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Hedgehog signalling in foregut malignancy

D.N. Watkins*, C.D. Peacock

School of Medicine, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD 21231, USA

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Abstract

Hedgehog (Hh) signalling mediates axial patterning and stem cell fate in development. This is mediated by Sonic, Desert and Indian Hedgehogs whose morphogen gradients determine the level of signalling in recipient tissues. Aberrant, cell autonomous, ligand-dependent Hh signalling has recently been demonstrated in small cell lung cancer (SCLC), as well as in upper gastrointestinal malignancies arising from pancreas, esophagus and stomach. These tumors lack mutations in the Hh receptor PATCHED, identifying a mechanism of pathway activation distinct from Gorlin's syndrome associated neural and skin tumors. We believe that this phenomenon represents a conserved mechanism for establishing niche-independent stem cell fates in cancer which is essential for malignant transformation and metastasis. Specific inhibition of Hh signalling by the naturally occurring plant alkaloid cyclopamine provides the opportunity for pharmacologic assessment of the role of Hh signalling in these tumors. Cyclopamine inhibits growth of SCLC and a wide range of foregut derived malignancies both in vitro and in vivo. This demonstrates an ongoing requirement for Hh signalling in these highly lethal and aggressive tumors. A novel therapeutic strategy is proposed using pharmacologic targeting of Hh dependent tumors with high potency pathway antagonists.

Keywords: Hedgehog; Cancer; Foregut; Stem cell; Therapy; Cyclopamine

1. Introduction

Originally identified as a mediator of segment polarity in the fly [1], the Hedgehog (Hh) pathway is essential for normal animal development [2]. The three mammalian orthologues of the *Drosophila* gene *Hedgehog*, Sonic (Shh), Indian (Ihh) and Desert (Dhh) Hedgehog establish morphogenic gradients essential for axial patterning of the mammalian embryo [2–4]. Shh is the predominant signalling molecule in lung, brain and limb development and is the most extensively studied Hh protein in vertebrates [5–8].

The active Shh signalling peptide is formed by an auto-processing reaction that converts a 45 kDa precursor into a 19 kDa signalling peptide that is doubly lipid modified, with palmitate and cholesterol residues at the N and C termini, respectively [9,10]. While cholesterol modification occurs during auto-processing, palmitylation of Hh protein is mediated in flies by the acyl transferase Skinny Hedgehog [11]. Efficient generation and reception of the Hh ligand signal requires these lipid modifications [9,10], the transporter-like function of the transmembrane protein Dispatched [12], and an appropriate heparin sulfate peptidoglycan environment [13,14].

The Hh receptor Patched (Ptch) is a 12-transmembrane protein with homology to the resistance, nodulation, division (RND) bacterial transporter family [15]. Ptch acts catalytically to inhibit the 7-transmembrane protein Smoothened (Smo), rendering the pathway inactive in the absence of Hh ligand [15]. Evidence points to Ptch acting as a transporter of a small molecule which regulates Smo activity, although the identity of such a regulatory molecule remains elusive [15]. Binding of Hh ligand inactivates Ptch, de-repressing Smo and resulting in positive Hh pathway signalling [2,7,16].

Recent elucidation of events downstream of Smo in *Drosophila* cells has suggested potential mechanisms in mammalian cells. Activation of pathway involves a multi-protein cytoplasmic complex scaffolded by the atypical kinesin Costal2 [17–19]. This complex contains the serine/

Abbreviations: Dhh, desert hedgehog; Hh, hedgehog; Ihh, Indian hedgehog; RND, resistance nodulation division; SCLC, small cell lung cancer

* Corresponding author. Tel.: +1 410 955 8506; fax: +1 410 614 9884.
E-mail address: nwatkins@jhmi.edu (D.N. Watkins)

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threonine kinase Fused (Fu), and a novel protein Suppressor of Fused (SuFu) [18]. Hh signalling promotes association of this complex with the C terminus of Smo, which in turn regulates the activity of the latent zinc finger transcription factor *Cubitus interruptus* (Ci) through proteolytic processing and nuclear translocation events [17,20–23]. Such processing is enhanced by Ci phosphorylation, which can mediated by protein kinase A, shaggy/GSK3β and casein kinsase 1, all of which represent important negative Hh pathway regulators [14,24–28]. The processed form of Ci acts as a transcriptional repressor of Hh pathway genes. Hh signalling results in the accumulation of unprocessed, full length Ci which acts a transcriptional activator. Following nuclear translocation, the full length Ci induces transcriptional activation of Hh target genes, which include Ptc itself [2,16].

