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Overview

Hedgehog signaling and cross-talk therapeutic potential

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INTRODUCTION

Normal and tumor cells use many pathways for survival, proliferation and communication with environment. Many defects during lifetime occur and consequently many other sets of alternative pathways switch on, in a fighting for survival, repair and reproduction. In such dynamic processes depending on a moment, environment, conditions and events, cells learn how to survive. To find effective tools to attack particular sets of those pathways in particular cells is main strategic road to treat and fight with cancer.

Accumulating wide lines of experimental evidence revealed that aberrant activation of Hedgehog–Gli (Hh-Gli) pathway and pathways involving receptor tyrosine kinases (RTK), such as the EGF signaling, frequently occur during cancer initiation and progression, and these tumorigenic cascades may cooperate through multiple signaling cross-talks to the malignant transformation of cells, treatment resistance and disease relapse.

In this context, the most relevant issue for clinical application is: How to attack molecular mechanisms and specific downstream signaling elements that may contribute to the cooperative or synergistic interactions of the Hh-Gli and RTK signaling pathways, including EGFR, in cancer and metastasis-initiating cells?

Moreover, it is of great therapeutic interest to define drug resistance-associated molecules, including ABC transporters modulated through the inhibition of Hh and/or EGFR pathways, that could be targeted for reversing the chemoresistance of cancer and metastasis-initiating cells.

In view of the promising results from preclinical studies, targeting the Hh cascade seems to represent a therapeutic strategy of great clinical potential.

Misregulation of molecular signaling pathways

Misregulation of molecular signaling pathways that control fundamental cellular processes such as growth and cell death has been directly associated with a variety of inherited and sporadic diseases. Targeting such pathways, as is the Hedgehog (Hh) signaling pathway represents a promising new paradigm for drug discovery. Cyclopamine, plant-derived steroidal alkaloid, was the first discovered inhibitor for this pathway, shown to bind to the heptahelical transmembrane part of Smo, inhibiting its activity.

