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

Running Title
Primary Cilia in Cancer: Impact on Hedgehog–Targeted Therapy

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ABSTRACT
Abnormal Hedgehog (Hh) pathway activity has been reported in many cancers including basal cell carcinomas, medulloblastomas, rhabdomyosarcomas, glioblastomas, breast and prostate cancers. For this reason the Hh pathway is a flourishing area for development of anti-cancer drugs such as Hh ligand antagonists (e.g. 5E1, robotnikinin), Smo inhibitors (e.g. GDC-0449, IPI-926) and Gli transcriptional activity inhibitors (e.g. GANT58, GANT61). In vertebrate cells it is now clear that primary cilia are required for activation of the Hh pathway in normal cells. It is in the primary cilium that both positive and negative effectors of the Hh pathway are processed by post-translational modifications. In many cancers, preliminary results suggest that primary cilia are lost. As drugs are developed that inhibit different steps of the Hh pathway, it is important to consider how these drugs will function in the context of primary cilia in the tumor environment. We will 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 for improving their efficacy. Future work is needed in this area to maximize the potential of these exciting therapeutic targets.

BACKGROUND
Primary Cilia: Form and Function. The primary cillum 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, demonstrating its importance in developmental biology and human diseases including cancer. Here we discuss the importance of understanding cilia in cancers when choosing targeted cancer therapeutics, specifically Hedgehog (Hh) pathway inhibitors.

There are two categories of cilia, primary and motile cilia. Epithelial cells that are the ‘cancer-initiating cell’ generally have primary cilia rather than motile cilia; therefore, we will focus this discussion on primary cilia. Cells that have primary cilia only have 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 into the plasma membrane. The basal body acts to nucleate the microtubule bundles that extend up the cilium (Fig. 1).

Hundreds of proteins have been identified that make up the primary cilium (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 through sensing extracellular signals including developmental morphogens; for example, the Hh ligand receptor localizes to the cilium. At the core of both ciliogenesis as well as ciliary sensory function is a highly regulated and active process known as intraflagellar transport (IFT) (10, 11). The Kinesin-2 motor complex transports the IFT complex as well as other protein ‘cargo’ for anterograde movement of proteins to the tip of the cilium (towards the plus end of microtubules) (Fig. 1). The cytoplasmic Dynein 2 motor complex transports the IFT complex plus ‘cargo’ for retrograde movement from the tip of the cilium towards the cell body (towards the minus end of microtubules) (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 classified as ciliopathies. Ciliopathies include Joubert syndrome (JBTS), polycystic kidney disease (PKD), Bardet-Biedl syndrome (BBS), and nephronophthisis (NPHP) (13). Loss of cilia or ciliary function in these ciliopathies results in deregulation of developmental signaling pathways.

**Hedgehog Signaling and Primary Cilia.** While cilia have been implicated in numerous signaling pathways important in development and disease, including the Hh, Wnt and PDGF pathways (14-19). The mechanism by which cilia regulate the Hh signaling pathway is the best characterized. Therefore Hh signaling will be 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 et. al. (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. Transport of proteins into and out of the cilium is via
active transport. At the base of the cilium the transition zone contains transition fibers and other protein complexes such as the septin ring complex that are thought to restrict passive diffusion (Fig. 1) (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.

The details of the dynamic regulation of entry and exit of Hh pathway proteins into and out of the cilia are just now being unraveled. There are three Gli proteins (Gli1, Gli2 and Gli3) encoded by distinct genes. These Gli proteins are post-translationally processed into repressor (with the exception of Gli1) and activator forms in the absence and presence of Hh, respectively. Loss of cilia mutations results in abnormal processing of both the repressor and activator forms of Gli proteins suggesting that both are dependent on cilia. In the absence of Hh, the Hh pathway stays in an ‘off’ state via processing of Gli transcription factors to the repressor form which blocks transcriptional activation of Hh genes (Fig. 1). This appears to be cilia-dependent through localization of Patched (Ptch1 or Ptc1), a negative regulator of the pathway, to the ciliary membrane. In contrast, the Hh pathway activator protein Smoothened (Smo) is kept out of the cilium in the absence of Hh (Fig. 1) (14, 23). Another mechanism by which Gli proteins are regulated in a cilia-dependent manner is through the protein Suppressor of Fused (SuFu). SuFu is involved in the formation of both the repressor and activator forms of Gli proteins (24). In the absence of Hh, SuFu sequesters Gli protein to the cytoplasm and keeps it in the repressor form. In the presence of Hh, the Hh pathway is in an ‘on’ state via processing of Gli transcription factors into their activator forms, also in a cilia-dependent manner (Fig. 1). In the presence of Hh, its receptor, Ptch1, moves out of the cilium and Smo is phosphorylated and translocated into the cilium where it functions to promote Gli activation (Fig. 1) (14, 25, 26). Additionally, SuFu, in the presence of Hh, accumulates in the cilium but dissociates from Gli, promoting Gli’s conversion to the activator form and allowing it to enter the nucleus to activate Hh target genes (24, 27).