In vertebrate cells, Ci function has diverged into three Gli proteins which vary in their level of processing, and in their transcriptional activity [7,29]. The oncoprotein Gli1, itself a transcriptional target of mammalian Hh signalling, is a strong positive regulator of Hh pathway targets [30–32], and is thought not to be regulated by processing. Gli2 and Gli3 possess both transcriptional activation and repression properties [8,30–37], and Gli3 is significantly processed in response to Hh signalling in vertebrates [37]. A simplified diagram illustrating the mammalian Hh pathway is shown in Fig. 1.

2. Hedgehog signalling in foregut development

The mammalian foregut primordium gives rise to the lungs and proximal gut through interactions between endodermal cells and the splanchnic mesoderm [38,39]. Studies in *Shh* null mouse embryos have demonstrated a requirement for the Hh pathway in multiple aspects of foregut development [4,39]. In both lung and gut development, the prevailing model is one of epithelial-mesenchymal interactions mediated by endodermally derived Hh ligands [38,40,41]. In the embryonic lung, Shh synthesized in the distal lung buds signals to the adjacent mesenchyme to induce expression of Hh target genes and mesenchymal proliferation [38,39,42,43]. Patterning of the bronchial tree is thought to occur through Hh mediated regulation of fibroblast growth factor signalling from the mesenchyme, and bone morphogenic protein 4 expression in the airway epithelium [42,43].

Epithelial–mesenchymal interactions mediated by Hh signalling in the developing gut are no less complex. Studies in mutant mice have shown that both Shh and Ihh participate in radial axis patterning in the intestine by specifying the ratio of lamina propria and submucosa to smooth muscle and enteric neuronal cell neurons [40,41]. In addition, correct regional epithelial cell identity is dependent on the Hh pathway during embryogenesis of the proximal gut [41]. This is most dramatically illustrated in pancreatic exocrine development, which is dependent on the absence of Shh ligand [44,45]. Transgenic expression of Shh under the control of a pancreas specific promoter results in arrest of pancreatic exocrine development and conversion of pancreatic mesenchyme to an intestinal phenotype [44–47], whereas blockade of Hh signalling results in marked expansion of pancreatic tissue [45,48].

Give the weight of evidence that Hh signalling in foregut derived structures is epithelial–mesenchymal, how might we explain the importance of this pathway in regulating gut and pancreatic epithelial identity? One possible mechanism is that instructive signals by Hh ligands results in mesenchymal derived signals which in turn drive epithelial differentiation. An alternative explanation is reception of Hh signal within the epithelial compartment itself, and evidence is now emerging to support this idea. Analysis of Ptch reporter mice demonstrates Hh pathway activation in lung neuroendocrine progenitors during development, as well as in basally located epithelial cells of the adult conducting airway [49]. In the adult gut, similar intraepithelial signalling is prominent within the gastric epithelium [50–52]. More recent studies have demonstrated a role for the Hh pathway within colonocytes as a negative regulator of Wingless pathway in crypt cell growth and differentiation [53].

3. Hedgehog signalling and progenitor cells in cancer and development

Regulation of organ size and cell proliferation by Hh signalling has also been proposed to explain some aspects of limb [5,37,54] and forebrain development [55]. This concept is illustrated in cerebellar development, which is one of the best understood model of Hh regulated cell growth and proliferation [56–58]. Here, a Shh gradient established by Purkinje cells regulates expansion and proliferation of granule cell precursors [57,58]. More recent studies have illustrated the capacity of Hh signalling to regulate stem cell fates [7,59]. Inactivating mutations in Ptch result in aberrant Hh pathway activation, and are associated with medulloblastoma, a pediatric brain malignancy arising in the cerebellum [56,57,60,61]. These studies suggest that cancer may represent a developmental abnormality resulting in aberrant organogenesis, in which progenitor/stem cells escape dependence on niche signals through mutation in genes such as *Ptch*, or through persistent activation of progenitor cell pathways [7,59].

