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ORIGINAL ARTICLE

Deregulation and cross talk among Sonic hedgehog, Wnt, Hox and Notch signaling in chronic myeloid leukemia progression

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Deciphering the BCR-ABL-independent signaling exploited in chronic myeloid leukemia (CML) progression is an important aspect in cancer stem-cell biology. CML stem-cell compartment is dynamic as it progresses to terminal blast crisis where myeloid and lymphoid blasts fail to differentiate. We demonstrate cross-regulation of signaling network involving Sonic hedgehog (Shh), Wnt, Notch and Hox for the inexorable blastic transformation of CD34$^+$ CML cells. Significant upregulation in Patched1, Frizzled2, Lef1, CyclinD1, p21 ($P \leqslant 0.0002$) and downregulation of HoxA10 and HoxB4 ($P \leqslant 0.0001$) transcripts in CD34$^+$ cells distinguish blast crisis from chronic CML. We report Shh-dependent Stat3 activation orchestrates these mutually interconnected signaling pathways. Stimulation of CD34$^+$ CML cells with either soluble Shh or Wnt3a did not activate Akt or p44/42–mitogen activated protein kinase (MAPK) pathways. Interestingly, unlike dominant negative Stat3β, introduction of constitutive active Stat3 in CD34$^+$ CML cells induces cross-regulation in gene expression. Additionally, Shh and Wnt3a-dependent regulation of cyclin-dependent kinase inhibitors (CDKI) in CML suggests their role in the network. Taken together, our findings propose that deregulation in the form of hyperactive Shh and Wnt with repressed Notch and Hox pathways involving Stat3, Gli3, β-catenin, CyclinD1, Hes1, HoxA10 and p21 might act synergistically to form an important hub in CML progression.
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Introduction

Chronic myeloid leukemia (CML) is a stem-cell disorder characterized by chronic and blast-crisis phases. Emerging evidence indicates that leukemia stem-cell (LSC) compartment including that of CML is dynamic owing to its exquisite adaptability.[1–3] Interestingly, presence of BCR-ABL-containing CD34$^+$ cells in patients with cytogenetic remission under imatinib[4] suggests involvement of BCR-ABL-independent signaling exploited by the LSCs. However, the molecular program controlling hematopoietic stem-cell (HSC) self-renewal, Notch, Sonic hedgehog (Shh), Wnt and Hox signaling are also involved in oncogenesis.[5,6] Additionally, the cell cycle regulators p21 and p27 control HSC quiescence and progenitor proliferation, respectively.[7,8] Nevertheless, global elucidation of the molecular mechanisms orchestrating these multiple signaling pathways in the stem-cell self-renewal transcriptional program might explore CML progression.

Deregulation in Hedgehog signaling, which regulates tissue patterning and stem-cell maintenance, is associated with different forms of human cancer.[9] Shh may even function as a regulator of HSC depending on downstream signals.[10] Then again, aberrant Notch signaling is also involved in hematopoietic malignancies.[11] Direct protein–protein interactions between Stat3 and Hes1 have been suggested to link Jak–Stat and Notch–Hes pathways.[12] Moreover, contemporary studies suggest regulatory role of Notch in repressing p27 activity.[13,14] Notably, enhanced clonogenicity and poor prognosis have been associated with activation of canonical Wnt signaling in several types of leukemia.[15,16] Moreover, results arising from the experimental interference in some of these pathways signify the importance of the network in designing therapeutic modalities.[17–19]

Nevertheless, to address the dynamic stem-cell compartment, we compared the gene expression and signaling for this apparently diverse but mutually interconnected self-renewal-associated genetic programs of CD34$^+$ chronic phase and blast-crisis CML cells. We demonstrate that Stat3, Gli3, β-catenin, CyclinD1, Hes1, HoxA10 and p21 play important role in the signaling network.

Materials and methods

Reagents

Cytokines and recombinant proteins. Stem-cell factor (SCF), Flt-3-ligand, interleukin-3 (IL-3), recombinant Wnt3a and N-Shh (C24II) were purchased from R&D Systems Inc., Minneapolis, MN, USA.

Antibodies

Rabbit anti-pY705-Stat3/Stat3, anti-pT308-Akt/Akt, anti-pT202/Y204-p44/42 mitogen activated protein kinase (MAPK)/p44/42, anti-pS9-GSK-3β/GSK-3β, anti-β-catenin, anti-β-actin and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA).

Drugs

Forskolin was purchased from Calbiochem (Merck Ltd, USA) and Cyclopamine from Cyanochem, and they were dissolved in dimethyl sulfoxide and ethanol, respectively.

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Plasmids
The p27-PF-luc and pGVB2-luc reporter constructs were obtained from T. Sakai (Molecular-Targeting Cancer Prevention, Japan). The 1745-cyclinD1-luc and pA2-luc reporters were gifted by C Albanese (Lombardi Cancer Center, WA). The pTOPFLASH and pFOPFLASH reporters were obtained from H Clevers (Hubrecht Laboratory, Utrecht, The Netherlands). Stat3C and M67-luc were gifted by J Bromberg (Memorial Sloan-Kettering Cancer Center, NY). We also obtained dominant negative Stat3β from J Clifford (Meso scale Discovery, NY). In addition, different constructs of Notch1 were from J Aster (Harvard Medical School, MA, USA) and T Kadesch (University of Pennsylvania, School of Medicine, PA, USA).

CML patient samples and CD34$^+$ progenitor culture
Bone marrow and peripheral blood samples were obtained from chronic-phase (CP) and blast-crisis (BP) CML patients after informed consent and following institutional ethical guidelines. Conventional cytogenetics and BCR-ABL-specific quantitative real-time polymerase chain reaction (qRT–PCR) for both b3a2 and b2a2 transcripts were carried out for confirmation. Characteristics of individual CP/BP samples are provided in the Supplementary Table 1. We considered CP as with $<10\%$ of blast cells and BP having $>30\%$ blasts in peripheral blood or $>50\%$ blasts plus promyelocytes in bone marrow. Leukemic blasts were isolated by Ficoll-Paque (1.077, Sigma, St Louis, MO, USA), and CD34$^+$ progenitors were then enriched by immunomagnetic separation (Miltenyi Biotec, Bergisch, Gladbach, Germany). CD34$^+$ populations were $>95\%$ pure as determined by immunostaining with CD34-FITC antibody (Miltenyi Biotec) and subsequent fluorescence-activated cell sorting (FACS) analysis. Freshly isolated CD34$^+$ cells were cultured in Iscove's Modified Dulbecco's Medium in presence of 10% fetal calf serum (FCS) supplemented with SCF (100 ng/ml), Flt-3-ligand (100 ng/ml), IL-3 (10 ng/ml) and Wnt3a (500 ng/ml) or N-Shh (3 μg/ml) (All from Stem cell technologies Inc., Canada). Details are available in Supplementary Information.

