Molecular Pathways: The Role of Primary Cilia in Cancer Progression and Therapeutics with a Focus on Hedgehog Signaling

Nadia B. Hassounah¹, Thomas A. Bunch², and Kimberly M. McDermott¹,²,³

Abstract

Abnormal Hedgehog (Hh) pathway activity has been reported in many cancers, including basal cell carcinomas, medulloblastomas, rhabdomyosarcomas, glioblastomas, and breast and prostate cancers. For this reason, the Hh pathway is a flourishing area for development of anticancer drugs such as Hh ligand antagonists (e.g., 5E1 and robotnikinin), Smo inhibitors (e.g., GDC-0449 and IPI-926), and Gli transcriptional activity inhibitors (e.g., GANT58 and GANT61). It is now clear that primary cilia are required for activation of the Hh pathway in normal vertebrate cells. It is in the primary cilium that both positive and negative effectors of the Hh pathway are processed by posttranslational modifications. In many cancers, preliminary results suggest that primary cilia are lost. As drugs that inhibit different steps of the Hh pathway are developed, it will be important to consider how these drugs will function in the context of primary cilia in the tumor environment. Here, we discuss why some of the Hh inhibitors may be ineffective if primary cilia are lost on cancer cells. Understanding the relationships between clinical inhibitors of the Hh pathway and the presence or absence of primary cilia may turn out to be critical for targeting these therapeutics to the correct population of patients and improving their efficacy. Further work is needed in this area to maximize the potential of these exciting therapeutic targets.

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Background

Primary cilia: form and function

The primary cilium is a microtubule-based organelle that protrudes from the plasma membrane and acts much like an antenna to sense extracellular signals. Recent studies have taken this once forgotten organelle from obscurity to the forefront of cutting-edge research, showing its importance in developmental biology and human diseases such as cancer. Here we discuss the importance of understanding the role of cilia in cancers when choosing targeted cancer therapeutics, specifically Hedgehog (Hh) pathway inhibitors.

Cilia can be divided into 2 categories: primary and motile. Epithelial cells that are the cancer-initiating cells generally have primary cilia rather than motile cilia; therefore, we will focus this discussion on primary cilia. Cells that have primary cilia have only a single cilium. Primary cilia are usually immotile but can sense physical and chemical signals. At the base of the primary cilium is the basal body (also known as the mother centriole), which is anchored in the plasma membrane. The basal body acts to nucleate the microtubule bundles that extend up the cilium (Fig. 1). Hundreds of proteins that make up the primary cilium have been identified (1–9). Many of these proteins are involved in ciliogenesis, the formation of a new cilium. Other proteins localized to the cilium are involved in the sensory or signaling functions of the primary cilium. Cilia act like antennae by sensing extracellular signals such as developmental morphogens; for example, the Hh ligand receptor localizes to the cilium. At the core of both ciliogenesis and ciliary sensory function is a highly regulated and active process known as intraflagellar transport (IFT), as described by Rosenbaum and Whitman (10) and by Scholey (11). The Kinesin-2 motor complex transports the IFT complex as well as other protein cargo in an anterograde direction (toward the plus end of the microtubules) to the tip of the cilium (Fig. 1). The cytoplasmic Dynein 2 motor complex transports the IFT complex as well as other protein cargo in a retrograde direction (toward the minus end of the microtubules) toward the cell body (Fig. 1). The IFT complex is made up of several proteins, and mutations in IFT genes cause loss of ciliary assembly and consequently result in loss of sensory functions (12). Many mutations in genes required for ciliogenesis have been identified and are now known to be causal for a large number of genetic disorders that are classified as ciliopathies, including Joubert syndrome, polycystic kidney disease, Bardet-Biedl syndrome, and nephronophthisis (13). Loss of cilia or ciliary function in these ciliopathies results in the deregulation of developmental signaling pathways.
Hh signaling and primary cilia