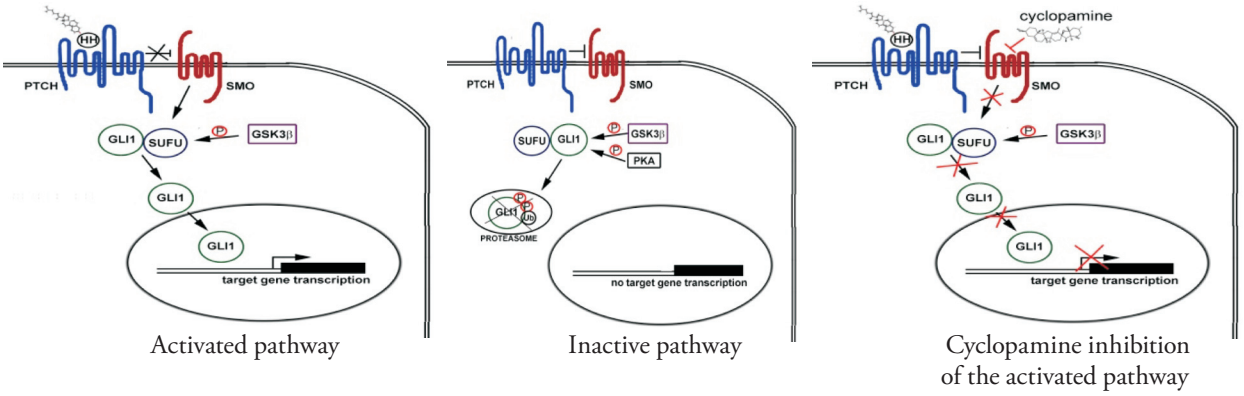

Figure 1. *Schematic presentation of main steps in Hh-Gli signaling pathway. Pathway is activated when the ligand Hh binds to 12-transmembrane receptor Ptch (Activated Pathway), or it is inactive when the ligand is not present (Inactive pathway); and the activated pathway can be blocked by cyclopamine inhibition (Cyclopamine inhibition of the activated pathway). The interactions of the components of the Hh-Gli pathway occur in the primary cilia of cells (10). Functional Hh protein is generated in a two-step process that involves autocatalytic cleavage of a precursor molecule to release a cholesterol-modified N-terminal signaling domain, followed by addition of palmitate to the N terminus. This protein is then secreted from the membranes of the producing cells and initiates the Hh signaling cascade upon binding to the Patched (Ptch) (11). In the absence of the Hh ligand, the Ptch receptor inhibits the activity of the downstream co-receptor Smoothened (Smo), which in general topology resembles G-protein-coupled receptors (GPCRs). It is proposed that Ptch effects this inhibition by excluding the ciliary localization of Smo (12). Binding of Hh protein to Ptch causes Smo, stored in intracellular vesicles, to move to the cilium and activate signal transduction. Active Smo then signals via a cytosolic complex of proteins including Suppressor of Fused (SuFu), and the cascade culminates by triggering activation of the glioma (Gli) family of transcription factors and their translocation to the nucleus. This activation results in the expression of specific genes that promote cell proliferation and differentiation.*

After that, small molecule Hh-Gli regulators have become very rapidly expanding field. They come in varieties depending on their source (synthetic versus natural products), as well as on the locus of action: those that inhibit SMO versus those that act downstream of SMO, including those that may block GLI function directly. Blocking antibodies, peptides and small RNA inhibitors are more recent aspects of new targeting tools.

The Hedgehog-Gli (Hh-Gli) signaling pathway

The Hh-Gli signaling pathway is a developmental pathway, which is often found aberrantly active in various tumors. The Hh pathway is a key regulator of patterning, growth, and cell migration during embryonic development *(1, 2)*, and inhibition of the Hh pathway at this stage has been shown to cause severe birth defects such as cyclopia *(3)*. In adult organisms, the Hh pathway contributes to homeostasis and regeneration of certain tissues such as skin and bone, it is active almost exclusively in somatic stem cells, but aberrant activation of the Hh pathway has been linked to tumorigenesis in various and severe types of cancers *(4)*. The fact that it is frequently activated in cancer stem cells makes it an interesting target for future therapies.

Hedgehog (Hh) signaling pathway was first discovered in Drosophila in early 1980s *(5)*. The pathway's name originates from the observations that mutations in the gene encoding the secreted protein, one of the key regulators of the pathway in fruit flies, give rise to an unusual spiky-haired phenotype. In mammals, the proteins are Sonic hedgehog (Shh), named after the popular video game hero, Indian hedgehog (Ihh) and Desert hedgehog (Dhh), the latter two named after existing species of living hedgehogs. Sonic hedgehog (Shh) is the most widely characterized of the three vertebrate Hedgehog homologs, and is essential for proper embryonic development.

The pathway activation begins when the secreted Shh protein binds to its receptor, Patched (Ptch1), a twelve transmembrane protein, resulting in the de-repression of Smoothened (Smo) a seven transmembrane protein, that has a function of co-receptor. This triggers a cascade of events in the cytoplasm leading to activation of the transcription zinc finger factors Gli and transcription of their target genes. Several components of the Hh-Gli pathway (PTCH, GLI1, GLI2 and HHIP) are Gli transcriptional targets that induce positive or negative feedback *(6)*. The Gli proteins are regulated by the Suppressor of Fused (SuFu), Protein Kinase A (PKA), Glycogen Synthase Kinase 3β (GSK3β) and Casein Kinase 1 (CK1). GLI targets mediate various cellular responses, notably enhanced cell proliferation and survival by upregulating D-type cyclins and antiapoptotic proteins *(7, 8, 9)*.