qRT-PCR
RNA was isolated from bone marrow samples of individual CP CML patients and from either bone marrow or peripheral blood of BP CML patients. Total RNA was extracted from CD34$^+$ cells using Tripure isolation reagent (TRIZOL, Roche, GmbH, Mannheim, Germany) and subsequently treated with RNAse-free DNAse (DNAfree Ambion, Austin, TX, USA) and quality-assayed (Eppendorf BioPhotometer, Germany). The cDNA was prepared by reverse transcribing 50 ng of total RNA with Taqman reverse transcriptase reagents (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was subsequently performed using SYBR Green core PCR reagents (Applied Biosystems) and HPRT1 was used as the endogenous control. The qRT–PCR reactions and analyses were carried out in 7500 Sequence Detection System (Applied Biosystems). Details are available in Supplementary Information and sequences of respective intron spanning primer pairs are included in the Supplementary Table 2.

Western blot analysis
Cells were harvested and lysates recovered by centrifugation. Estimated amount of proteins were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on nitrocellulose. Blots were probed with respective primary and HRP-conjugated secondary antibodies and developed by using ECL detection system (Amersham Biosciences, Pittsburgh, PA, USA). Densitometry was used for quantitation (Bio-Rad, Hercules, CA, USA).

Nucleofection
CD34$^+$ primary cells were nucleofected either with reporter constructs or plasmids according to the manufacturer's recommendations (AMAXA Inc, Gaithersburg, MD, USA). Details are available in Supplementary Information.

Luciferase assay
For determining the luciferase activity, CD34$^+$ cells were cotransfected with respective reporter constructs (5 μg) together with phRL control vector (1 μg). Relative luciferase unit (RLU) was determined by normalizing phRL-null using Dual luciferase assay kit (Promega, MA, USA).

Statistical analysis
The gene expression levels in CP and BP CML were compared by unpaired two-tailed *t*-test. Only a $P<0.001$ was considered statistically significant. In addition, results obtained from multiple experiments were reported as the mean $\pm$ s.e.m.

Results

Distinct gene expression and reporter activity in CD34$^+$ chronic and blast-crisis CML cells
Several self-renewal-associated genes are differentially expressed in CD34$^+$ CP and BP CML cells as assessed by qRT–PCR (Supplementary Table 3). In the BP CML, Ptc1 and Lef1 are most abundant with fold induction values 20 or above ($P\leqslant0.0001$) (Figure 1a). Compared with CP CML cells, the BP samples contain significantly higher levels of Fdz2, β-catenin, p21 and CyclinD1 ($P\leqslant0.0002$) (Figure 1b). However, BP CML showed significant downregulation of HoxA10, HoxB4 ($P\leqslant0.0001$) and Hes1, Gli3 and c-Myc ($P<0.001$) transcripts (Figure 1a and b). The increase in Lef1 and CyclinD1 transcripts in blast crisis is correlated with enhanced pTOPFLASH reporter and CyclinD1 promoter activity as determined by the luciferase assay (Figure 1c and d). However, we did not observe change in p27 promoter activity in CD34$^+$ CP and BP CML cells (Supplementary Figure 1).

Deregulation of Shh signaling in CD34$^+$ CML
Presence of high level of Ptc1 in blast crisis prompted us to investigate the Shh signaling. Recombinant Shh (N-Shh) induces dose-dependent and time-dependent increase in Gli1 transcript in CD34$^+$ CP CML cells (Figure 2a and b). We analyzed RNA from CD34$^+$ CP and BP CML cells cultured in the presence or absence of Shh (3 μg/ml) for 24 h. Compared to CP, Shh induces upregulation in CyclinD1 and p21 transcripts in BP CML cells (Figure 2c). Unlike in BP, Hes5 expression was induced in CD34$^+$ CP CML cells (Figure 2c). Surprisingly, expression of CyclinD1, Ptc1 and Gli1, but neither Gli3 nor p21, was repressed when CD34$^+$ CP CML cells were cultured in presence of forskolin (200 μM) (Figure 2d).

Shh signaling regulates Stat3 activation in CD34$^+$ CML cells
To address the deregulation observed in Shh-induced gene expression, we analyzed downstream signaling pathways.