4. Pharmacologic inhibition of the hedgehog pathway

Pregnant sheep that consume the plant *Veratrum californicum* give birth to lambs with holoprosencephaly, a

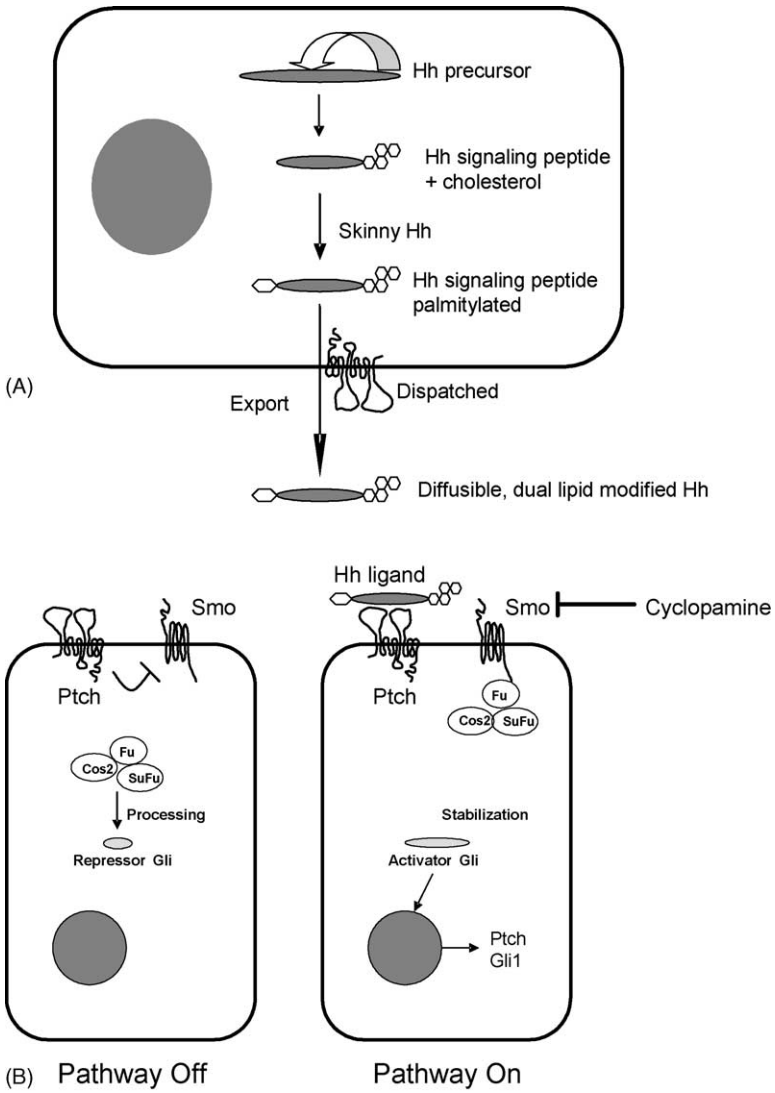

Fig. 1. Simplified model of hedgehog (Hh) signalling, combining data from fly and vertebrate studies. (A) Synthesis of Hh signalling peptide in the sending cell. Full length Hh precursor peptide autocatalyses its own cleavage to a 19 kDa signalling peptide with cholesterol modification at the new C terminus. Processing is completed by the enzyme Skinny Hedgehog, which adds a palmitate to the N terminus. Export of the Hh signalling peptide requires the transporter-like molecule Dispatched. (B) Reception of Hh signalling. In the absence of ligand the receptor Patched (Ptch) constitutively inhibits Smoothened (Smo). This promotes processing of the Gli transcription factors by a multi-protein, microtubule-associated complex consisting of the kinase Fused (Fu), the novel protein Suppressor of Fused (SuFu) and Costal2 (Cos). Processing favors repressor forms of Gli proteins, which silence the Hh transcriptional program. Binding of Hh ligand inhibits Ptch. This in turn de-represses Smo, releasing unprocessed Gli proteins which can promote transcription of Hh targets in the nucleus, including Gli1 and Ptch. Similar pathway activation is achieved in the absence of Ptch function through mutation or epigenetic silencing. Cyclopamine inhibits Hh signalling by direct binding to Smo.

malformation syndrome which includes cyclopia as one of its most striking manifestations [62]. *Veratrum* teratogenesis dramatically resembles the Shh knockout mouse [4,63], and the *Veratrum* alkaloid cyclopamine can reproduce this phenotype in developing chick embryos [64]. Cyclopamine is a naturally occurring alkaloid which specifically inhibits the Hh pathway when activated by Shh ligand, or by mutations in *Ptch* [7,65]. Cyclopamine, and a series of novel compounds do this by directly binding to the heptahelical bundle of the Smo molecule [66,67].