Many studies have shown that the activity of GLI proteins can be additionally modified by integration of distinct signals, such as the MEK/extracellular signal-regu-

TABLE 1

Small molecule Hh-Gli pathway inhibitors and indicative targets in clinical trials (some of the data from Mas and Ruiz i Altaba 2010-*(16)*).

Compound	Target	Cancer type	Status
GDCO449	SMO	medulloblastoma, glioblastoma, BCC	
		Colorectal cancer, stomach, ovarian, pancreatic	phase II
GDCO449			
+gemcitabine	SMO+DNA Replication	metastatic pancreatic	‘’
BM-833923	SMO	BCC, BCNS, small cell lung	phase I
BM-833923	SMO	small cell lung	‘’
+carboplatin	DNA alkylation	small cell lung	‘’
+etoposide	Topo II	small cell lung	‘’
BM-833923	SMO		
+cisplatine	Topo II	metastatic gastric and esophageal	‘’
BM-833923	SMO	multiple myeloma	

lated kinase (ERK) and phosphinositide-3 kinase (PI3K)/AKT pathway, and they have been described as noncanonical Hh-Gli activators in cancer.

However, signaling events immediately downstream of Smo are still not clearly understood. Accumulating evidence from several groups indicates an important but not yet fully defined step: mammalian Smo is during signaling translocated to primary cilia. This was found in most vertebrate cells *(10)*.

The pathway is a highly coordinated and orchestrated network, linking events from ligand binding on the membrane, toward events in cytoplasm and transcription factors Gli. Therefore, it deserved the name Hh-Gli signaling pathway, today in predominant use.

Hh-Gli pathway inhibition

The first small-molecule inhibitor of the Hh-Gli pathway, natural product alkaloid cyclopamine, achieves inhibition by direct binding to the seven-transmembrane alpha-helical bundle of the Smoothened, the co-receptor Smo *(13)*. The majority of Hh-Gli pathway inhibitors target Smo, and this has led to the identification and development of many other Smo antagonists and derivatives of cyclopamine. Since a number of cancer cells have been found insensitive to Smo inhibition, because of the mostly acquired resistance to Smo antagonists through mutations in SMO that prevent binding of the antagonist *(14, 15)*, there was a need to target downstream effectors. Today there is a long list of small molecule inhibitors of the Hh-Gli pathway, acting from the level of attacking the ligand (any of Hh varieties), or from the Smo level, or acting on downstream targets in cytoplasm. Some of them are promising and are in clinical trials (table 1). Also, there is a long list of many new potential antagonists and agonists of the pathway, some of them listed in table 2.

TABLE 2

Some of known Hh-Gli antagonists and agonists.

Antagonists	Target	References
cyclopamine	Smo	17, 18, 19
KAAD-cyclopamine	Smo	20
Robotnikinin	Shh	21
SANT1,2,3,4	Smo	22
SANT74, SANT75	Smo	23
Cur-61414	Smo	24
GANT58	Gli	25
GANT61	Gli	25
Hh-Ag	Smo	26
SAG	Smo	22

Even though GLI1 is a transcription factor and thus a priori a bad target, it is a rather unusual factor with multiple lives in different cellular compartments *(25)*. GLI1, and the other GLIs, are exquisitely regulated at different levels, including phosphorylation, acylation, sequestration and degradation *(27–31)*. Each of these steps, as well as the partners that physically interact with the Gli proteins, provides possible sites for small molecule action. Therefore, Gli1 is not only a valid target but so far it is also the only reliable and general marker of a cell’s response to Hh signaling. Measuring GLI1 levels in relevant human cells is thus a requisite *(32)*.