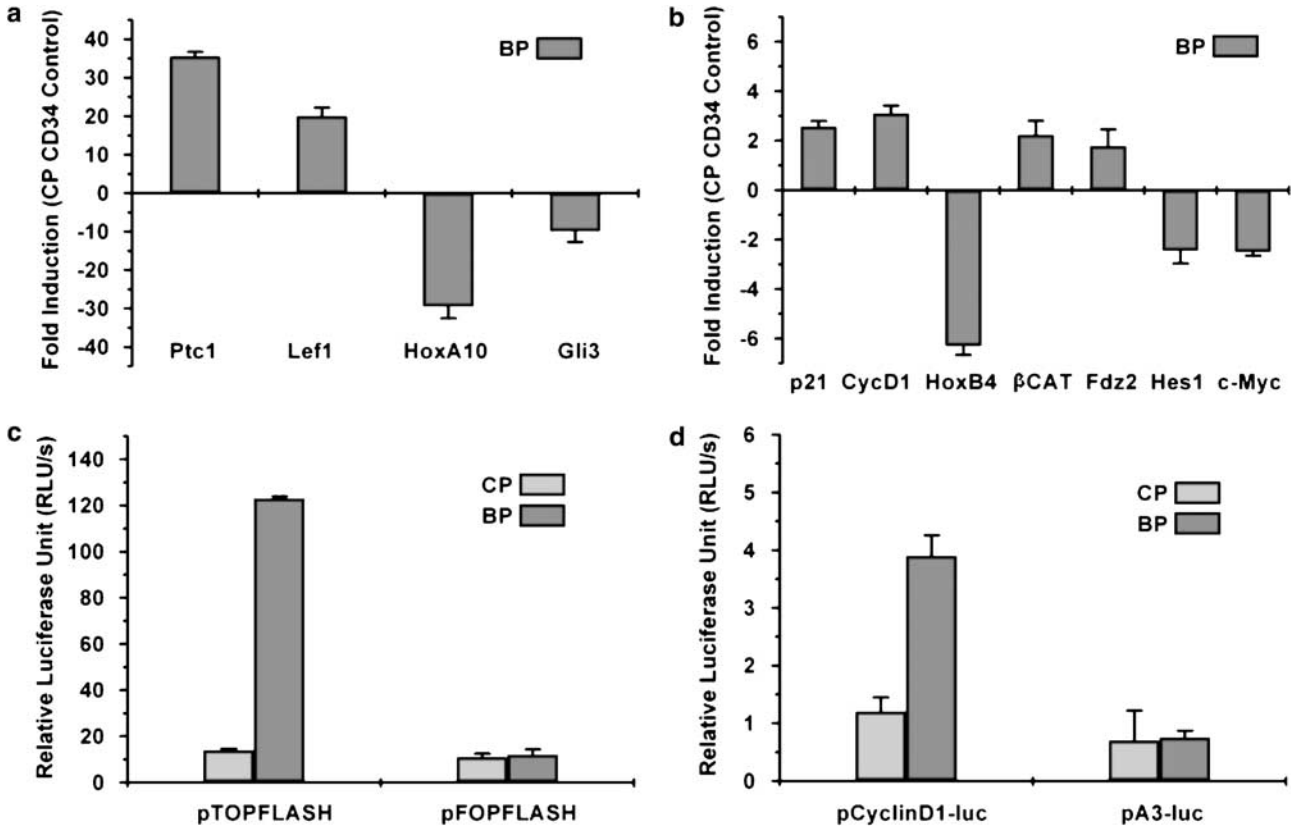

Figure 1 Relative gene expression and reporter assay in CD34$^+$ CP and BP CML cells. (**a**) Ptc1 and Lef1 transcripts are upregulated in BP by more than 20-fold ($P \leqslant 0.0001$). Significant downregulation was observed in HoxA10 ($P \leqslant 0.0001$). (**b**) p21 and CyclinD1 along with β-catenin and Fzd2 transcripts ($P \leqslant 0.0002$) are increased in the BP CML. However, BP CML cells showed downregulation in HoxB4 ($P \leqslant 0.0001$), Hes1, Gli3 and c-Myc ($P < 0.001$) RNA. The relative expression levels were determined by normalizing the ΔC_t values against the average ΔC_t values for CD34$^+$ CP CML cells for the specified genes. (**c**) Tcf4/Lef1 reporter (pTOPFLASH) activity in CD34$^+$ CP and BP CML cells determined after 24 h of nucleofection. pFOPFLASH was used as the empty control vector. Data represent the mean $\pm$ s.e.m. of separate experiments performed on three individual patient samples. (**d**) CyclinD1 promoter activity in CD34$^+$ CP and BP CML cells measured after 24 h of nucleofection. pA3-luc was used as the empty control vector.

Western blot analysis-revealed Shh signaling induces Stat3 phosphorylation (Y705) in CD34$^+$ CP CML cells that could be inhibited by cyclopamine (40 μM) (Figure 3a and b). Low dose of Shh (100 nM) was even able to promote Stat3 phosphorylation (data not shown). Again, total Stat3 protein remains unaltered (Figure 3a). However, Shh signaling did not induce phosphorylation of Akt or p44/42-MAPK in CD34$^+$ CP CML cells (Figure 3b). Moreover, Shh induces dose-dependent activation of M67-luciferase, containing Stat3-binding sites (Figure 3c).

Stat3-dependent gene expression induces cross talk in CD34$^+$ CML cells

Shh-induced Stat3 activation prompted us to investigate the role of Stat3 in regulating downstream gene expression. Unlike dominant negative Stat3β, expression of constitutive active Stat3 (Stat3C) in CD34$^+$ CP CML cells induced expression of Wnt3a, Lef1, Gli1, CyclinD1 and p21 (Figure 4). However, it neither induced HoxA10, HoxB4 nor Notch target genes: Hes1, Hes5 and Hey1. We also noticed Stat3C-driven increase in Shh transcript in one of the CML samples studied (data not shown).

Deregulation of Wnt signaling in CD34$^+$ CML

To investigate Wnt signaling, CD34$^+$ CML cells were cultured in presence or absence of saturating concentration of soluble Wnt3a (500 ng/ml) for 24 h. Compared to CP, BP CML cells showed significant increase in both CyclinD1 and c-Myc transcripts, however, expression of β-catenin and Lef1 was unaltered (Figure 5a). Interestingly, Wnt3a induced expression of Gli1 and Gli3 transcripts in chronic phase, but it did not induce expression of Hes5 and HoxA10 (Figure 5a). In addition, compared to chronic phase, expression of Ptc1 was more induced in CD34$^+$ BP CML cells as a result of Wnt3a signaling (Figure 5a). Western blot analysis reveals upregulation of β-catenin in CD34$^+$ CP CML cells within 24 h of treatment with Wnt3a (Figure 5b). This increase in β-catenin protein is accompanied by concomitant rise in phosphorylated glycogen synthetase kinase-3β (GSK-3β), which suggests activation of the canonical Wnt/β-catenin axis (Figure 5b). However, identical dose of Wnt3a did neither induce phosphorylated-Stat3 (Y705) nor total Stat3 protein in CD34$^+$ CP CML cells (Figure 5b).

Discussion

The objective of CML stem-cell biology is to decipher BCR-ABL-independent signaling exploited by the progenitors to reacquire self-renewal characteristics. Previous studies have highlighted the role of Hox pathway in HSC maintenance.[20–24] It has been suggested that overexpression of HoxA10 might have both prodifferentiation and antidifferentiation properties in a dose-

