 of gestation [7]. Jervine and cyclopamine were two important teratogenic compounds isolated from *Veratrum californicum* (Fig. 12.1, compound **2** and **6**, respectively), while numerous nonteratogenic but toxic *Veratrum* alkaloids were also present such as veratramine and muldamine [42–46]. Of note, the maternal ewes do not suffer ill effects from ingestion of the plants or cyclopamine [7], with birth defects being confined to a specific window of time during fetal development [103]. Because of its steroidal structure, cyclopamine was originally proposed to antagonize putative hormones involved in regulation of specific genes [42]. The pharmacology of cyclopamine remained dormant for nearly 30 years until genetic studies revealed that mutations in the Hedgehog pathway impacted development.

The role of the hedgehog pathway in development was first discovered in the fruit fly Drosophila by Nüsslein-Volhard and Wieschaus [72] and was ultimately recognized by a Nobel Prize in 1995. Their groundbreaking mutational analysis of genes in Drosophila that control segmentation and polarity elucidated a pathway that, when mutated, resulted in larvae with spiculated cuticles on their skin, resembling the spines of a hedgehog. Subsequent identification of the specific gene products revealed a unique signaling pathway with related orthologues in vertebrate organisms [37]. Mutations generated in the Hh pathway in vertebrates resulted in animals with cyclopic features [14]. These findings were substantiated in humans,

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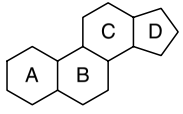
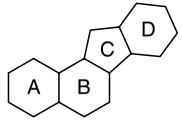
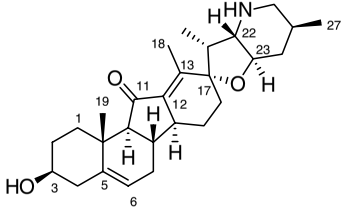
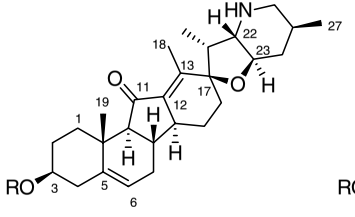


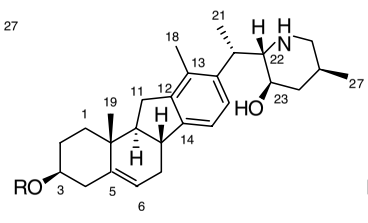
General carbon framework of steroid

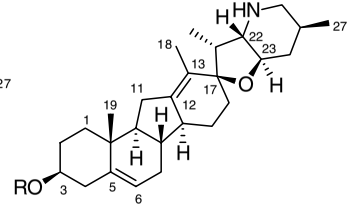


General carbon framework of C-Nor D-Homo steroid









1 Jervine (Originally proposed structure) **2** jervine (revised structure, R = H)
3 pseudojervine (R = Glu)

4 veratramine (R = H)
5 veratrosine (R = Glu)

6 cyclopamine (R = H)
7 cycloposine (R = Glu)

Fig. 12.1 General structure of steroid and C-nor D-homo steroids. Chemical structures of a subset of *Veratrum* alkaloids

where mutations in Shh were linked to holoprosencepaly, which can include cyclopic features [5, 80]. Thus, all inhibitors of the Hh pathway, whether derived from cyclopamine or not, would be expected to impact embryogenesis.

Studies of the Hh pathway in multiple developmental systems have identified key signaling proteins and genes that are regulated in response to ligand activation. Important links between Drosophila genetics and vertebrate biology led to breakthroughs in our understanding of the Hh pathway. One such link, as described above, was the discovery of the plant-derived alkaloid cyclopamine that is found in *Veratrum californicum*. Cyclopamine was subsequently found to antagonize the Hh pathway [15, 32] and to exert its inhibitory effects by binding to Smoothened (Smo) [12, 88]. The natural product cyclopamine, while not active against Drosophila Smo, has served as a powerful tool to help understand the role of the Hh pathway in many aspects of mammalian physiology and disease. While the entire book describes how the Hh pathway is involved in development and cancer, this chapter will review several aspects of the chemistry, pharmacology, and therapeutic potential of cyclopamine and its derivatives.

Cyclopamine, a Natural Steroidal Alkaloid from *Veratrum* Species: Extraction, Isolation, and Structure Elucidation

As early as in the 1870s, a number of alkaloids with the C-nor D-homo steroid skeleton have been isolated from plants of the lily family such as *Veratrum* species. Over the years, a subset of alkaloids shown in Fig. 12.1 has triggered interest because of either their relative abundance and/or their pharmacological effects. Jervine was first isolated from *Veratrum album* [108, 109]. Since this pioneering work by Wright, several extraction and isolation methods for jervine have been described [82, 83] and its structure determination has been debated over several decades. Originally, a number of proposed structures placed jervine in the category of regular steroids constructed with a 6-6-6-5 tetracyclic framework [34, 35, 106]. However, careful exploration of the chemical reactivity of the C–D ring α/β-unsaturated ketone and degradation studies provided evidence that "this alkaloid does not have a normal steroid nucleus" [22]. The structure as depicted in **1** was then proposed, but there continued to be uncertainty concerning the C17 configuration [107]. Further characterization work [54] and X-ray crystallographic data of related alkaloids [79] subsequently showed that the configuration at the C17 position was inverted in the original assignment and that structure **2** was indeed jervine's accurate molecular structure. The structure elucidation of jervine served as a cornerstone to the discovery of other *Veratrum* alkaloids.

A jervine congener, cyclopamine **6** (11-deoxojervine), was isolated independently from *Veratrum glandiforum* (aka *Veratrum album*) collected in the Tokachi district of Hokkaido in Japan [65] and from *Veratrum californicum* collected in the western US [44, 46]. As was the case originally for jervine, the C17 configuration was wrongly assigned initially but corrected when more data became available on jervine.

Early after a reliable isolation method was published for cyclopamine by the USDA research group, they also reported the isolation of the glycosylated form of cyclopamine called cycloposine **7** [47]. Several years after pioneering work by Masamune and Keeler, improved methods to extract and isolate cyclopamine were published [36, 50, 73, 86], and it is clear that *Veratrum californicum* is rich in cyclopamine from all of these reports.

Synthetic Chemistry of Cyclopamine

The amazing structural constitution of jervine and cyclopamine classified these plant natural products as C-nor D-homo steroids. Both compounds have 27 carbons arranged in four carbocycles (A–D rings) and dimethyloctahydrofuro[3,2-b] pyridine (E–F rings), the latter being spiro-connected to the D-ring at the C17 position. Stereochemical complexity in a form of ten stereogenic centers, two of which are quaternary centers, can be found in jervine and cyclopamine. Two polar functions (C3-alcohol and basic piperidine nitrogen) are on the two opposite sides of the lipophilic steroidal skeleton, which is unsaturated at two positions (B-ring and D-ring). Jervine is only different from cyclopamine by the presence of the C11-keto function, which is conjugated with the alkene on the D-ring. Relative to conventional steroids, the preparation of C-nor D-homo steroids has received far less attention from the synthetic community [11]. Nevertheless, a few strategies were exploited to access small quantities of jervine and cyclopamine (Fig. 12.2).