**Primary Cilia, Hedgehog 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 in the development of many cancers including glioblastoma, basal cell carcinoma, medulloblastoma, as well as, breast, prostate, melanoma, lung and pancreatic cancers (28). In human cancers, the Hh pathway is upregulated either through mutations of pathway proteins such as Ptch1, Smo and SuFu or through overexpression of Hh. Experiments in mouse models of cancer demonstrate that cilia can play a dual role in promoting and preventing tumorigenesis through regulation of the Hh pathway (29, 30). 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 (29, 30). 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 Smo-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 show that presence of cilia allows for the formation of the repressor form of Gli protein (Fig. 2B). 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 is 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 in regulating the Hh pathway in normal cells and that they can play a dual role during tumorigenesis, it is critical that we 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 papers 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 has been observed in clear cell renal cell carcinoma (31), breast cancers (32), melanoma (27), basal cell carcinoma (22), medulloblastoma (23) and pancreatic cancer (33). Pancreatic intraepithelial neoplastic lesions were also devoid of cilia, suggesting that loss of cilia may occur early in tumorigenesis (25). In the medulloblastoma study (23), 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 renal, pancreatic and melanoma studies 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 (31, 33, 34).

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 cancer types are needed. Reported sample sizes are small (ranging from 8 to 38 patient samples) and there is a general lack of quantitation and statistical analysis.
Comparison to normal tissue is 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 (35). 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 (20). Not 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 further explore these ranges by asking if 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 that cilia frequency be correlated with markers of relevant pathways such as Hh target genes to begin to understand if there is a causal association between cilia and human cancers.

Primary Cilia and Clinical Inhibitors of the Hedgehog Pathway. Hh-targeted drugs are expected to be effective as anti-cancer drugs through killing cancer cells as well as through targeting stromal cells associated with tumorigenesis (e.g. inhibition of angiogenesis). In this section, our focus is on the role cilia play in the efficacy of Hh-targeted drugs specifically on cancer cells; however, the same concepts are likely to apply to inhibition of Hh pathway signaling in stromal cells. While all of the Hh-targeted drugs mentioned show pre-clinical 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 et. al. (36)). We predict that the efficacy of Hh-targeted anti-cancer drugs will rely on whether activation of the Hh pathway is upstream (cilia-dependent) or downstream (cilia-independent) of cilia, as well as if the cancer cells are positive or negative for primary cilia. Hh pathway activation in cancer can be divided into two groups: 1. Hh ligand-driven and 2. mutation-driven. As we will describe below, Hh ligand-driven cancer can only be cilia-dependent, while mutation-driven cancers can be cilia-dependent or cilia-independent.

Ligand-Driven Hh pathway Activation (Cilia-Dependent): Overexpression of Hh ligands have been documented 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, 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 through administering a ligand antagonist (e.g. 5E1 or robotnikinin), Smo antagonist (e.g. GDC-0449, IPI-926), or Gli-processing inhibitor (e.g. HPI-2,3) would be predicted to be effective only if the tumor cells have cilia (Fig. 2A). For example, HPI-2,3 have both been shown to reduce Hh pathway signaling in response to Hh ligand in vitro using cell lines. Pre-clinical 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 is 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 in stimulating tumor growth at an earlier stage when cilia were present. If the tumor cells no longer have cilia then 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 Hh ligand (Fig. 2D). Emerging data suggests 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 documented that are downstream of cilia and would therefore allow the Hh pathway to be turned on even in the absence of cilia. SuFu is a protein known to sequester the Gli proteins in the cytoplasm and thereby exerts a negative effect on Hh pathway activation (41). Mutations in SuFu have been documented 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) (44). Glioblastomas have been found to have amplification of Gli1 (36). Mutations in Gli1 and Gli3 have been documented 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 (29, 30, 45) (Fig. 2D). 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 indicates 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 cilia-independent may be an effective treatment for some patients.

GANT61 inhibits Hh signaling downstream of cilia by inhibiting Gli transcriptional activity and has indeed been demonstrated 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 using the 22RV1 prostate cancer cell line that was shown to lack cilia, GANT61 treatment resulted in tumor regression (46). This pre-clinical data supports 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 Ptch1 (negative regulator) and constitutive activation of Smo (positive regulator) are upstream of cilia
(cilia-dependent) (Fig. 2A). While these mutations require the presence of cilia for activation, the cancer cells no longer rely on Hh ligand for pathway activation. Inactivation or loss of heterozygosity mutations in the Ptch1 receptor or activating Smo mutations are common in basal cell carcinoma with 90% of basal cell carcinomas having loss of function of Ptch1 and 10% having activation of Smo (36, 37). Ptch1 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 a 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. Ptch1 or Smo), and the tumor has cilia then inhibiting the Hh pathway with Smo inhibitors such as GDC-0449 is predicted to be effective (Fig. 2A). If the tumor carries Ptch1 or Smo mutations, but does not have primary cilia, we predict that the Hh pathway activation is no longer dependent on the Ptch1 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 Ptch1 or Smo mutations may have been important in an earlier stage in cancer progression similar to 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). Based on this rationale, we predict that only ciliated cancer cells could be responsive to GDC-0449. Of patients with basal cell carcinoma containing Ptch1 or Smo mutations, 66% responded to the GDC-0449 Smo inhibitor in a phase I clinical trial (47). We hypothesize that the 66% that were responsive to GDC-0449 have cancer cells that express cilia. The non-responsive tumors may lack ciliated cancer cells. This is consistent with the finding that ~63% of the primary human basal cell carcinoma patient samples that were tested had cilia (29). Further studies are needed to determine if this correlation holds true and if presence of cilia on basal cell carcinoma is predictive of responsiveness to GDC-0449 treatment. It is also possible that many of the non-responsive patients are no longer dependent on the Hh pathway for survival.