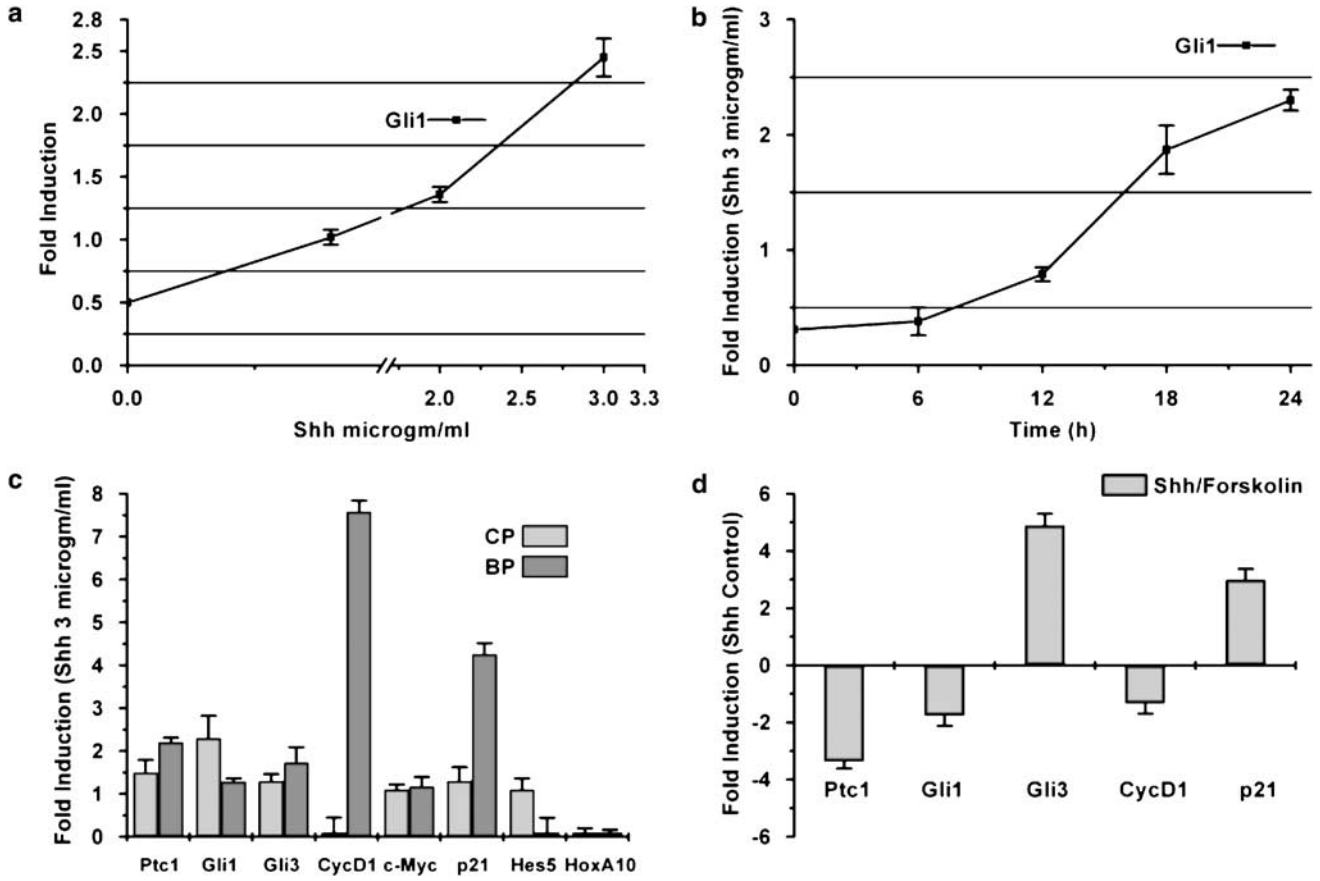

Figure 2 Shh signaling in CD34+ CML cells. (**a**) Shh (24 h) induces dose-dependent and (**b**) time-dependent (Shh = 3 μg/ml) increase in Gli1 transcript in CD34+ CP CML cells as determined by qRT–PCR. (**c**) CD34+ CP and BP CML cells were cultured in presence or absence of Shh (3 μg/ml) for 24 h and gene expression was determined using qRT–PCR. Individual data were normalized with respect to absence of Shh in the media for both chronic and blast phase. Data represent the mean ± s.e.m. of separate experiments performed on three individual patient samples. (**d**) Effect of forskolin (200 μM for 24 h)/Shh (3 μg/ml) on gene expression of CD34+ CP CML cells compared to Shh (3 μg/ml) alone. Data represent the mean ± s.e.m. of separate experiments performed on three individual patient samples.

dependent spatiotemporal manner.[21,22] In contrast, overexpression of HoxB4 in HSC enhances *in vivo* lympho-myeloid regeneration without impairing normal differentiation or inducing cellular transformation.[23,24] Given that CML blast crisis is characterized by differentiation blockage, the reduced HoxA10 and HoxB4 transcripts in CD34+ BP CML cells might reflect the deregulation in Hox pathway that warrants future investigation. In addition, we detected upregulation in Fzd2, Lef1, β-catenin, CyclinD1 and Ptc1 transcripts in BP CML. Transcriptional increase in Fzd2 and Ptc1, the receptors for Wnt and Shh signaling, respectively, indicate activation of these pathways in blast crisis as Ptc1 itself is a target of the Shh signaling. Furthermore, significant increase in Ptc1 transcript in blast crisis is not always associated with that of Gli3, which is another target of Shh pathway activation. Taken together, this indicates that CML progression is associated with deregulation in Shh signaling.

We compared Shh and Wnt3a-induced signaling in CD34+ CP and BP CML cells cultured *in vitro*. Interestingly, we find Shh signaling induces Stat3 activation in chronic phase cells that could be inhibited by the smoothened inhibitor cyclopamine. However, unlike Shh-dependent Akt activation, as observed in NIH3T3 cells,[25] we did not get either PI3K-Akt or p44/42-MAPK activation. This indicates that Shh-dependent Stat3 activation is a specific event. Again, we could not detect Shh-induced Stat3 activation in blast crisis possibly owing to the presence of high level of phosphorylated Stat3.[26] However, the precise mechanism by which Shh induces Stat3 phosphorylation still remains to be identified. Interestingly, forskolin, an activator of protein kinase A (PKA) and thus inhibitor of Shh signaling, inhibits activation of Ptc1 and Gli1, but maintains upregulated Gli3 transcript. This may suggest that the complicated regulation of Gli3 transcription is indirect and that is either independent or upstream of PKA function.

In addition, compared to CP, Shh induces significant upregulation of CyclinD1 and p21 transcripts in CD34+ BP CML cells. Forskolin inhibition was not even able to repress induction of CyclinD1 transcript. We compared constitutive active Stat3C-dependent regulation of gene expression in CD34+ CP CML cells with respect to the dominant negative Stat3β. Remarkably, Stat3C induced expression of Wnt3a, Lef1, CyclinD1, p21, p27 and Gli1. However, it did not induce HoxA10, HoxB4 and Notch target genes: Hes1, Hes5 and Hey1. Interestingly, Stat3C-dependent increase in Wnt3a could be the plausible way of transformation from paracrine to autocrine signaling. Recently, it has been suggested that Stat3 transcriptionally regulates CyclinD1 in breast carcinoma cells.[27] Moreover, nuclear accumulation of β-catenin is associated with Stat3 activation in colorectal cancer.[28] Altogether, our data demonstrates that Shh-dependent Stat3 activation and Stat3 dependence