The rearrangement of regular steroids to C-nor D-homo system discovered by Hirschmann [31] provided the basis for the semisynthesis of jervine **2** from hecogenin **8** [67] and more recently of cyclopamine **6** from dehydroepiandrosterone **9** [24]. One of the first successful approaches to jervine started with hecogenin **8** that was converted to intermediate **11** by a six step sequence [69]. Alternatively, the same intermediate could be synthesized from Hagemann ester **10** by a series of annulation reactions [38]. Conversion of the tetracyclic intermediate **11** to jervine involves addition of the E–F ring system in multiple steps [55, 67]. Cyclopamine can be obtained from jervine through a Wolff–Kishner reduction of the C11-ketone [87]. For nearly three decades following these reports, the synthetic community paid little attention to the synthesis of in *Veratrum* alkaloids. The elucidation of the mode of action of cyclopamine coupled with its therapeutic potential revamped interest for this wonderful but complex molecule. Nowadays, many synthetic groups have active research programs aimed at the synthesis of cyclopamine. Recently, a semisynthetic approach to cyclopamine from dehydroepiandrosterone was described [24]. While this approach utilized essentially the same rearrangement pioneered by Hirschmann (*vide infra*), this key transformation was performed on the advanced intermediate **12** bearing the extremely challenging E-ring with the correct configuration at C17 (Fig. 12.2). This approach is very powerful because the spiro-γ-lactone in intermediate **12** can be readily installed and ultimately serves as a versatile building block for the construction of the piperidine ring.

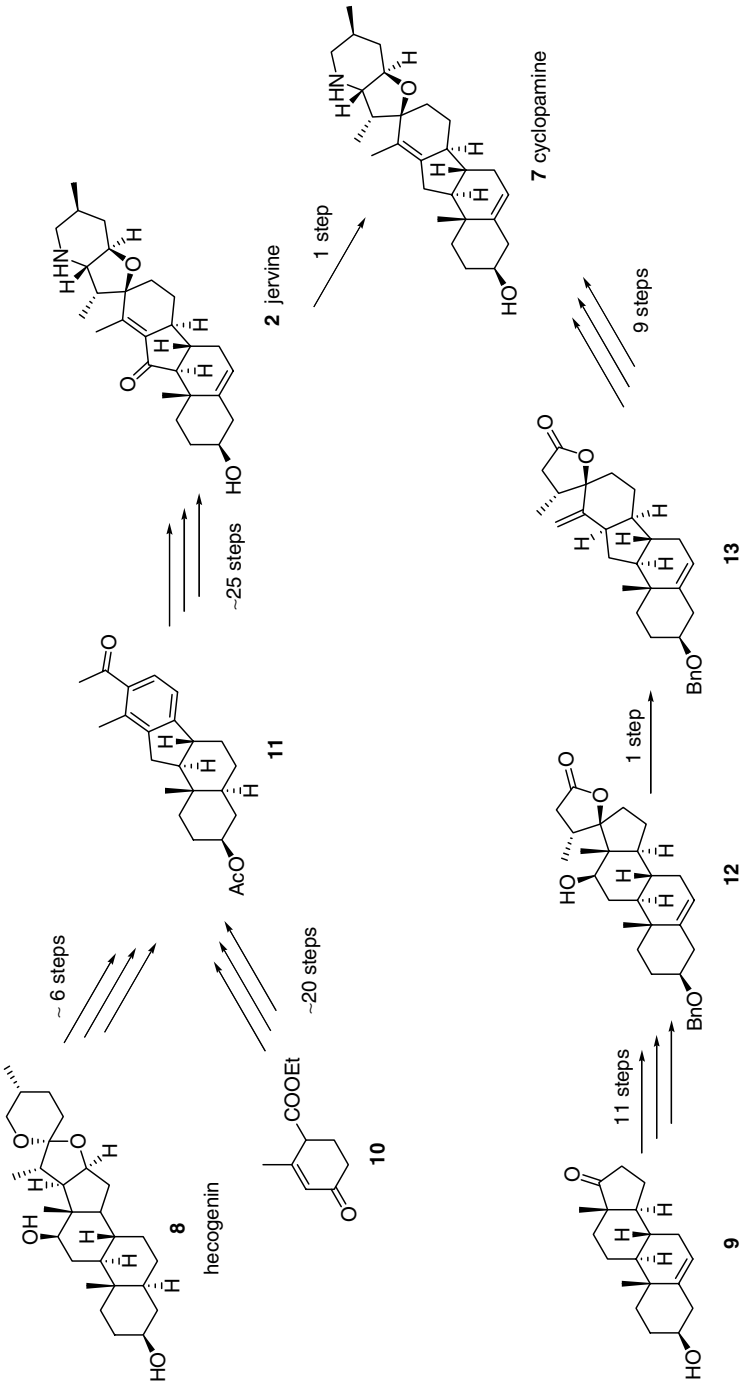


Fig. 12.2 Synthetic approaches to jervine and cyclopamine through rearrangement of C/D ring of conventional steroids to C-nor D-homo steroids or by total synthesis

Evaluation of Cyclopamine as Drug Lead: Its Drug Properties

With the emerging role of the Hh pathway in disease in the late 1990s, the need for selective modulators to test their therapeutic potential became apparent. The discovery of small molecule modulators of the Hh pathway has been comprehensively reviewed elsewhere [62, 77, 95] and the optimization of cyclopamine analogs is the focus of this book chapter. By virtue of its role in target validation of Smo and its availability from nature, cyclopamine is a very interesting starting point for the discovery of modulators of the Hh pathway. Cyclopamine can be readily isolated in high yield from *Veratrum californicum* [36, 50, 73, 86]. This plant species is naturally occurring in the western US, particularly in Utah and Idaho [49], where it was once the target for eradication due to the harmful effects on livestock. Alternatives to field cultivation can already be envisioned for *Veratrum* plant species. Successfully applied to taxol, a therapeutic compound isolated from the yew tree [52], a plant cell culture technique has been reported to generate green plants from embryonic calli of *Veratrum californicum* [61]. Most importantly, these green plants produced veratramine and traces of cyclopamine when grown in suspension media in the presence of naphthalene acetic acid. To complement the latter approach, alternative production platforms such as metabolic engineering are emerging [56] and may be applicable to *Veratrum* alkaloids. Toward that end, important steps in the biosynthetic pathway of cyclopamine have been elucidated [39, 40], which may supplement the development of in vitro techniques to produce cyclopamine. In addition to biomass production and processing, cyclopamine can be obtained by semisynthetic approaches. As one example, jervine can be converted to cyclopamine via Wolff–Kishner reduction [87] (Fig. 12.2). Another example is the single step conversion of cycloposine **7** to cyclopamine **6** by deglycosylation methods [36]. These one step transformations from other C-nor D-homo steroids provide additional source of starting materials.

While cyclopamine represents a very interesting starting point for the discovery of modulators of the Hh pathway, its clinical development was hampered mainly by poor pharmaceutical properties and suboptimal potency. Cyclopamine has low water solubility, which initially impeded the development of formulations for administration to animals. A variety of cyclopamine formulations, including ethanol [3, 50], triolein/EtOH (4:1, v/v) [6, 89], dimethylformamide [98], and dimethylsulfoxide [96], have recently been reviewed [59]. The addition of high concentrations of complexing agents such as 2-hydroxypropyl-β-cyclodextrin resulted in better aqueous formulations of cyclopamine [75, 99]. Alternatively, decreasing the concentration of 2-hydroxypropyl-β-cyclodextrin and thereby the viscosity can be achieved by using the hydrochloride salt of cyclopamine to generate chemically and physically stable formulations [21, 92]. In addition to the hydrochloride salt, water soluble tartrate salts of cyclopamine have recently been described in the literature [103, 110].