**FUTURE DIRECTIONS AND SUMMARY**

Further work is needed to determine if the predicted relationships between the presence of cilia and responsiveness to specific Hh pathway inhibitors are clinically relevant. If they are, then the presence or absence of cilia is another tool available to 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 the focus will need to be on developing additional inhibitors that are downstream of cilia (cilia- and ligand-independent). These therapeutics are currently underrepresented amongst Hh clinical inhibitors and would be predicted to target a much broader range of tumor cells.
FIGURE LEGEND

Figure 1. Regulation of the Hedgehog Pathway by Primary Cilia in Normal Cells.
Structure of Primary Cilium: The primary cilium contains microtubule bundles (9 doublets arrayed as a cylindrical structure) that are nucleated from the basal body. The microtubule bundles are enclosed in a ciliary membrane that is continuous, but distinct, from the plasma membrane. At the base of the cilium are transition fibers localized in the transition zone. This transition zone is known to restrict passive diffusion of proteins in and out of the cilium. Kinesin 2 moves the IFT complex and its ‘cargo’ (e.g. Gli, Ptch and Smo) towards the plus-end of microtubules (ciliary tip). Dynein 2 moves the IFT complex and its ‘cargo’ towards the minus-end of microtubules (cell body).
Hh Regulation: In the absence of Hh (left side) Gli protein is converted to its repressor form (GliR). Also in the absence of Hh, Ptch1 is localized to the ciliary membrane and Smo is kept out of the cilium. In the presence of Hh (right side) Gli protein levels increase in the cilium and Gli is processed into the activator form (GliA) for transport out of the cilium and into the nucleus where it activates Hh target genes. In the presence of Hh, Ptch1 moves out of the cilium and Smo moves into the cilium where it promotes formation of the activator form of Gli (GliA).

Figure 2. The Role of Cilia in Hedgehog 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 GliA levels if cilia are present. If cilia are present, then inhibitors targeting Hh ligand, Smo, and Gli trafficking (grey boxes) will be effective. Inhibitors that target Gli activity downstream of cilia (white box) will also be effective in reducing the Hh pathway in this context. B. Cancer-associated overexpression of Gli1 (GliA) in the presence of cilia will result in low levels of Hh pathway activation. In this context, cilia make the repressor form of Gli (GliR) counterbalancing GliA to reduce over activation of the Hh pathway. Mutations in REN(KCTD11) can also result in increased GliA activity. As this activation is downstream of cilia only the downstream Gli targeting inhibitors (white box) are predicted to be effective. C. Cancer-associated overexpression of Hh ligands or mutations in genes such as Ptc 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 (GliA) or mutations in Ren(KCTD11) have been documented in cancers and will turn on the Hh pathway in the absence of cilia due to high GliA and low GliR. Therefore, only downstream Gli targeting inhibitors (white box) are predicted to be effective in this scenario.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
No potential conflicts of interest were disclosed.

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REFERENCES


Cancer: Mutation-driven or ligand-dependent (Upstream of cilia)

- **5E1 Robotnikinin**
- Hh
- Overexpression

Cancer: Mutation-driven, ligand-independent (Downstream of cilia)

- GDC-0449
- IPI-926
- SANT1, 2, 3, 4
- CUR-61414
- HhAntag691
- BMS-833923
- PF-04449913
- LDE-225
- TAK-441
- LEQ506

Hh targets

Cancer: Mutation-driven or ligand-dependent (Upstream of cilia)

- GANT58, GANT61
- HPI-1, physalin F, B
- arsenic trioxide

Cancer: Mutation-driven, ligand-independent (Downstream of cilia)

- GANT58, GANT61
- HPI-1, physalin F, B
- arsenic trioxide

Hh targets ON

Hh targets Low

Cancer: Mutation-driven, ligand-independent (Upstream of cilia)

- Overexpression
- Smo
- Basal body
- Hh targets OFF

Cancer: Mutation-driven, ligand-independent (Downstream of cilia)

- Overexpression
- Smo
- Basal body
- Hh targets ON
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