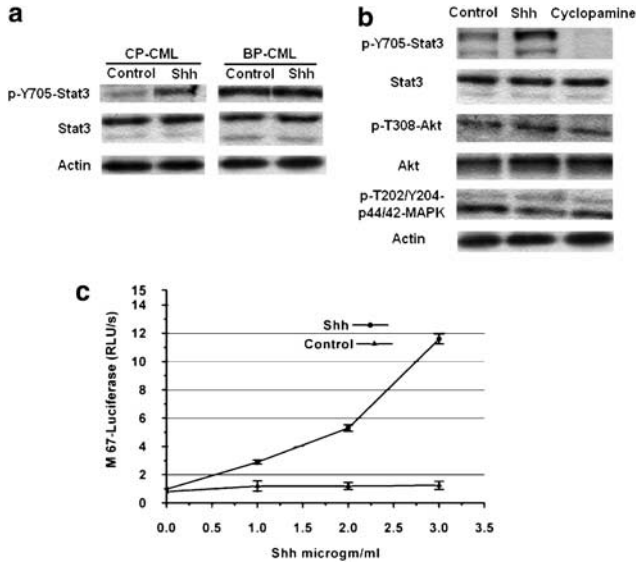

Figure 3 Shh induces Stat3 activation in CD34$^+$ CML cells. (**a**) Western blot analysis performed on CD34$^+$ CP and BP CML cells, cultured in presence or absence of Shh (3 μg/ml) for 24 h. The Western blot is a representative of separate experiments performed on five individual CP CML and three individual BP CML patient samples. (**b**) Western blot analysis performed on CD34$^+$ CP CML cells, cultured in presence or absence of Shh (3 μg/ml) or cyclopamine (40 μM) for 24 h. The Western blot is a representative of separate experiments performed on three individual CP CML patient samples. (**c**) Shh (dose)-dependent increase in M67-luc (RLU/s) in CD34$^+$ CP CML cells after 24 h of nucleofection. Data represent the mean$\pm$s.e.m. of separate experiments performed on three individual patient samples.

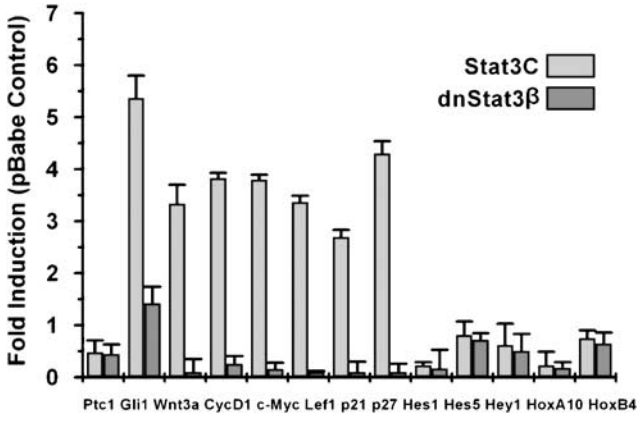

Figure 4 Stat3 induces cross talk in gene expression. CD34$^+$ CP CML cells were nucleofected with constitutive active Stat3C or dominant negative Stat3β or pBabe control vector and were cultured for 24 h. Relative fold induction in gene expression with respect to the pBabe control was determined using qRT–PCR. Data represent the mean$\pm$s.e.m. of separate experiments performed on four individual patient samples.

of CD34$^+$ CML cells not only interconnect Shh, Wnt, Notch and Hox pathways, but also paves BCR-ABL-independent signaling pathway for CML progression (Figure 6).

We report increase in p21 transcript in untreated CD34$^+$ BP CML cells. Furthermore, Shh, Wnt and Stat3 signaling independently regulate expression of p21 and p27. Although high level of p21 mRNA has been reported in bone marrow mononuclear cells,[7] it is not increased in CD34$^+$ cells during normal

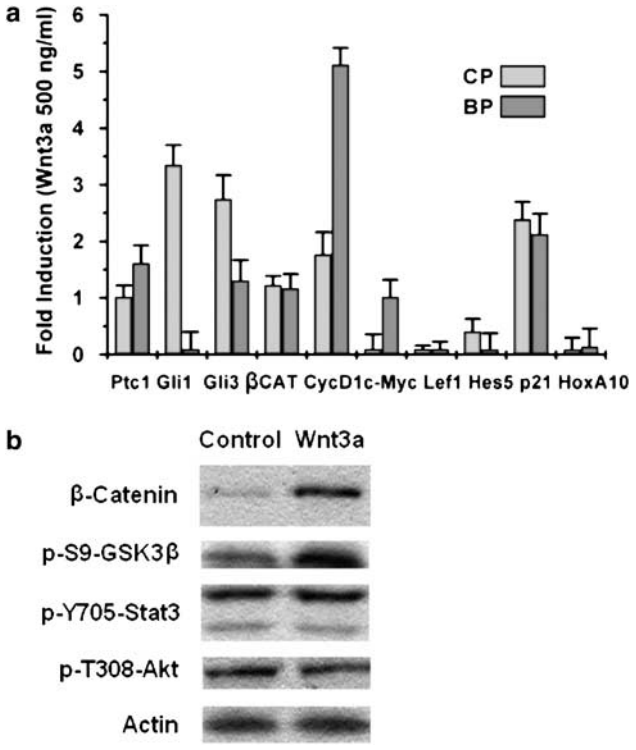

Figure 5 Wnt signaling in CD34$^+$ CML cells. (**a**) CD34$^+$ CP and BP CML cells were cultured in presence or absence of Wnt3a (500 ng/ml) for 24 h and gene expression was determined using qRT–PCR. Analogous to Shh treatment, individual data were also normalized with respect to absence of Wnt3a in the media for both chronic and blast phase. Data represent the mean$\pm$s.e.m. of separate experiments performed on three individual patient samples. (**b**) Western blot analysis performed on CD34$^+$ CP CML cells cultured in presence or absence of Wnt3a (500 ng/ml) for 24 h. The Western blot is a representative of separate experiments performed on three individual CP CML patient samples.

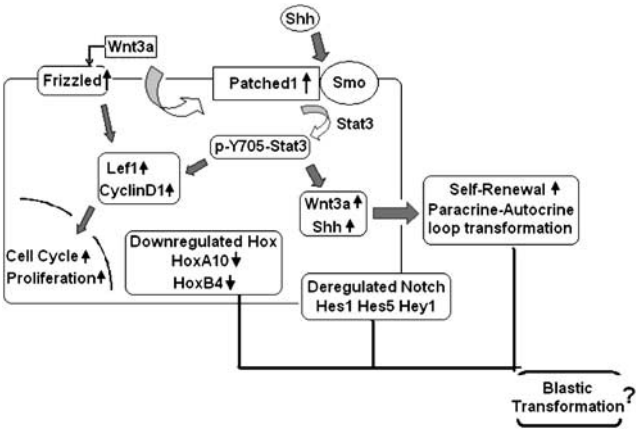

Figure 6 Model of self-renewal-associated signaling crossregulation in CML progression. Blast-crisis CML is associated with proactive Shh and Wnt signaling, downregulated Hox and deregulated Notch pathways. Shh-dependent Stat3 activation in CD34$^+$ CML cells necessitates BCR-ABL-independent mechanism in connecting Shh and Wnt pathways through upregulation of CyclinD1 and Lef1, which eventually leads to uncontrolled proliferation. It might also lead to paracrine (generally signaling cues are provided by the bone marrow microenvironment) to autocrine loop transformation by inducing Wnt3a itself. In addition, Wnt3a-mediated upregulation in Ptc1 could be another plausible mechanism of such regulatory cross talk in CML progression.