Although cilia have been implicated in numerous signaling pathways that are important for development and disease, such as the Hh, Wnt, and platelet-derived growth factor (PDGF) pathways (14–19), the mechanism by which cilia regulate the Hh signaling pathway is the best characterized. Therefore, Hh signaling is the focus here. Due to space limitations, we only provide an overview of cilia and Hh signaling; for a recent and thorough review, please refer to Goetz and Anderson (20). In vertebrate cells, Hh signaling requires primary cilia. Hedgehogs are a family of secreted proteins that include Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh). These Hh ligands activate the downstream Gli family of transcription factors that translocate into the nucleus to activate Hh target genes. The cilium itself is a subcellular compartment in which key Hh pathway components, including Gli proteins, are brought together differentially depending on the absence or presence of Hh. Proteins are actively transported into and out of the cilium. At the base of the cilium, the transition zone contains transition fibers (Fig. 1) and other protein complexes, such as the septin ring complex, that are thought to restrict passive diffusion (21, 22). A likely mechanistic function of the cilium is to regulate the Hh pathway by increasing the local concentration and bringing pathway components together for key protein–protein interactions required for Hh pathway regulation.

Details about the dynamic regulation of the entry and exit of Hh pathway proteins into and out of the cilium are just now being unraveled. Three Gli proteins (Gli1, Gli2, and Gli3) are encoded by distinct genes. These Gli proteins are post-translationally processed (with the exception of Gli1) into repressor and activator forms in the absence and presence of Hh, respectively. Loss of cilia mutations results in abnormal
Primary cilia, Hh signaling, and cancer

The Hh pathway is an important regulator of cell growth and differentiation during development. Abnormal activation of the Hh pathway is critical for the development of many cancers, including glioblastoma, basal cell carcinoma, medulloblastoma, and breast, prostate, melanoma, lung, and pancreatic cancers. In human cancers, the Hh pathway is upregulated either through mutations of pathway proteins, such as Ptc1, Smo, and SuFu, or through overexpression of Hh. Experiments in mouse models of cancer showed that cilia can play a dual role in promoting and preventing tumorigenesis through regulation of the Hh pathway. This paradox is due to the primary cilium’s role in turning the Hh pathway “on” in the presence of Hh (by processing Gli proteins into the activator form) and keeping the Hh pathway “off” in the absence of Hh (by processing Gli proteins into the repressor form).

In these mouse models of basal cell carcinoma and medulloblastoma, an active form of Smo was ectopically expressed in wild-type mice and mice genetically modified to have mutant primary cilia. As expected, the mice with active Smo and wild-type cilia developed basal cell carcinomas and medulloblastomas, and those with active Smo but mutant cilia did not develop tumors. As described above, the mammalian Hh pathway requires cilia for Smoothened-dependent activation of Gli proteins (Fig. 2A). Therefore, without cilia, the active Smo could not activate the Hh pathway and tumors were unable to form (Fig. 2C). When the Hh pathway was activated downstream of cilia, by ectopically expressing an activator form of one of the Gli proteins in the absence of cilia, the absence of cilia resulted in a significant increase in tumorigenesis. Follow-up studies showed that the presence of cilia allowed for the formation of the repressor form of Gli protein (Fig. 2B). The cilia-generated repressor form of Gli protein is predicted to balance out the exogenously expressed activator form of Gli protein to slow tumor growth. In the absence of cilia, the repressor form of the Gli transcription factor was reduced and unable to counteract the activator Gli protein, resulting in increased tumorigenesis (Fig. 2D).

Clinical–Translational Advances

Primary cilia expression in human cancers

Given that cilia are important for regulating the Hh pathway in normal cells and can play a dual role during tumorigenesis, it is critical to learn more about which human cancers make functional primary cilia and which cancers have ciliary dysfunction. Moreover, depending on which step of the Hh pathway is functioning to sustain tumorigenesis, the presence or absence of cilia may have a significant impact on the effectiveness of targeting different steps of the Hh pathway (upstream or downstream of cilia).