Inhibitors of the pathway

Because of its accessibility on the membrane and its importance in regulation of the pathway, SMO has been the primary focus in the development of small-molecule inhibitors of the Hh-Gli pathway. GDC-0449 (vismodegib; Genentech) is an orally administered agent that selectively suppresses SMO activity and was the first SMO

inhibitor to progress to clinical trials. It has produced promising antitumor responses in patients with advanced basal cell carcinoma and medulloblastoma *(33, 34)*, but resistance has been reported *(15, 35)*. The resistance to SMO inhibitors highlights the therapeutic need to target downstream effectors. So, the small molecule GANT61 was identified as a specific inhibitor of GLI1 and GLI2. It suppresses the DNA-binding capacity of GLIs and inhibits GLI-mediated transcription. GANT61 reduces proliferation and induces apoptosis in a GLI-specific fashion in prostate cancer *(25)*, colon carcinoma *(36, 37)*, oral squamous cell carcinoma *(38)*, pancreatic cancer *(39)*, neuroblastoma *(40)*, and chronic lymphocytic leukemia *(41)*. However, today it is generally recognized that this inhibitor is not really specific inhibitor for Gli, and unfortunately the Hh-Gli pathway has no specific inhibitors created yet.

MicroRNA regulation

MicroRNAs (miRNA), small RNA molecules which bind to regulatory elements in the mRNA molecules and control their stability, are crucial post-transcriptional regulators of gene expression, cell differentiation and proliferation. They are involved in normal cell development and in development of various types of tumors. The role of miRNA in regulation of Hh-Gli signaling pathway has been suggested using screening approaches and bioinformatics, and a direct link between these two mechanisms has been investigated in various cancers. Downregulation or even misregulation of specific miRNAs allows high levels of Hh-dependent gene expression leading to tumor cell proliferation, sustaining cancer development *(42)*. Specific miRNAs involved in the regulation of the Hh signalling (miR-125b, miR-324-5p and miR-326), downregulated in medulloblastoma, target the activator components of the pathway, Smo and Gli1, thereby suppressing tumor cell growth. This was the first discovered mechanism of regulation of Hh signaling through miRNA-mediated control of Smo and Gli1 and of involvement of miRNA-mediated control of the Hh pathway in malignancy. The concept is still under research, particularly for severe types of cancer *(43)*, and may have promising implications for miRNA based therapies *(44)*.

Link between developmental biology and cancer

First discoveries related to the Hh-Gli pathway and human disorders were made on a range of PTCH1 alteration profiles, including genetic mutation, LOH, and promoter hypermethylation, and the two-hit theory was explored to dissect all possible genetic and epigenetic mechanisms *(45–49)*.

At this level, the key player in the pathway is PTCH1. Inactivation of PTCH1 allows hedgehog ligand-independent activation of SMO, causing a downstream activation of the pathway that may lead to neoplastic growth. Mutations in the PTCH (PTCH1) gene are the underlying cause of nevoid basal cell carcinoma syndrome (NBCCS) or Gorlin syndrome. And thanking to this syndrome, tumor suppressor PTCH was cloned, its role in development and cancer was unraveled. Cancers driven by mutations within the Hh signaling, mostly BCC and tumors described within Gorlin syndrome *(50, 51)*, had not been in wider focus, until aberrant activation of the pathway and its inhibitors (i.e. the natural alkaloid, cyclopamine) were described. After that, various studies through *in vitro* and *in vivo* models explored and attempted to explain mechanisms of ligand-dependent, ligand-independent, autocrine, canonical and non-canonical Hh-Gli pathway activation in multiple tumors.

MECHANISMS OF HH-GLI SIGNALING PATHWAY DEREGULATION THAT MAY LEAD TO CANCER DEVELOPMENT

The first and widely described, ligand-independent mechanism, usually involves mutations in which loss of PTCH1 or its functionality leads to loss of suppression, whereas SMO mutations create a constitutively active form of the protein *(20, 52, 53, 54)*.

But it was also shown that PTCH1 function can be lost through methylation *(48, 55, 56)*.

Amplifications or mutations of genes downstream of Ptch contribute to activation of the Hh-Gli pathway, e.g. high amplification of Gli1 was reported in glioblastomas *(6)*.