hematopoiesis. This may reflect the complex biochemical role p21 plays as either a requisite participant in the formation of a cyclin-dependent kinase (CDK) complex, necessary for G1-S progression, or as a CDK inhibitor (CDKI). On the other hand, p27 is regulated both at the transcriptional and posttranslational level, and earlier we have shown that modifying p27 could have a therapeutic role in CML.[19] All these suggest that p21 and p27 play distinct roles depending on the subcompartments of the hematopoietic cascade. Nevertheless, LSC may exploit these subtleties necessary for HSC maintenance[29] in making deregulated p21 and p27 for CML progression.

Additionally, enhanced level of Lef1, β-catenin and CyclinD1 transcripts along with increased Tcf4-dependent reporter activity are consistent with the activation of Wnt signaling in blast crisis. In contrast, we did not observe increase in c-Myc transcript, which is another important target of canonical Wnt pathway. Recently, c-Myc has been identified as a direct and major Notch1 target in T-cell leukemia.[30] Notably, Notch pathway has recently been shown to regulate embryonic neural stem-cell number by modulating Stat3 activation.[31] Here, the gene expression data demonstrate that Notch signaling is not hyperactivated, if not downregulated, in CD34$^+$ BP CML cells. Taken together, this suggests that even though Wnt/β-catenin axis is activated, owing to deregulated Notch signaling, c-Myc transcript could not get increased. Moreover, Wnt3a-induced upregulation in c-Myc transcript in blast crisis is an indicative of overcoming the Notch-dependency. Furthermore, Wnt3a-induced expression of Gli1 and Gli3 selectively in CP CML reflects the cross talk similar to the *de novo* gene expression pattern.

Altogether, we have identified a distinct gene expression profile of self-renewal-associated signaling of CD34$^+$ CML cells. Unlike CP CML cells, some samples of BP were isolated from peripheral blood rather than bone marrow. Earlier study conducted on normal HSC indicates that in contrast to bone marrow resident CD34$^+$ cells, circulating peripheral CD34$^+$ population consists of a higher number of quiescent stem and progenitor cells.[32] However, it has been recently demonstrated that in BCR-ABL$^+$ CML cells, proliferating status and gene expression pattern are similar and extremely well correlated irrespective of the origin of these CD34$^+$ cells.[33,34] Furthermore, discrepancies may also arise from the differential karyotype of CML and efforts were therefore made to ensure that analysis were carried out only on CML samples containing more than 95% Philadelphia (Ph) chromosome in metaphase. Recent studies have focused on the gene expression of CML using microarray indicating biphasic nature of the disorder and the involvement of the Wnt/β-catenin pathway that corroborates our findings.[34,35] However, considering posttranscriptional and posttranslational modifications, gene expression signature might not be the only choice to pursue. Significantly, the novel findings of our study based on an integrated approach do not merely represent the gene-by-gene or signal-to-signal quantitation but put a step toward contemporary molecular dissection of tumor cells to understand the complexity and subtlety of the signaling networks that drive and maintain them. Nevertheless, there remains an immense scope for studying the consequence of such ramified signaling network particularly in the field of regenerative and preventive medicine.

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Mini-review

Cross-talk between miRNA and Notch signaling pathways in tumor development and progression

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ABSTRACT

Notch signaling pathways are known to regulate many cellular processes, including cell proliferation, apoptosis, migration, invasion, and angiogenesis, and is one of the most important signaling pathway during normal development. Recently, emerging evidences suggest that microRNAs (miRNAs) can function as key regulators of various biological and pathologic processes during tumor development and progression. Notch signaling has also been reported to be regulated through cross-talk with many pathways and factors where miRNAs appears to play a major role. This article will provide a brief overview of the published evidences for the cross-talks between Notch and miRNAs. Further, we summarize how targeting miRNAs by natural agents could become a novel and safer approach for the prevention of tumor progression and treatment.

1. Notch signaling

In recent years, we have witnessed the sudden explosion in the literature regarding the role of Notch signaling in tumor progression. It has become clear that Notch signaling is involved in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs [1]. Notch genes encode proteins which can be activated by binding of a family of its ligands. The four members of Notch receptors have been identified to date in mammals, including Notch-1 to -4. Five Notch ligands have been found in mammals: Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1 and Jagged-2 [1]. Notch signaling is initiated by binding of the Notch transmembrane receptors with their specific ligands between two neighboring cells. Upon activation, Notch is cleaved, releasing the Notch intracellular domain (NICD) through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase complex. The NICD can subsequently translocate into the nucleus for transcriptional activation of Notch target genes [2] (Fig. 1). Inhibiting γ-secretase function would prevent the cleavage of the Notch receptor, resulting in blocking the Notch signal transduction signaling [3]. Therefore, γ-secretase inhibitors could be useful for the treatment of human malignancies, which are being tested in clinical trials (see website: www.clinicaltrials.gov). In the absence of NICD, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL (C protein binding factor 1/Suppressor of Hairless/Lag-1). When NICD enters the nucleus, it binds to CSL and recruits transcription activators to the CSL complex, leading to convert it from a transcriptional repressor into a transcription activator complex [3]. A few Notch target genes have been identified, some of which are dependent on Notch signaling in multiple tissues, while others are tissue specific. Notch target genes include Hes (Hairy enhance of split) family, Hey (Hairy/enhancer of spit related with YRPW motif), nuclear factor-kappa B (NF-κB), vascular growth factor receptor (VEGF),

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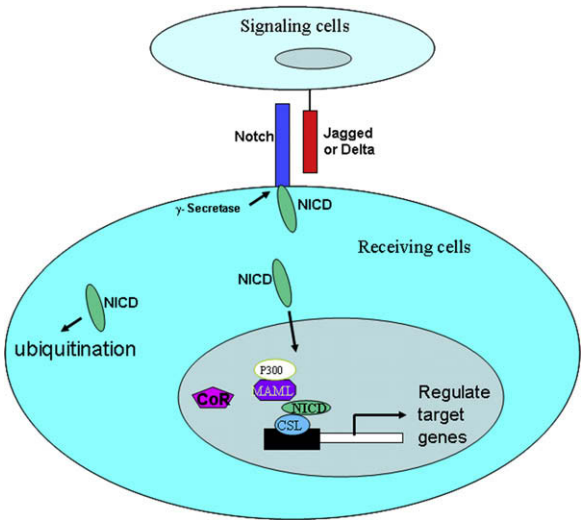