This remarkable series of studies demonstrates the capacity of mechanism-based drug discovery and targeted drug design to specifically disable Hh signalling with compounds of increasing potency [66,67]. Moreover, the hypothesis that aberrant Hh signalling is an important feature of some cancers could be tested directly using such pharmacologic strategies [65]. The first demonstration of an ongoing requirement for the Hh pathway in cancer was in medulloblastoma [68]. In this setting, ligand-independent Hh pathway activation mediated by genetic and epigenetic inactivation of Ptch function was required for growth of these tumors in vitro and in vivo [68]. Hedgehog pathway blockade also resulted in a change in the transcriptional profile of these tumors from expression of classical primitive neuroectodermal tumor genes such as *nestin* and *Math1*, to expression of more mature

neuronal markers such as *NeuroD* [68]. These data suggest that in cancer, specification of progenitor cell fates by Hh signalling occupies a critical, ''master-regulator'' role in controlling the malignant behavior.

5. Hedgehog signalling drives foregut derived malignancy

The potential therapeutic and biologic impact of aberrant Hh signalling in cancer was broadened substantially by three publications in the last 12 months demonstrating that ligand-dependent pathway activation was required for a variety of highly aggressive malignancies arising from foregut derived endodermal epithelium [49,50,69]. These studies show that in cancer of the lung, esophagus, stomach and pancreas, aberrant Hh signalling is mediated by expression of either Shh or Ihh, accompanied by reception of the same signal within the same tumor cells. Moreover, Hh pathway blockers inhibited growth of these tumors when pathway activation was demonstrated by expression of Hh target genes, as well as by activation of Hh pathway-specific reporters [49,50].

Is ligand-dependent Hh pathway activation an aberrant feature of these cancers, or does this phenomenon reflect persistent activation of homeostatic Hh signalling that normally regulates the progenitor cell niche of such adult epithelia? One important clue may come from studies in regeneration of adult airway epithelium following acute Clara cell injury in mice [49]. During this repair process, transient activation of the Hh pathway was observed within the normally quiescent bronchial epithelium, reflected in the co-expression of Shh and Gli1 within the regenerating epithelial pool [49]. This mode of Hh pathway activation persists in small cell lung cancer (SCLC), a lethal airway malignancy strongly associated with tobacco exposure. This tumor characteristically exhibits a neuroendocrine phenotype, possibly reflecting a primitive airway epithelial precursor [70] which normally uses the Hh pathway to regulate the size of the airway epithelial progenitor pool.

Such a process may well also occur during adult gut epithelial turnover [50–52]. Moreover, although Hh signalling is not seen in adult or embryonic esophagus and pancreas, adenocarcinomas arising from these organs demonstrate dramatic ligand-dependent pathway activation [50,69]. Pancreatic cancer in particular demonstrates levels of Ptch mRNA expression several thousand fold that of normal adult pancreas [50]. Perhaps this pathway activation is indicative of a primitive endodermal phenotype rather than a vestige of organ specific pathways normally only active during development? This concept is supported by the findings of Thayer et al. [69], where transgenic expression of Shh within pancreatic epithelium induces a dramatic phenotypic shift towards structures more consistent with intestinal epithelium and mesencyhme. As these animals age, this intestine-like phenotype progresses to lesions which resemble pancreatic intraepithelial neoplasia (PanIN), a probable precursor lesion in human pancreatic cancer [69].

6. Conclusions

An emerging view of cancer is that it represents either a defect in epithelial stem cell behavior, or an abnormal recreation of an organ specific stem cell microenvironment [7,59,70]. Proper regulation of epithelial stem cells during homeostasis in the adult may require a cellular niche in which appropriate signals are directed to a stem cell within the context of the correct epithelial and mesenchymal feedback [7,59]. Perhaps escape from such niche-dependent signalling is a feature of the cancer, since abnormal activation of embryonic patterning pathways such as Notch, Hedgehog and Wingless have been described in many types of malignancies [7,59]. The availability of specific Hh pathway inhibitors has allowed us to directly address this question in a substantial number of highly aggressive foregut derived malignancies for which there are few therapeutic options. This requirement for Hh signalling in foregut derived cancers may represent an ''Achilles heel'' which can be exploited for therapeutic benefit.

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