Only a limited number of studies have examined the expression of primary cilia on human cancer cells from primary patient samples. A reduction in cilia frequency relative to adjacent normal tissue was observed in clear cell renal cell carcinoma, breast cancers, melanoma, basal cell carcinoma, medulloblastoma, and pancreatic cancer. Pancreatic intraepithelial neoplastic lesions were also devoid of cilia, suggesting that loss of cilia may occur early in tumorigenesis. In a study of medulloblastoma, the presence of cilia correlated with desmoplastic medulloblastoma (better prognosis), and the absence of cilia was associated with anaplastic medulloblastoma (poor prognosis).

Cellular proliferation rates can influence the presence of cilia. Therefore, it was an important finding in the studies of melanoma, renal cell carcinoma, and pancreatic cancer that primary cilia loss was independent of Ki67 staining (a cell proliferation marker). This suggests that loss of cilia is not a result of altered cellular proliferation rates and that primary cilia dysfunction in cancers may be due to another mechanism, such as loss of a gene required for ciliogenesis, potentially resulting from mutagenesis or genomic instability.

These studies suggest that cilia dysfunction is a common event in cancers and that it may occur early during the tumorigenic process. However, further work on these and additional types of cancer is needed. The reported sample sizes are small (ranging from 8 to 38 patient samples), and there is a general lack of quantitation and statistical analysis. Comparisons with normal tissue are also needed. Normal tissue adjacent to cancer has been used and may provide interesting findings; however, field effects in tissue surrounding cancer have been documented. Also, cilia lengths have not been examined in cancers or normal tissue for comparison. Cilia may be present in cancers, but if they have abnormal lengths, this may affect signaling.
Figure 2. Role of cilia in Hh pathway activation in cancer cells. A, cancer-associated overexpression of Hh ligands or mutations in genes such as Ptch1 or Smo, which lie upstream of cilia, will only result in activation of the Hh pathway by increasing Gli1\(^\text{a}\) levels if cilia are present. If cilia are present, then inhibitors targeting Hh ligand, Smo, and Gli trafficking (red boxes) will be effective. Inhibitors that target Gli activity downstream of cilia (white box) will also be effective at reducing the Hh pathway in this context. B, cancer-associated overexpression of Gli1 (Gli1\(^\text{a}\)) in the presence of cilia will result in low levels of Hh pathway activation. In this context, cilia make the repressor form of Gli (Gli1\(^\text{r}\)), counterbalancing Gli1\(^\text{a}\) to reduce overactivation of the Hh pathway. Mutations in REN(KCTD11) can also result in increased Gli1\(^\text{a}\) activity. Because this activation is downstream of cilia, only the downstream Gli targeting inhibitors (gold box) are predicted to be effective. C, cancer-associated overexpression of Hh ligands or mutations in genes such as Ptch or Smo, which lie upstream of cilia, will not activate the Hh pathway in the absence of cilia. D, cancer-associated mutations downstream of cilia, such as overexpression of Gli1 (Gli1\(^\text{a}\)) and mutations in Ren(KCTD11), have been found in cancers and will turn on the Hh pathway in the absence of cilia due to high Gli1\(^\text{a}\) and low Gli1\(^\text{r}\). Therefore, only downstream Gli targeting inhibitors (gold box) are predicted to be effective in this scenario.
surprisingly, there is a range in the percentage of patient samples with cilia and a range in the percentage of cells with cilia in independent tumors. It is important to explore these ranges further by asking whether specific levels of cilia frequency correlate with specific cancer subtypes and with clinical data such as survival, recurrence, and response to treatment. It is also critical to correlate cilia frequency with markers of relevant pathways, such as Hh target genes, to begin to understand whether there is a causal association between cilia and human cancers.

**Primary cilia and clinical inhibitors of the Hh pathway**

Hh-targeted drugs are expected to be effective as anticancer drugs by killing cancer cells as well as by targeting stromal cells associated with tumorigenesis (e.g., inhibiting angiogenesis). In this section, our focus is on the role cilia play in the efficacy of Hh-targeted drugs, specifically with regard to cancer cells; however, the same concepts are likely to apply to inhibition of Hh pathway signaling in stromal cells. Although all of the Hh-targeted drugs mentioned have shown preclinical efficacy in cell lines and, in some cases, mouse models, efficacy in clinical trials is mixed, ranging from full or partial response to no efficacy [for a detailed review, please see Ng and colleagues (36)]. We predict that the efficacy of Hh-targeted anticancer drugs will rely on whether activation of the Hh pathway is upstream (dependent) or downstream (independent) of cilia, as well as whether the cancer cells are positive or negative for primary cilia. Hh pathway activation in cancer can be divided into 2 groups: Hh ligand driven and mutation driven. As we describe below, Hh ligand–driven cancer can only be cilia dependent, whereas mutation-driven cancers can be cilia independent or independent.