Another under widely explored, ligand-dependent mechanism, can be achieved through ligand hyperproduction or by downstream activation processes.

Ligand-dependent Hh-Gli signaling has been reporteded in different stages of carcinogenesis in different tumors: pancreatic cancer, lung cancer, esophageal cancer, prostate cancer, breast cancer, gastric cancer, colon cancer, ovarian cancer and hepatocellular cancer (58–66), suggesting that Hh-Gli signaling has significant role in carcinogenesis of these tumors.

In support of these findings, transgenic mice with pancreatic-specific expression of SHH or GLI2 develop pancreatic tumors *(59, 67)*. In some other tumors (gastric, prostate cancer) Hh signaling activation is associated with cancer progression, and consistent with these findings, inhibition of Hh signaling in prostate and gastric cancer cells reduces cell invasiveness *(63, 68)*. Also, it was published that Hh signaling is required for development and progression of melanoma, gliomas, breast cancer, ovarian cancer, leukemia and B-cell lymphomas *(69, 70)*.

In addition, the modes of Hedgehog signaling in cancer development may be variable. Activated Hh-Gli signaling can act in an autocrine or paracrine manner. In the

autocrine manner Hh is produced by the cancer cells themselves. In the paracrine manner (various studies in pancreatic, lung, esophageal cancer) stromal tumor cells are included in receiving signals. Even more, it was demonstrated that tumor-infiltrating monocytes or macrophages secrete ligand Shh, that activates Hh-Gli pathway in cancer cells *(71, 72, 73)*. Shh or Ihh ligands secreted by the tumor cells activate Hh signaling in the stromal cells *(74)*. It is also evident from studies of Dierks et al 2009 *(74)* and Zhao et al 2009 *(75)* that Hh signaling is required for maintenance of cancer stem cell population.

In our research we have observed hyperproduction of the Shh ligand by tumor cells in ovarian cancer, that lead to cell proliferation, as an example of the autocrine activation (*76*). These results indicate that in ovarian tumors pathogenesis through SHH gene expression differs in borderline tumors and carcinoma. Also, it was shown by others that the Hh-Gli signalling pathway plays an important role in ovarian tumorigenesis as well as in the activation of cell proliferation, thus could be as molecular target of new treatment strategies for ovarian carcinoma *(66)*.

On the other hand, in breast cancer we observed a cross-talk between Hh-Gli signaling (Shh ligand) and estrogen receptors creating an autoregulatory loop *(77, 78*). Furthermore, in colon cancer we observed hyperactivation of the regulatory kinase GSK3β that leads to overproduction of activator form of Gli3 and to the pathway hyperactivation *(79)*. This suggests a major role for the interplay of GSK3and Gli3 in the regulation of this pathway in colon cancer (publication in preparation).

Such examples from recent research of our group and many others document various ways of Hh-Gli signaling activation in many types of cancer, indicating different tumors have different modes of interaction with the pathway. Therefore, this pathway might indeed be a suitable target for cancer therapy.

CANCER THERAPY TARGETING HH SIGNALING

Today it is generally recognized that Hh-Gli signalling pathway is activated in various types of cancer and at various levels, and contributes to cancer proliferation, progression and invasiveness, so this pathway is anticipated to provide a new avenue for cancer therapy.

There are probably more than hundred compounds disclosed to have inhibitory effects on Hh signalling. Some are under clinical trials. Hh-signaling inhibitors are mainly targeting three sites in Hh-Gli pathway: Ligand Hh (by neutralizing antibodies, Robotnikinin), Smo protein (cyclopamine and its derivates) and Gli inhibitors. Several Smo inhibitors have been proposed as potential candidates for cancer therapy either as a single agent or in combination regimens with conventional chemotherapy. Most pathway inhibitors can be divided into three groups: natural products (cyclopamine), novel synthetic compounds and Hh-signaling modulators.