In addition to its poor aqueous solubility, cyclopamine is acid labile and readily converts to veratramine (**4**, Fig. 12.1) [48] as well as other isomeric products [104]. Although structurally related to cyclopamine, veratramine does not act as a Smo

antagonist but affects several receptors [70, 90] and causes hemolysis [2]. This liability may not be problematic for oral administration of cyclopamine in preclinical species where the stomach pH may not promote conversion, but in humans the stomach pH ranges between 1.5 and 3 [20] and gastrointestinal transit time is typically longer (20–30 h) than other laboratory animals [41].

In some of the early studies with cyclopamine in sheep, observation of the teratogenic effects required daily oral (p.o.) administration of 2–3 g/animal (average weight of animal ~50 kg; 40–60 mg/kg) [42, 45, 50]. This need for high and frequent doses could be due to poor pharmacokinetic properties and/or potency; some recent studies have shed some light on both aspects. In sheep, a short elimination half-life (1.1 ± 1 h) was measured when cyclopamine tartrate salt (1.6 mg/kg) was administered intravenously (i.v.) [103]. In rodents, the oral (p.o.) bioavailability of cyclopamine is modest (33% relative to intraperitoneal (i.p. administration [59]) and the poor pharmaceutical properties are likely responsible for this observation.

Many cellular assays have been developed to evaluate the potency of Hh pathway inhibitors. Cyclopamine inhibits Hh-dependent processes including (a) HNF3β-induction in chick embryo neural plate assay (IC_{50} of 10 nM; [33]), (b) Gli-reporter assay (IC_{50} of 300 nM; [12]), and (c) C3H10T1/2 differentiation (IC_{50} of 300–400 nM [18]). While the teratogenic effect of cyclopamine is associated with on-target activity on Smo and the Hh pathway, there is evidence that cyclopamine may not be entirely selective. High concentrations of cyclopamine (10 μM) have shown cytotoxic effect on cancer cells that do not express Smo, indicating that the observed effect was off-target. This phenomenon is not unique to cyclopamine since another small molecule Hh antagonist (Cur61614) that is not structurally related to cyclopamine has shown the same behavior [114]. The use of cancer cells grown in culture for the evaluation of Hh pathway antagonists has been extremely controversial. There have been reports in the literature revealing that cancer cell lines grown in culture lose their dependence on the Hh signaling pathway for growth [85, 101]. For this reason, in vitro assessment of a therapeutic window related to treatment with cyclopamine needs to be interpreted carefully. Recent studies have provided some insights into the toxicity of cyclopamine in vivo [59]. The observed toxicity is dependent on the route of administration and could be circumvented by infusion of the drug. This mode of administration delivers cyclopamine at steady state concentrations, whereas bolus intraperitoneal (i.p.) and oral (p.o.) dosing resulted in high and transient plasma peak concentrations that lead to severe dystonia and lethargy, respectively. The relatively rapid onset of the observed toxicity (between 2 and 6 h postdose for i.p. and p.o., respectively) suggests that off-target mechanisms are likely the cause. However, it is unclear if this toxicity is due to the parent drug or a metabolite of cyclopamine. Indeed, high plasma concentration of cyclopamine (20 μM) are well tolerated by the animals dosed i.p., whereas toxicity is observed in orally dosed animals with relatively low (2 μM) plasma concentration of cyclopamine. In summary, derivatives of cyclopamine with improved solubility, stability, and potency are highly desirable to address some of the issues outlined above.

Medicinal Chemistry of Cyclopamine Analogs

Well before the elucidation of cyclopamine's biological target, scientists have been intrigued by the structure–activity relationship (SAR) of naturally occurring jervine [9, 10] and cyclopamine, [48], in particular with respect to their teratogenic potential. However, the determination of biological effects required gram quantities of material which obviously hampered the ability to obtain SAR data. Moreover, the teratogenic potential measured in vivo encompasses both pharmacodynamic and pharmacokinetic properties of the molecules, which makes it difficult to draw conclusive SAR. With that caveat in mind, given that both jervine **2** and cyclopamine **6** are active teratogens, one may conclude that the 11-keto group is not necessary for this activity. However, this functional group plays a role in increasing the stability of D/E-ring system by reducing acid-mediated opening of the ether bridge [9]. Interestingly, cyclopamine-4-en-3-one **14** [66] (Fig. 12.3) was found to be at least twofold more bioactive than jervine as demonstrated independently by its teratogenity in hamsters [9, 23] and its inhibitory effect of Shh signaling in chick neural plate [33]. Also shown in these two assays, the reduction of C5–C6 and C12–C13 double bonds produced a weaker teratogen tetrahydrojervine **15** [68], whereas substantial activity was retained for 12β,13α-dihydrojervine **16**. Cyclo-posine **7** bearing a glucosyl group at the C3 position has also shown teratogenic activity [45], but its effects on the Shh signaling in the chick neural plate was modest [33]. It is plausible that the glycosyl group of cycloposine could be hydrolyzed to liberate cyclopamine in vivo [103], thus explaining the difference between these two results. In addition to alterations of the steroidal skeleton and substitution at the C3 position, the piperidine

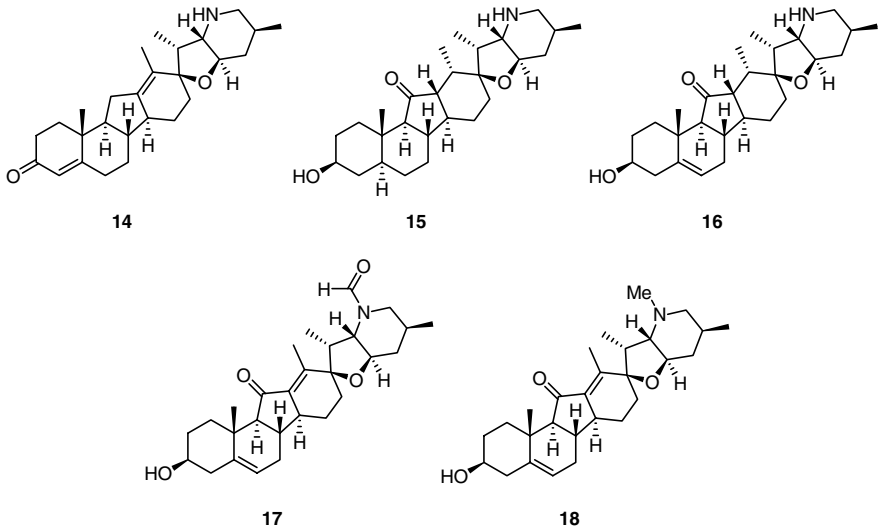


Fig. 12.3 Representative set of early analogs of cyclopamine and jervine that have shown teratogenic activity

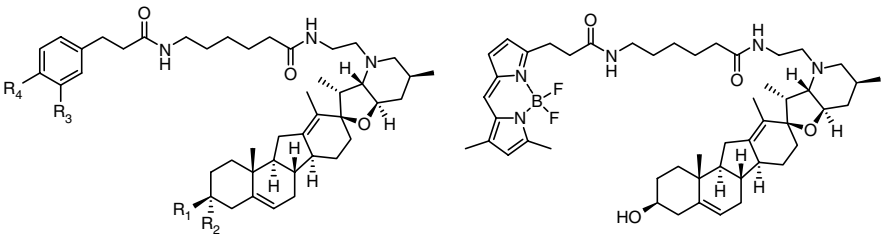


Fig. 12.4 Semisynthetic analogs and probes of cyclopamine based on the natural steroid skeleton

nitrogen was also subjected to modifications [10]. Notably, *N*-formyl (**17**) and *N*-methyl (**18**) jervine analogs were determined to be active teratogens in hamsters, while quaternization of the nitrogen or bulkier alkyl substituents almost completely eliminates the biological activity. In summary, early studies around the structure–teratogenicity relationships of jervine and cyclopamine revealed that these structures were amenable to changes while retaining the biological response. However, improvement of the drug-like properties of these compounds (*vide infra*) would require further investigations.