Fig. 1. Schematic of Notch signaling. Notch signaling is initiated by binding of the Notch transmembrane receptors with their specific ligands between two neighboring cells. Upon activation, Notch is cleaved, releasing the Notch intracellular domain (NICD) through cleavage by γ-secretase complex. The NICD can subsequently translocate into the nucleus for transcriptional activation of Notch target genes. When NICD enters the nucleus, co-repressors associated with CSL (CBF1/Suppressor of Hairless/Lag-1) are displaced and a transcriptionally active complex consisting of CSL, NICD, Mastermind, and other co-activators is formed, which converts CSL from a transcriptional repressor into an activator, leading to activation of Notch target genes.

mammalian target of rapamycin (mTOR), cyclin D1, c-myc, p21, p27, Akt, etc. [4–7].

It has been well documented that Notch signaling maintains the balance between cell proliferation, differentiation and apoptosis. Therefore, alterations in Notch signaling are considered to be associated with tumorigenesis. Indeed, it has been reported that Notch genes are abnormally regulated in many human malignancies [8–10]. These observations suggest that dysfunction of NICD prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation. Interestingly, it has been shown that the function of Notch signaling in tumorigenesis could be either oncogenic or anti-proliferative, and the function could be context dependent [11]. Notch signaling has been shown to be anti-proliferative in a limited number of tumor types, including skin cancer, human hepatocellular carcinoma and small cell lung cancer [11–13]. However, most of the studies have shown oncogenic function of Notch in many human carcinomas. In summary, emerging evidence suggest that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer [6,14–17].

Moreover, patients with tumors expressing high levels of Jagged-1 or Notch-1 had a significantly poorer overall survival compared with patients expressing low levels of these genes [18–20]. Jagged-1 was also found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues [18]. Furthermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, suggesting that Jagged-1 may be a useful marker in distinguishing indolent *vs.* aggressive prostate carcinomas [18]. Notch signaling pathway has also been reported to cross-talk with multiple oncogenic signaling pathways, such as NF-κB, Akt, Sonic hedgehog (Shh), mTOR, Ras, Wnt, estrogen receptor (ER), androgen receptor (AR), epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) [14,21–25], and thus it is believed that the cross-talk between Notch and other signaling pathways may play critical roles in tumor aggressiveness. The main features of these pathways and cross-talk with Notch signaling have recently been reviewed, and thus the readers who are interested in learning more on the cross-talk between these pathways and Notch pathway are referred published review articles [1,6,7,24,25]. Recently, microRNAs (miRNAs) have been reported to cross-talk with Notch pathway for its regulation [26–30], suggesting that the post-transcriptional and/or translational regulation of genes by miRNAs are becoming critically important. Therefore, in the following sections, we have attempted to summarize the functional role of miRNAs in Notch signaling pathway.

2. miRNAs

In recent years, a large body of literature has emerged documenting the biological significance of miRNAs in tumor progression [31–33]. Over 4500 miRNAs have been identified in vertebrates, flies, worms, plants and viruses after the first miRNA, which was discovered in 1993 while studying *Caenorhabditis elegans* [34]. It is well known that miRNAs work as integral players in cancer biology. The miRNAs elicit their regulatory effects in post-transcriptional regulation by binding to the 3′ untranslated region (3′ UTR) of target messenger RNA (mRNA). Either perfect or near perfect complimentary base pairing results in the degradation of the mRNA, while partial base pairing leads to translational inhibition to functional proteins [35]. The miRNAs have been implicated in a wide array of cell functions in many normal biological processes, including cell proliferation, differentiation, apoptosis, and stress resistance [36]. It has also been shown that miRNAs are key players in human cancer. The reason why miRNAs are connected with cancer is that miRNAs are involved in the biological processes of cell proliferation and apoptosis, the two intimately linked processes that are critically involved in the development and progression of human malignancies. It has been reported that there are aberrant expression of miRNAs when comparing various types of cancer with normal tissues [37]. It is very important to note that some miRNAs are thought to have oncogenic activity while others have tumor suppressor activity as indicated earlier. Oncogenic miRNAs are up-regulated in cancer and contribute to its pathology through various mechanisms such as targeting tumor suppressor genes. In contrast to the oncogenic miRNAs, other miRNAs are considered to have tumor suppressor activity and are down-regulated in cancer [38,39]. However, these distinctions may not be so strict,

suggesting that some miRNAs may express either activity, depending on the biological context and tissue type.

Recent studies also suggest that miRNAs could have diagnostic, prognostic, and therapeutic value. For example, up-regulation of miR-21 is strongly associated with both a high Ki-67 proliferative index and the presence of liver metastasis [40]. High expression of miR-196a-2 had a median survival of 14.3 months compared with a median of 26.5 months for those with low expression in pancreatic cancer [41], suggesting that miR-196a-2 could be important predictor of survival. Moreover, high expression of miR-15b was significantly associated with poor prognosis and tumorigenesis in melanoma [42]. Furthermore, Patients whose liver tumors had low miR-26 expression had shorter overall survival [43]. Many other published papers showed that miRNAs expression profiling not only can be used in diagnosis, but can also be used as prognostic markers in cancer [37]. Although the research studies for the role of miRNAs in cancer have exploded in recent years, the question remains whether the alteration in miRNAs expression could be ascertained as the cause or the consequence of cancer development [37]. It is not clear for the specific targets and functions of miRNAs although there are several excellent review articles published documenting the role of miRNAs in human cancers [31–37,44], and thus we will not discuss the functions of miRNAs in cancers in this article rather we will present evidence regarding the cross-talk regulation of Notch and miRNAs in cancer development and progression.

3. Cross-talk between Notch and miRNAs

Recently, it has been reported that miRNAs play critical roles in Notch signaling pathway. Several miRNAs have been shown to cross-talk with Notch pathway. However, the role of miRNAs in the Notch pathway remains unclear. Therefore, in this article, we will discuss the effect of miRNAs in the Notch signaling pathway and their cross-talk in tumor development and progression.