Cyclopamine is Hh-Gli pathway inhibitor on the level of direct interaction with Smo *(3)*. Some derivates of cyclopamine differing in solubility (IPI-926) or in structure (GDC-0499, LDE225, BMS-833923), or inhibitors of the transformation of inactive Smo into active Smo (SANT 74-75), and more others have been developed, and some are in clinical use *(80, 81, 82)*. Most drug development programs and recent clinical trials are focused on Smo inhibitors.

However, it was also shown that on the level of Gli-mediated transcription, which constitutes the final step in the pathway, some tumors could be selectively inhibited (GANT58 and GANT 61) *(25)*. Another recently identified Gli inhibitor, Gli-antagonist, is arsenic trioxide (ATO), which FDA approved as a drug for the treatment of acute promyelocytic leukemia. ATO binds directly to Gli1 inhibiting its transcriptional activity and suppressing tumor growth *in vitro* and *in vivo (83, 84)*.

Rapid advancement in the discovery of novel Hh signaling inhibitors has provided many opportunities for developing novel cancer therapeutic strategies. It is not surprising to learn that several major challenges still exist to prevent the use of Hh signaling inhibitors in clinics. These challenges include a lack of basic understanding of the molecular mechanisms by which Hh signaling mediates carcinogenesis; no clear criteria to identify the right tumors for therapeutic application; only a few reliable, physiologically relevant, and reproducible mouse models for cancer metastases to test and optimize drug dosages in order to minimize side effects; and a lack of clear strategies to mitigate drug resistance. Over the last years, research in this area has greatly improved. It is anticipated that additional novel therapeutic strategies will be developed for cancer clinical trials using Hh signaling inhibitors in the next years.

INTERACTIONS BETWEEN HH-GLI SIGNALING AND OTHER PATHWAYS OR CROSS-TALK

We may assume that pathways that enable particular cell to survive are interacting among themselves, and that in many cases Hh-Gli signaling pathway is involved.

Examples include regulation of SHH expression by Ras, NFkB and ERalfa, as well as regulation of Ihh by Msx2 *(67, 85)*. Also, expression of Gli1 is regulated by TGFbeta, Ras and Jun oncoprotein *(86, 87)*. Furthermore, the interaction between PKC and Hh signaling varies depending on PKC isoforms and cell types; although PKC alpha is shown to activate Hh signaling, PKC delta inhibits it *(88)*.

Particularly interesting are the interactions with another developmental pathway, Wnt pathway, which is also often active in some cancers, like colon cancer. Hh and Wnt signaling can form a positive or negative feedback loop depending on tissue content. In gastric cancer Hh signaling can exert negative effects on Wnt signaling through elevated expression of Wnt inhibitor sFRP-1 *(89)*. But in Hh-mediated skin carcinogenesis Wnt signaling is required (90), mostly trough beta-catenin expression *(91)*.

Many studies have shown p53 pathway collaboration with Hh pathway in skin carcinogenesis. In melanoma it was shown that p53 negatively regulates Gli1 expression through MDM2 *(92);* this feedback regulatory loop is required for maintaining stem cell number and cancer cell number.

Synergistic effects with Hh-Gli signaling was shown for some growth factors (IGF-I, VEGF, PDGF alpha, EGF) and their receptors, affecting MEK/ERK/JUN pathway *(93)*. This raises the question whether targeting Hh signaling with inhibitors of the pathway is also a good target for growth factor pathways, and could such strategy make contribution to better treatment of cancer (inhibitors of Hh-Gli pathway and EGF/EGFR, IGF inhibitors etc).

The importance of the Hh-Gli signaling pathway investigations related to its role in cross-talk is underlined by the estimates that the pathway may be active in one third of all cancers. Better understanding of the modes of Hh-Gli pathway regulation and tumor response, as well as of interactions of the pathway with other signaling pathways, has an obvious potential for development of better therapies that would be based on combined effects of the Hh-Gli and other pathways inhibitors.

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