A synthetic analog of cyclopamine named KAAD-cyclopamine **19** (IC_{50} = 20 nM) was determined to be one order of magnitude more potent than cyclopamine (IC_{50} = 300 nM) in the Shh-Light Gli-reporter assay [13] (Fig. 12.4). This compound played a significant role in establishing Smo as the target of cyclopamine. A radiolabeled and photoaffinity derivative of KAAD-cyclopamine (**20**) (IC_{50} = 150 nM, Shh-Light) was used in the target identification studies [12] and BODIPY derivative **21** (IC_{50} = 150 nM, Shh-Light) is commonly used to assess binding affinity of Smo antagonists. A number of related analogs have also been reported in the patent literature [4]. These studies showed that the potency of cyclopamine could be improved through chemical modifications.

A diversity-oriented approach was designed to generate a focused library of carbohydrate cyclopamine conjugates with improved aqueous solubility [113]. As an example, compound **22** bearing L-rhamnose moiety has better aqueous solubility and similar anticancer activity (IC_{50} = 33 μM) relative to cyclopamine in A549 lung cancer cell line. Although no binding data on Smo were reported for these compounds, it is notable that polar solubilizing groups on the piperidine nitrogen are tolerated while KAAD-cyclopamine **20** bears highly lipophilic side-chain at the same position.

Aiming to develop a targeted therapy for prostate cancer and perhaps move away from potential on-target effects of cyclopamine on the normal stem cell niche, prodrug derivative **23** was designed and synthesized (Fig. 12.5) [53]. In this approach, a peptide carrier susceptible to selective hydrolysis by the active form of prostate-specific antigen (PSA) was grafted to cyclopamine. While the prodrug itself had no significant activity, cyclopamine was indeed released in presence of PSA and biological response was observed in DU-145 prostate cell line. The same concept,

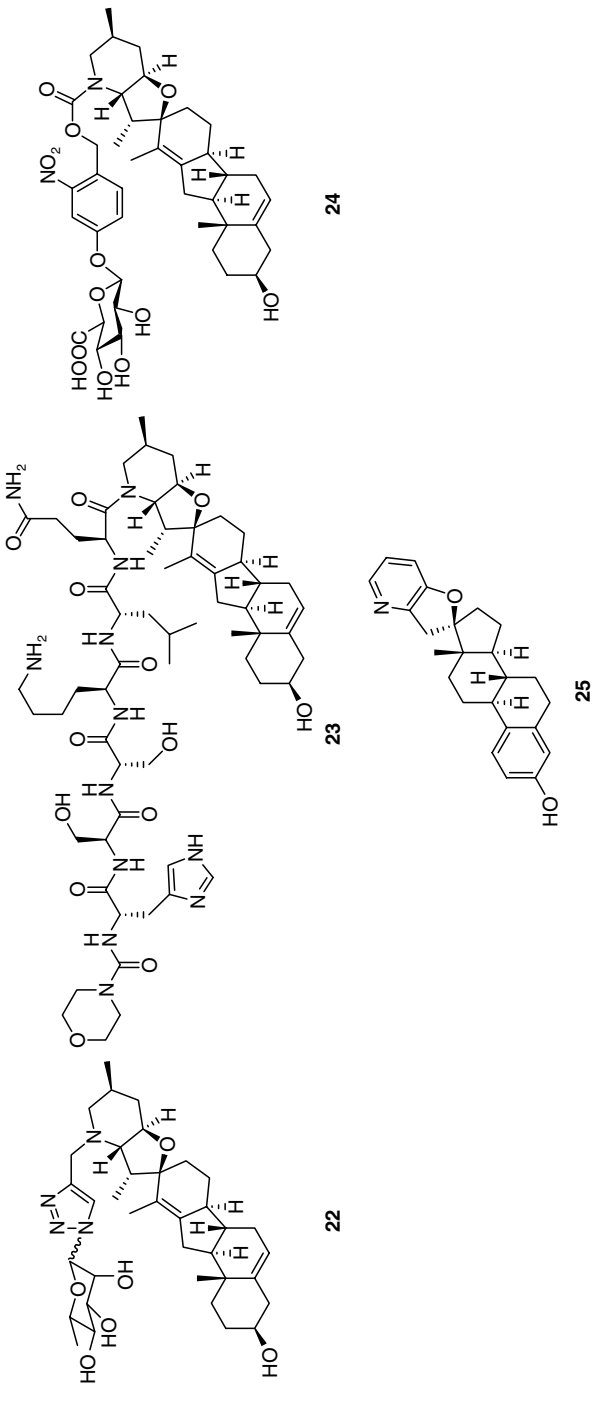


Fig. 12.5 Semisynthetic analogs of cyclopamine based on natural steroid skeletons

with potentially broader scope, led to the design and synthesis of the carbamate cyclopamine analog **24**. Carbamate prodrug **24** was not active by itself, but recapitulated cyclopamine's growth inhibition in glioma U87 cell line when exposed to β-glucuronidase, which is typically present in high levels in the tumor vicinity [26]. These two distinct approaches demonstrated that cyclopamine prodrugs offer the opportunity to selectively deliver cyclopamine to tumor environments.

Readily available steroidal synthetic starting points provided novel cyclopamine analogs that were evaluated for their effect on the Hh pathway [105]. For instance, estrone derivative **25**, which displays a simplified E/F ring system, was shown to inhibit Shh-induced proliferation of mouse granule neuron precursors as well as Shh-Light2 cells at 10 μM. This study revealed that simplification of the core structure of cyclopamine could result in analogs with desirable properties, but more work needs to be done to understand the consequence of these simplifications on the binding to Smo. As a complementary approach, the synthesis of cyclopamine from dehydroepiandrosterone (Fig. 12.2) could deliver very interesting analogs, particularly on the E/F rings, which would be otherwise challenging to obtain from the natural product [28, 29].

Discovery and Development of IPI-926, a Semisynthetic Cyclopamine Derivative in Clinical Trials

The first strategy to improve the pharmaceutical properties of cyclopamine was to address the acid lability. The approach was to alter the influence of the D-ring allylic ether on the cleavage of the spirotetrahydrofuran E-ring, with minimal change to the D-ring geometry. It was originally hypothesized that the oxygen of the allylic ether could direct a chemo- and stereo-selective cyclopropanation of the D-ring double bond. Serendipitously, it was found that the D-ring could be expanded synthetically by cyclopropanation and subsequent acid-catalyzed rearrangement. The resulting 7-membered ring cyclopamine analogs, exemplified by compound **26** (Fig. 12.6), were found to have increased chemical stability [93]. Reminiscent of the case of cyclopamine (*vide infra*), the change in oxidation state from homoallylic alcohol to the α/β-unsaturated ketone in the A/B-ring system brings significant improvement of potency and, in this case, solubility. Also, the structure–activity relationships for N-substitution on compound **26** and cyclopamine closely track, suggesting that expansion of the D-ring did not cause major changes in the binding mode. Compound **26** is equipotent to cyclopamine, yet has improved aqueous solubility and stability relative to cyclopamine. The α/β-unsaturated ketone system found in compound **26** is a very common and structurally important functionality in endogenous steroid hormones. However, this functionality on compound **26** was found to be readily metabolized to the corresponding saturated alcohol and glucuronide conjugate after oral administration in cynomolgus monkeys [64]. The conversion of the half-chair A-ring system of compound **26** to a *cis*-ring fusion system provided a remarkable improvement (approximately tenfold) in potency of