**Ligand-driven Hh pathway activation (cilia dependent).**

Overexpression of Hh ligands has been shown in many cancers (37, 38). Hh-induced signaling in cancer can occur through autocrine (cancer-cancer) and paracrine (stroma-cancer) expression of Hh (39). Hh pathway activation from elevated ligand is upstream of cilia (cilia dependent). As described above, cilia are required to respond to ligand and activate the Hh pathway via Smo and Gli proteins (Fig. 2A). Therefore, therapeutically treating patients who have Hh pathway upregulation driven by elevated Hh ligand by administering a ligand antagonist (e.g., 5E1 and robotnikinin), Smo antagonist (e.g., GDC-0449 and IPI-926), or Gli-processing inhibitor (e.g., HPI-2 and HPI-3) would be predicted to be effective only if the tumor cells have cilia (Fig. 2A). For example, HPI-2 and HPI-3 have both been shown to reduce Hh pathway signaling in response to Hh ligand in vitro in studies using cell lines. Preclinical reports showing reduced Hh pathway signaling for HPI-2,3 were all tested on NIH-3T3 cells that are known to be ciliated (40). Clinical trial data are not currently available for HPI-2,3.

**Mutation-driven Hh pathway activation (cilia independent).**

Overexpression of Hh ligand does not mean that the cancer cells rely on ligand for Hh pathway activation. As cancers evolve through multiple stages of activation, the elevated Hh ligand levels observed may have been important for stimulating tumor growth at an earlier stage when cilia were present. If the tumor cells no longer have cilia, the high Hh ligand levels are no longer relevant to activation in the tumor cells. Instead, continued activation of the Hh pathway would require a secondary mutation downstream of cilia (cilia independent), which would relieve reliance on the Hh ligand (Fig. 2D). Emerging data suggest that many cancers have lost cilia. Therefore, in cancer cells without cilia, any observed Hh pathway activation must be driven by mutations that are downstream of cilia. Mutations in the pathway that are downstream of cilia allow the Hh pathway to be turned on even in the absence of cilia. There are several examples of Hh pathway mutations that have been shown to be downstream of cilia and would therefore allow the Hh pathway to be turned on even in the absence of cilia. SuFu is a protein that is known to sequester the Gli proteins in the cytoplasm and thereby exert a negative effect on Hh pathway activation (41). Mutations in SuFu have been found in medulloblastomas (36, 37), and loss of SuFu protein has been observed in prostate cancers. Additionally, loss-of-heterozygosity mutations in SuFu have been reported in rhabdomyosarcoma (36, 37, 42, 43). Medulloblastomas also have been shown to have loss of REN(KCTD11), a protein that antagonizes Gli-mediated transactivation of the Hh target genes, ultimately resulting in activation of the Hh pathway downstream of cilia (Fig. 2D; ref. 44). Glioblastomas have been found to have amplification of Gli1 (36). Mutations in Gli1 and Gli3 have been found in pancreatic adenocarcinoma (39). Murine studies suggest that activation of a downstream component of the Hh pathway will activate the Hh pathway, leading to enhanced tumorigenesis when cilia are absent (Fig. 2D; refs. 29, 30, 45). When cilia are present, the repressor form of Gli protein can still be made, which can counteract downstream activation of the pathway (Fig. 2B). Therefore, we predict that if the cancer cells lack cilia, it will be necessary to target inhibition of the Hh pathway downstream of cilia with a drug such as GANT58 or GANT61 (Fig. 1F). Emerging data indicate that many patients have a low frequency of ciliated tumor cells. This tumor heterogeneity suggests that combinatorial treatment with Hh-targeted drugs that are cilia dependent and independent may be an effective treatment for some patients.