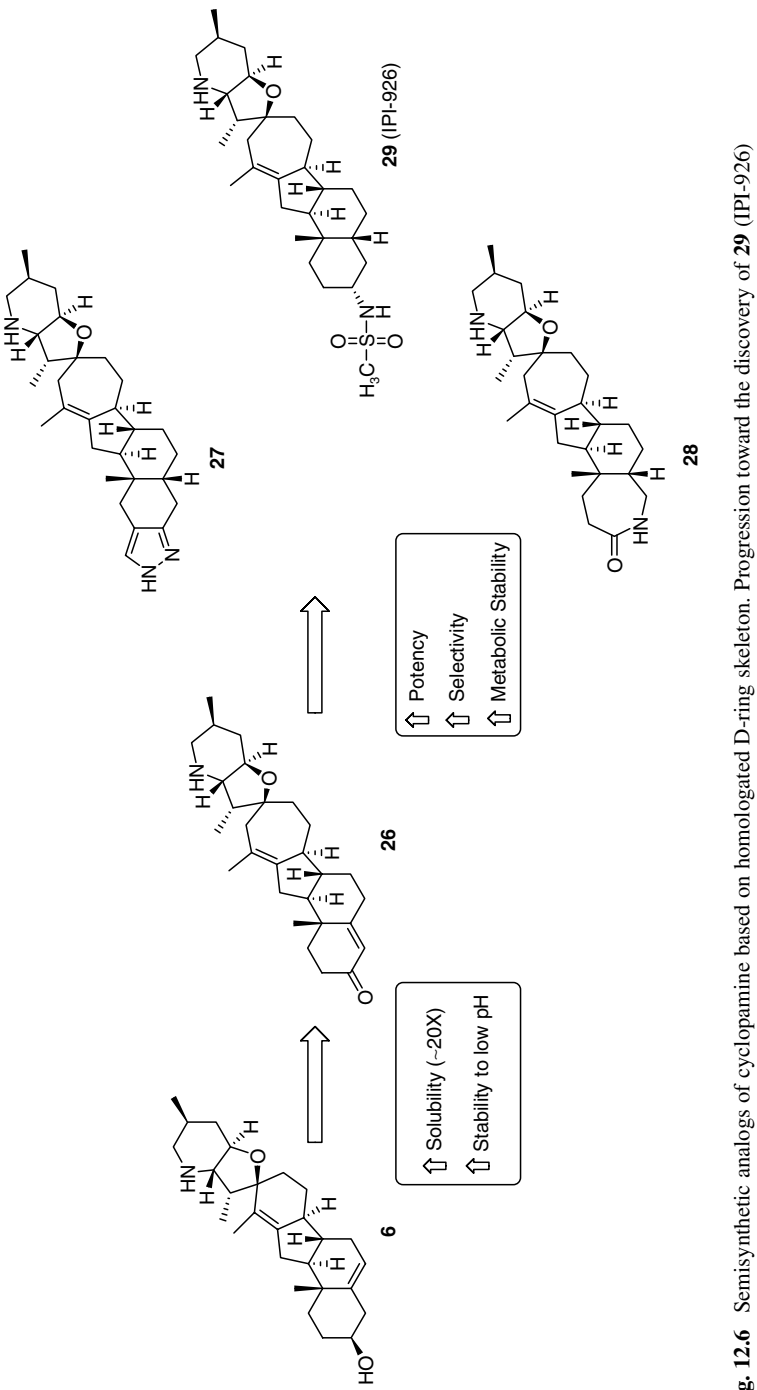


Fig. 12.6 Semisynthetic analogs of cyclopamine based on homologated D-ring skeleton. Progression toward the discovery of **29** (IPI-926)

these D-homo cyclopamine derivatives. Further modifications of the A-ring generated three new series of analogs pyrazole **27**, lactam **28**, and sulfonamide **29** with significantly greater potency and less susceptibility to metabolism than the first generation compound **26**. One key discriminating factor between the three series of analogs is their pharmacokinetic profiles. Although all three series displayed good exposure when administered orally to multiple species, the sulfonamide **29** showed a significant increase in plasma half-life due to low clearance and high tissue distribution (Table 12.1). These properties translated into greater tumor-free intervals following treatment and more robust efficacy than the two other lead compounds when studied in an Hh-dependent B837Tx tumor model, which is described in more detail below [94].

To support its clinical development, capabilities for robust large-scale production of compound **29** were established. First, sourcing of starting material and large-scale extraction and isolation of cyclopamine *Veratrum californicum* was developed and optimized. A robust process was developed to produce multiple kilograms of advanced intermediates (e.g., compound **26**) in fixed equipment. Likewise, the original conversion of cyclopamine to IPI-926 utilized multiple steps that involved potential throughput-limiting purification steps [93, 94].

Table 12.1 In vitro and in vivo profiling of cyclopamine analogs **27**–**29**[a]

Potency	**6** (cyclopamine)	**27**	**28**	**29** (IPI-926)
C3H10T1/2 (EC50)	300–400 nM	10–20 nM	30–40 nM	7–15 nM
Smo Binding (IC50)	114 nM	6 nM	6 nM	1–2 nM
DMPK	**6** (cyclopamine)	**27**	**28**	**29** (IPI-926)
Range F_{PO} (mouse, rat, dog, cynomolgus)	33%[b]	30–80%	>90%	50–100%
Range $t_{½}$ (mouse, rat, dog, cynomolgus)	4 h[b]	1–7 h	3–7 h	8–24 h
Range Vd (mouse, rat, dog, cynomolgus)	–	6–13 L/kg	2–5 L/kg	9–30 L/kg
Efficacy Ptc1$^{+/-}$/ Hic1$^{+/-}$ B837Tx allograft mouse model	**6** (cyclopamine) (40 mg/kg p.o.)	**27** (80 mg/kg p.o.)	**28** (30 mg/kg p.o.)	**29** (IPI-926) (40 mg/kg p.o.)
Tumor-free interval 21 days posttreatment	Not observed	19 days	10 days	21 days
Recurrence rate posttreatment	100%	40%	30%	0%

[a] From Tremblay et al. [94]

[b] From Lipinski et al. [59]. Species: Female C57BL/6J mice at 12–16 weeks of age

Following optimization, IPI-926 drug substance is produced without the involvement of chromatography [1]. Finally, IPI-926 drug product is a solid dosage form intended for oral administration, which was developed to support dose-escalation in the clinic. In a single molecule, IPI-926 (**29**) encompasses the structural features and pharmaceutical properties that overcome many of the deficiencies identified for cyclopamine – namely, solubility, stability, pharmacokinetic profile, and in vivo potency.

Preclinical Pharmacology of IPI-926

The activity of cyclopamine and analogs (e.g., **27**–**29**) were assessed in an assay that measures activation and inhibition of Hh pathway-dependent cellular differentiation. This assay is a Smo-mediated differentiation of the murine mesenchymal cell line C3H10T1/2; it has been demonstrated that cells exposed to either Hh ligands or oxysterols will differentiate to osteoblasts and that cyclopamine will inhibit this differentiation [18, 71, 100]. Cyclopamine inhibits the Smo-dependent differentiation of C3H10T1/2 with an IC_{50} of 300 nM. Derivatives of cyclopamine (**27**–**29**) are much more active than cyclopamine. A Smo binding competition assay was conducted with BODIPY-cyclopamine, as previously described above [12], to confirm that the inhibition of the Hh pathway is through targeting Smo. While cyclopamine blocks the binding of BODIPY-cyclopamine with an EC_{50} of 114 nM, the derivatives of cyclopamine bind more tightly (Table 12.1). IPI-926 (**29**) has an IC_{50} between 7 and 15 nM in the differentiation assay and an EC_{50} of ~2 nM in the Smo binding assay. The inhibition of Smo-driven differentiation correlates with inhibition of expression of Gli-1 regulated genes such as Gli1 and Ptch1. Similar inhibition by IPI-926 of Hh pathway gene expression is detected in the human mesenchymal cell line HEPM.

As described in previous chapters, some tumor types have mutations in Hh pathway members that lead to constitutive activity of the pathway, such as loss of Ptch function or activation of Smo. These types of mutations are found in BCC and some medulloblastoma. The activity of IPI-926 was investigated in a mouse model of medulloblastoma with the Hh pathway constitutively active due to heterozygous $Ptch1^{+/-}$. This mouse model is also heterozygous for Hic-1, the gene for *H*ypermethylated *I*n *C*ancer [8]. The B837Tx cell line was derived from a medulloblastoma in this mouse and passaged as a subcutaneous allograft in NOD/SCID mice. A single oral dose of IPI-926 leads to a dose-dependent decrease in Gli-1 in a dose-dependent manner (Fig. 12.7). A subsequent study determined the pharmacokinetic (PK) and pharmacodynamic (PD) relationship in this model. A single oral dose of either 4 or 40 mg/kg was administered to B837Tx tumor-bearing mice. Levels of Gli1 expression in the tumor and drug levels in the tumor and plasma were assessed at multiple time points postdose, taken over 7 days. The plasma drug concentration profile indicates exposure over many days after both low and high doses of IPI-926 (Fig. 12.8). Gli1 expression correlated well with tumor drug levels,

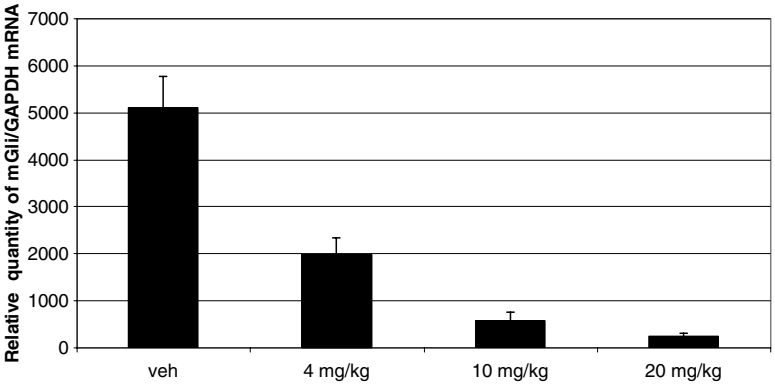


Fig. 12.7 Inhibition of Gli-1 expression at 8 h in B837Tx tumors following single oral administration of IPI-926

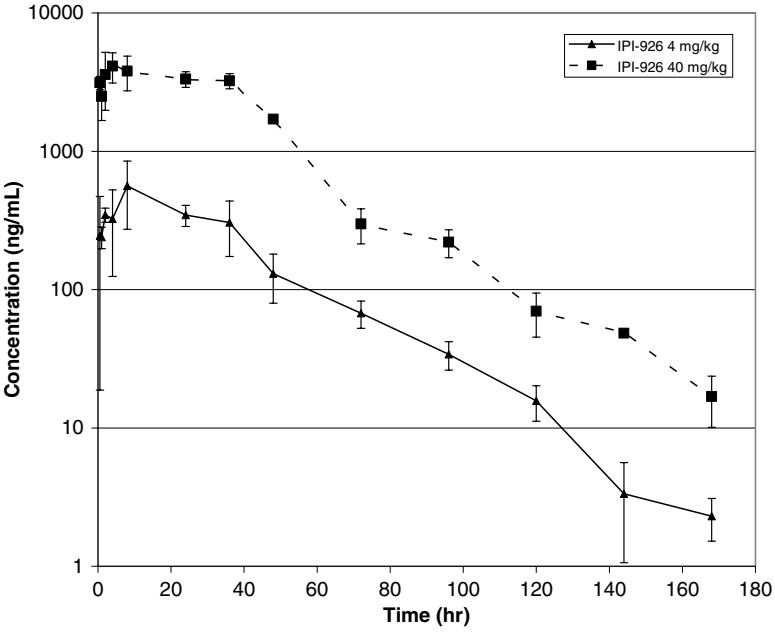


Fig. 12.8 Plasma concentration-time profile of IPI-926 following 4 and 40 mg/kg oral administration of IPI-926 to NOD/SCID mice bearing a B837Tx tumor

and a single dose of 40 mg/kg IPI-926 led to inhibition of Gli1 in the tumor beyond 6 days (Figs. 12.9 and 12.10) [78].

The antitumor activity of IPI-926 was also assessed in the medulloblastoma allograft. IPI-926 was administered orally to tumor-bearing animals daily at 4, 10, or 20 mg/kg for 21 days. During treatment, tumors regressed to undetectable levels in animals administered 10 or 20 mg/kg (Fig. 12.11), with the 4 mg/kg group showing

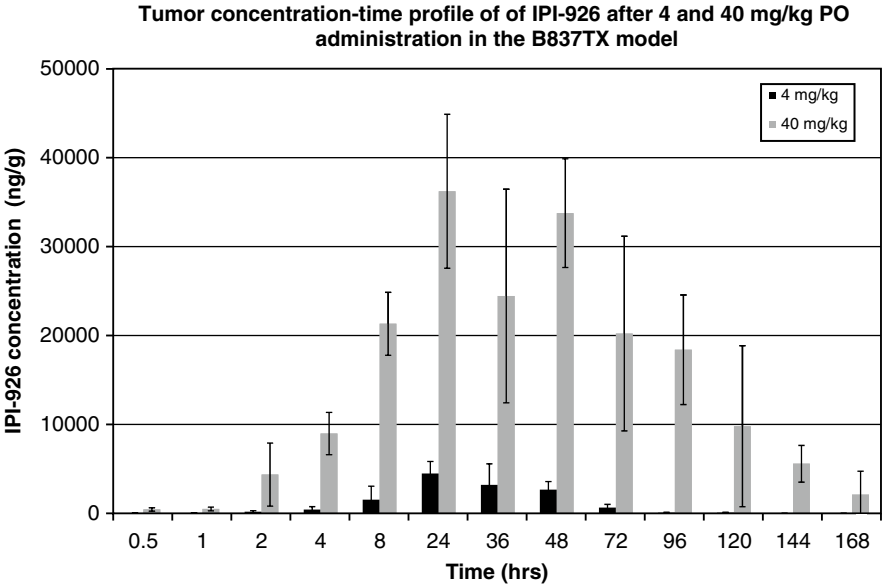


Fig. 12.9 Tumor levels of IPI-926 following single-dose administration of 4 and 40 mg/kg in B837Tx tumor-bearing mice