GANT61 inhibits Hh signaling downstream of cilia by inhibiting Gli transcriptional activity and has been shown to be effective at inhibiting the Hh signaling pathway in cells containing or lacking cilia. For example, GANT61 effectively inhibited Hh signaling in HEK293 and NIH-3T3 cells, which are both known to have cilia (46). In a prostate xenograft model with the 22Rv1 prostate cancer cell line that was shown to lack cilia, GANT61 treatment resulted in tumor regression (46). These preclinical data support our hypothesis that GANT61 is a cilia-independent inhibitor.

Mutation-driven Hh pathway activation (cilia dependent). Another mechanism by which the Hh pathway can be activated upstream of cilia involves mutations in Hh pathway proteins that require cilia for their regulation. Deactivation of Ptc1 (negative regulator) and constitutive
activation of Smo (positive regulator) are upstream of cilia (cilia dependent; Fig. 2A). Although these mutations require the presence of cilia for activation, the cancer cells no longer rely on the Hh ligand for pathway activation. Inactivation or loss-of-heterozygosity mutations in the Pch1 receptor or activating Smo mutations are common in basal cell carcinoma, with 90% of basal cell carcinomas having loss of function of Pch1 and 10% having activation of Smo (36, 37). Pch1 receptor mutations and loss of heterozygosity are also seen in medulloblastomas and rhabdomyosarcomas (36, 37).

As described above, murine studies have shown that activation of an Hh pathway component upstream of cilia, such as mutations in the Smo protein, requires primary cilia for Hh pathway activation (29, 30). If a patient is found to have a mutation in the Hh pathway upstream of cilia (e.g., Pch1 or Smo) and the tumor has cilia, then inhibiting the Hh pathway with a Smo inhibitor such as GDC-0449 is predicted to be effective (Fig. 2A). If the tumor carries Pch1 or Smo mutations but does not have primary cilia, we predict that the Hh pathway activation is no longer dependent on the Pch1 or Smo mutations but may now have Hh pathway activation due to a Hh pathway mutation at a later cilia-independent step (Fig. 2D). The Pch1 or Smo mutations may have been important in an earlier stage in cancer progression, similar to the elevated Hh ligand levels discussed in the previous paragraph. If the cancer cells do not have cilia, the tumor would not be responsive to Smo- or Gli-processing inhibitors and instead would need to be treated with downstream inhibitors, such as the Gli antagonists GANT58 or GANT61 (Fig. 1F). On the basis of this rationale, we predict that only ciliated cancer cells could be responsive to GDC-0449. In a phase I clinical trial, 66% of patients with basal cell carcinoma containing Pch1 or Smo mutations responded to the GDC-0449 Smo inhibitor (47).

We hypothesize that these patients had cancer cells that expressed cilia. The nonresponsive tumors may have lacked ciliated cancer cells. This is consistent with the finding in one study that ~63% of tested samples from patients with primary human basal cell carcinoma had cilia (29). Further studies are needed to determine whether this correlation will hold true and whether the presence of cilia on basal cell carcinoma is predictive of responsiveness to GDC-0449 treatment. It is also possible that many of the nonresponsive patients are no longer dependent on the Hh pathway for survival.

Future Directions and Conclusions

Further work is needed to determine whether the predicted relationships between the presence of cilia and responsiveness to specific Hh pathway inhibitors is clinically relevant. If they are, then the presence or absence of cilia will provide another tool for clinicians to use in choosing Hh-targeted drugs to treat individual cancers. On a more general note, if the trend continues that cancer cells lose cilia, then Hh inhibitors that are upstream of cilia may prove generally ineffective, and we will need to focus on developing additional inhibitors that are downstream of cilia (cilia and ligand independent). Such therapeutics are currently underrepresented among Hh clinical inhibitors and would be predicted to target a much broader range of tumor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: N.B. Hassounah, K.M. McDermott

Writing, review, and/or revision of the manuscript: N.B. Hassounah, T.A. Bunch, K.M. McDermott

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