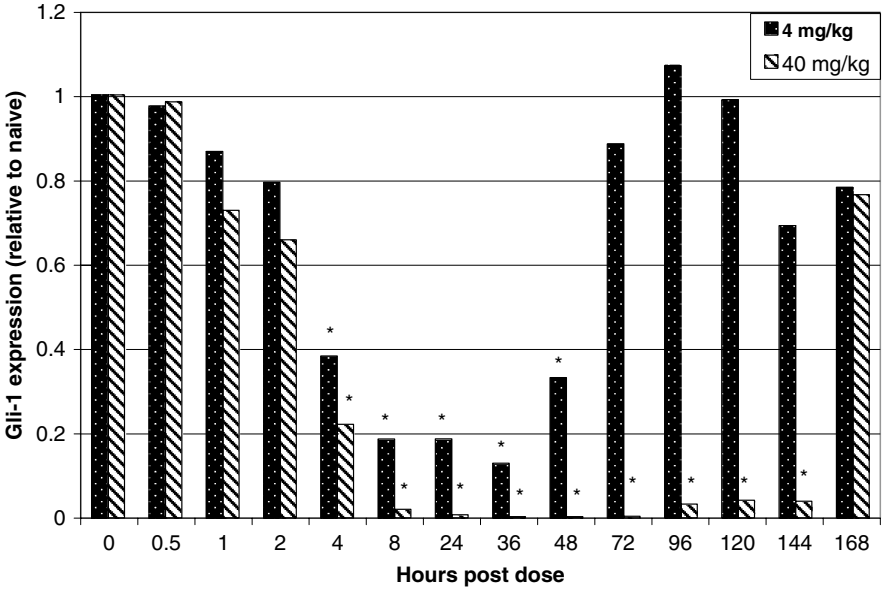


Fig. 12.10 Gli-1 mRNA expression in B837Tx tumors following administration of a single oral dose of IPI-926 (4 and 40 mg/kg); $p<0.05$

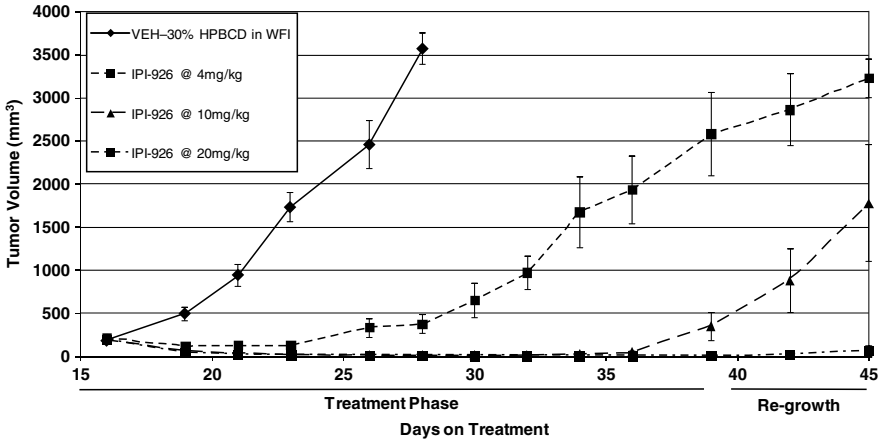
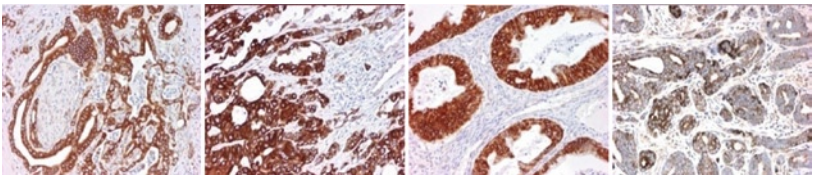


Fig. 12.11 Inhibition of B837Tx tumor growth with daily oral administration of IPI-926; $p<0.05$

50% tumor growth inhibition. In a separate study, daily administration of 40 mg/kg led to regression with no regrowth of the tumors posttreatment, thereby demonstrating that IPI-926 is active in inhibiting growth of Ptch mutant driven tumors [78].

The activity of IPI-926 was also determined in nongenetic tumors. These tumors are driven by malignant activation of the Hh pathway in a Hh ligand-dependent manner. Examples of this type of tumor include pancreatic, ovarian, prostate, and breast ([111] and also see Chaps. 7, 10, and 11). Utilizing a sensitive immunohistochemistry (IHC) method for detection of Sonic hedgehog (SHh) ligand, a high percentage of tumors of various types were found to express high levels of SHh (Fig. 12.12). In many tumor types, Hh signaling occurs with tumors secreting Hh ligand and the surrounding stroma cells responding. Blocking signaling with a Smo antagonist inhibits the Hh gene expression in the stroma and can lead to tumor growth inhibition. This has been detected in multiple tumor models with IPI-926 and other Smo inhibitors (e.g., Fig. 12.13; [112]).

An improved understanding of the mechanism of action of Hh pathway inhibition in ligand-driven tumors has been described in studies with a transgenic model of pancreatic cancer. This model is driven by activated Kras and loss of p53 [KPC] [30] and recapitulates the human disease, from PanIN lesions to the development of pancreatic adenocarcinoma (PDA) and liver metastases. The PDA tumors are highly desmoplastic, as are human PDAs, with an abundance of stromal cells producing collagen and fibronectin among the tumor cells. PDAs are not very susceptible to chemotherapy, including the standard of care, gemcitabine, in both humans and in the KPC model. It was demonstrated that treatment with IPI-926 decreased the stromal content and increased the microvascular density of the KPC tumors [74]. The effect of IPI-926 on the stroma enabled delivery of the chemotherapeutic gemcitabine to the tumor, leading to tumor growth inhibition and a doubling of the median survival of these mice. The IPI-926 plus gemcitabine-treated mice also had



Pancreas, ductal adenocarcinoma | Colon, adenocarcinoma | Ovary, cystadenocarcinoma | Prostate, adenocarcinoma

Cancer Type	Total # of Samples	SHh Positive	SHh Negative	Percent Positive
Pancreatic	92	65	27	71%
Colon	69	58	11	84%
Ovarian	68	32	36	47%
Prostate	73	56	17	77%

Fig. 12.12 Sonic Hedgehog is highly expressed in multiple tumor types. IHC for SHh shown in *top*; staining in tumor microarrays summarized in *bottom*

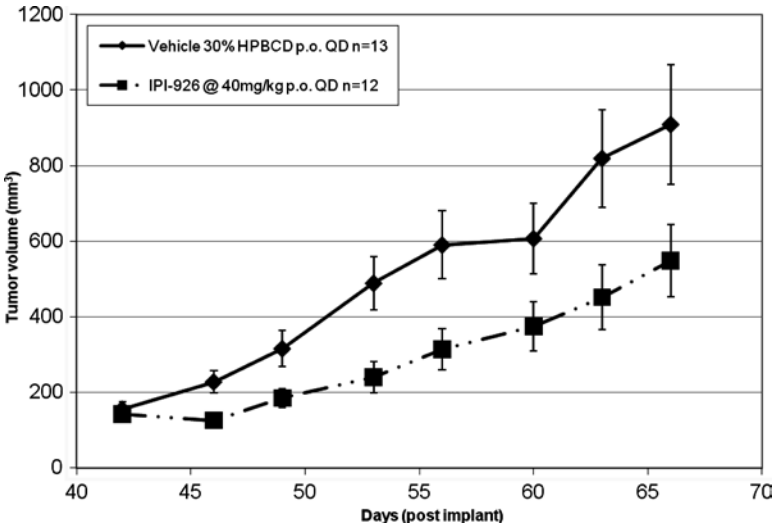


Fig. 12.13 Activity of IPI-926 in the pancreatic cancer xenograft model BxPC3, 40 mg/kg daily p.o.; $p < 0.05$

a lower incidence of liver metastases. These data provide rationale for evaluation of the combined treatment of chemotherapy and IPI-926 in pancreatic cancer.

The potential for a role for Hh pathway in tumor progenitor cells has been suggested in multiple indications (reviewed in [97]), in both hematological malignancies,

including multiple myeloma [76], acute lymphocytic leukemia [58] and chronic myeloid leukemia [17] and in solid tumors, such as glioma [3, 19] breast cancer [60] and pancreatic cancer [57]. It is believed that tumor progenitor cells are resistant to chemotherapy and therefore suspected to be responsible for disease relapse following treatment with conventional therapeutic agents. To address the role of Hh pathway in a model of minimal residual disease (MRD) postchemotherapy, IPI-926 was testing in a primary tumor model of small cell lung cancer (SCLC). Clinically, SCLC responds well to chemotherapy but then relapses within months with no further response to therapy (ref). A chemotherapy-sensitive primary tumor-derived xenograft model, LX22 was utilized to address whether IPI-926 would have an effect on time to relapse postchemotherapy [27]. The LX22 model responds well to chemotherapy and regresses, then regrows, resembling a clinical "complete response." Inhibition of Hh pathway alone in established tumors does not affect tumor growth. However, treatment with IPI-926 after SCLC tumors regress with chemotherapy leads to a significant delay in time to tumor reoccurrence (Fig. 12.14) [91]. Recently, we have determined, along with collaborators, that IPI-926 is active in additional models of minimal residual disease. Similar activity as seen in LX22 of IPI-926 in delaying tumor relapse postchemotherapy was detected in multiple primary ovarian tumor xenografts [25]. In addition, IPI-926 is also active posttumor reduction with targeted therapy, such as tyrosine kinase inhibitors (TKIs). Tumor reduction occurs in a cell line xenograft model of non-small cell lung cancer (NSCLC), NCI-H1650, with treatment with the TKI gefitinib. Maintenance therapy with

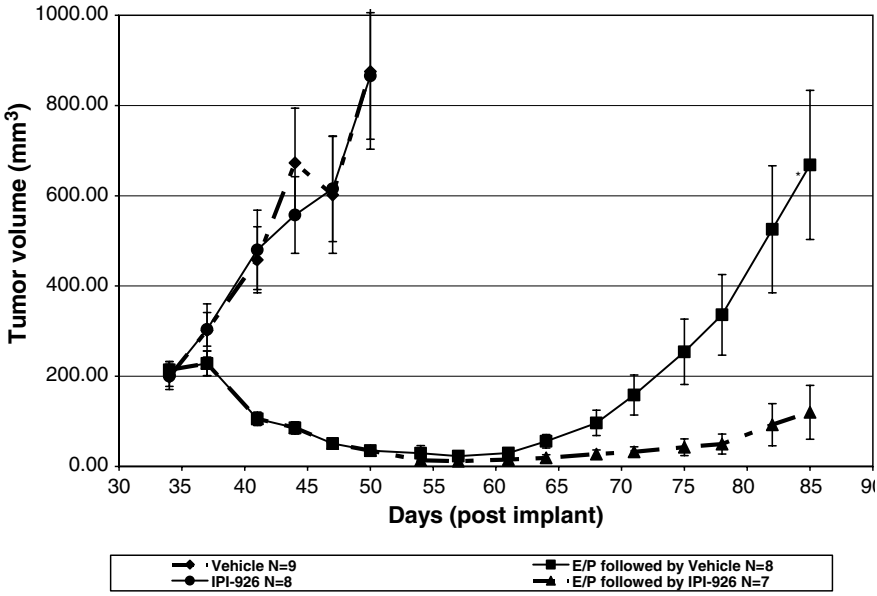


Fig. 12.14 Inhibition of the Hh pathway with IPI-926 delays LX22 SCLC growth following etoposide/carboplatin treatment. Etoposide: 12 mg/kg i.v. three consecutive doses; Carboplatin: 60 mg/kg i.v. once per week. IPI-926 40 mg/kg p.o. QD; $p = 0.0101$

IPI-926 post-gefitinib significantly delays time to tumor regrowth [63]. Finally, multiple primary tumor xenograft models of head and neck squamous cell carcinoma (HNSCC) that regress with cetuximab treatment are sensitive to IPI-926 posttumor reduction, with a delay in time to tumor regrowth [51]. These data suggests an important role for the Hedgehog pathway in tumor relapse, potentially through a cancer stem cell and provides rationale for evaluation of a Smo inhibitor such as IPI-926 in the minimal residual disease setting in the clinic.

Clinical Application

The role of the Hedgehog pathway in the preclinical settings described above suggests a broad potential for Smo antagonists in multiple clinical settings. The most straight-forward is in tumors driven by activating mutations in key Hh pathway members, such as in basal cell carcinoma and some medulloblastoma. Clinical proof of concept has been demonstrated in these indications [81, 102]. In addition, targeting the tumor microenvironment with a Hedgehog inhibitor could affect drug delivery to desmoplastic tumors such as pancreatic cancer and the studies in the KPC mouse model support evaluation of chemotherapy combined with IPI-926 in pancreatic cancer. The minimal residual disease setting is another example where the Hedgehog pathway plays a key role in relapse of tumors posttumor reduction with chemotherapy. While the precise mechanism is still being elucidated, whether Hh-dependent tumor initiating cells or a higher dependence on the microenvironment for tumor regrowth, the cells responsible for regrowth posttumor reduction are dependent on the Hh pathway and inhibited by IPI-926. There are potentially many uses for a Hh antagonist in the treatment of cancer and the trials with multiple Smo inhibitors will provide more insight into the role of Hh in these clinical settings.

Conclusion

Plant-derived natural products continue to play an important role in drug discovery for many therapeutic areas [84]. In oncology, several drugs that have greatly impacted the life of cancer patients are plant derived such as taxol, camptothecin, combrestatin, podophyllotoxin, and vinca alkaloids such as vinblastine and vincristine [16]. The discovery of the mode of action of plant-derived cyclopamine and the importance of this target in cancer has attracted the attention of many researchers to investigate the therapeutic potential of cyclopamine and its analogs. IPI-926, the focus of this chapter, is currently in clinical trials. Preclinical studies have shown that IPI-926 displays antitumor activity in both Hh ligand-independent and ligand-dependent models of malignant activation of the Hh pathway. Clinical evaluation of the first semisynthetic analog of cyclopamine will determine whether this approach will prove beneficial in an array of different clinical settings.

Acknowledgments Several data on IPI-926 summarized in this chapter were the fruit of the entire Infinity Hedgehog team who are specially acknowledged. Outstanding contributions made by Dr. Phillip A. Beachy and Dr. James K. Chen served as the starting point for Infinity's program. In addition, we gracefully acknowledged long-standing collaborators of Infinity Pharmaceuticals, Inc. who had a major impact on the work presented in this chapter: Dr. Neil Watkins (Monash Institute of Medical Research), Dr. David A. Tuveson (Cancer Research UK), Dr. André B. Charette (University of Montreal) and Mr. Steve Monsen (USFS retiree). Finally, the authors thank Drs. Margaret A. Read, Alfredo C. Castro, and Vito J. Palombella for critical review of the manuscript.

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