3.1. miR-1

It has been well known that some miRNAs have tumor suppressor activity and are down-regulated in cancer. One such miRNA which belongs to tumor suppressor group is the miR-1. In several studies investigating the expression levels of miR-1, the authors have found that the miR-1 was markedly reduced in primary human hepatocellular carcinoma (HCC), prostate cancer, head and neck, and lung cancer [45–49]. Datta et al. have shown that ectopic expression of miR-1 inhibited HCC cell growth and reduced clonogenic survival [47]. In prostate cancer cell lines, transfection with *miR-1* represses the expression of its target genes exportin-6 and protein tyrosine kinase 9 [45]. Nasser et al. reported that re-expression of miR-1 in lung cancer cells reversed their tumorigenic properties, including growth, migration, clonogenic survival, and tumor formation in nude mice. The anti-tumor effect of miR-1 in lung cancer may be mediated through down-regulation of oncogenic targets, such as MET, Pim-1, FoxP1, and HDAC-4. Further, ectopic miR-1 expression was found to induce apoptosis in lung cancer cells in response to the potent anticancer drug doxorubicin, suggesting that miR-1 has potential therapeutic application against lung cancers [48]. Interestingly, the exon 1 and intron 1 of miR-1-1 was methylated in HCC cell lines and in primary human HCC [47]. Recently, it has been reported that miR-1 regulated Notch signaling pathway. Kwon et al. reported that miR-1 directly targets the Notch ligand delta in *Drosophila* for repression [50]. Recently, it has also been found that Dll-1 protein levels are negatively regulated by miR-1 in mouse embryonic stem cells [51]. These results suggest that miR-1 could regulate the Notch signaling pathway; however further in-depth research is needed in order to fully understanding how miR-1 regulate the Notch pathway.

3.2. miR-34

Another important miRNA is miR-34, which has been found to participate in p53 and Notch pathways regulation consistent with tumor suppressor activity [29,52]. In mammalians, the miR-34 family is composed of three processed miRNAs: miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript. It has been reported that the expression of miR-34a was lower or undetectable in pancreatic cancer, osteosarcoma, breast cancer and non-small cell lung cancer [53–56]. Recently, the inactivation of miR-34a was identified in cell lines derived from some tumors including lung, breast, colon, kidney, bladder, pancreas and melanoma [57]. More recently, the inactivation of miR-34b/c due to CpG methylation was found in malignant melanoma, colorectal cancer, and oral squamous cell carcinoma [58–60]. Moreover, lower levels of miR-34a expression was correlated with higher probability of relapse in non-small cell lung cancer (NSCLC), suggesting that miR-34a could work as a novel prognostic marker in NSCLC patients [61]. All published data to-date suggests that the inactivation of the miR-34 is a common event in human malignancies.

The reports from several groups have shown that the members of the miR-34 family could direct p53 signaling. Expectedly, ectopic miR-34 inhibited cell proliferation, colony formation, and caused a cell cycle arrest in the G1 phase [53,62]. Moreover, re-expression of miR-34a induced apoptotic cell death [52]. It has been suggested that miR-34-mediated apoptosis could be suppressed by inactivation of p53 gene. It was also documented that miR-34a could target several mRNAs, such as SIRT1, Bcl-2, N-myc, cyclin D1, leading to translational repression of these genes [53,63,64]. Recently, Li et al. reported that transfection of miR-34a to glioma cells down-regulated the protein expression of Notch-1, Notch-2, and CDK6 [26]. More recently, Ji et al. reported that human gastric cancer cells with miR-34 restoration reduced the expression of target gene Notch [28]. Very recently, the same group reported that Notch-1 and Notch-2 is downstream genes of miR-34 in pancreatic cancer cells. They found that restoration of miR-34 expression in the pancreatic cancer cells down-regulated Notch-1 and Notch-2 [29]. They also re-

ported that pancreatic cancer stem cells are enriched with tumor-initiating cells or cancer stem cells with high levels of Notch-1/2 and loss of miR-34. These results suggested that miR-34 may be involved in pancreatic cancer stem cell self-renewal, potentially via the direct modulation of downstream target Notch [29]. Taken together, it may be possible to restore miR-34 function for cancer therapeutic for which novel and innovative research is warranted.

3.3. miR-146

The miR-146 was previously reported to function as novel negative regulators that help to fine-tune the immune response. Konstantin et al. described the role for miR-146 in the control of Toll-like receptor and cytokine signaling through a negative feedback regulation loop involving inhibition of TNF receptor-associated factor 6 protein and IL-1 receptor-associated kinase 1 levels [65]. Recently, it has been found that miR-146a/b acts as terminal transducers of TLR4 signaling by targeting NF-κB activation by TLR4 [66]. They also demonstrated a decrease in miR-146b in adult T-cell Leukemia cells. The decrease in miR-146b may lead to increased inflammation and decreased T-reg functions, resulting in leukemia [66]. Very recently, miR-146a has been found to have the strongest predictive accuracy for stratifying prognostic groups and have also shown superiority in predicting overall survival in lung squamous cell carcinoma [67]. The miR-146 has been reported to cross-talk with breast cancer metastasis suppressor 1 (BRMS1), a predominantly nuclear protein that inhibits metastasis without blocking orthotopic tumor growth. Specifically, BRMS1 significantly up-regulates miR-146a and miR-146b in breast cancer cells. Transduction of miR-146a or miR-146b into breast cancer cells decreased expression of epidermal growth factor receptor, down-regulated NF-κB activity, inhibited migration and invasion *in vitro*, and suppressed lung metastasis in experimental xenograft models [68,69]. These provided experimental support suggesting that the modulation in the levels of miR-146 could have therapeutic value in inhibiting breast cancer metastasis. Very recently, miR-146a was found to regulate Numb in C2C12 cells [27], which is interesting because Numb is known to regulate Notch signaling negatively through interaction with Notch and the subsequent ubiquitin-mediated protein degradation. Indeed, Notch activation and the loss of Numb expression were found in a large proportion of breast carcinomas [70,71]. It has been reported that over-expression of Notch-1 stimulates NF-κB activity in several cancer cell lines [72] and since miR-146 also regulate NF-κB activity, it clearly suggest that miR-146 could regulate NF-κB through Notch mediated signaling pathway. However, the role of miR-146 in Notch signaling pathway need further innovative investigations.

3.4. miR-199

It has been reported that miR-199a was down-modulated in ovarian cancer [73]. Murakami et al. also found that miR-199a was down-regulated in hepatocellular cancer. Moreover, they found that over-expression of miR-199a can introduce cell cycle arrest in G2/M phase [74]. Recently, It was reported that miR-199a and miR-199b were down-regulated after 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent tobacco-specific carcinogen, treated rats up to 20 weeks [75]. Very recently, miR-199b-5p was seen to be a regulator of the Notch pathway through its targeting of the transcription factor Hes-1 in medulloblastoma (MB) tumors. Inhibition of Hes-1 by miR-199b-5p negatively regulated the MB cell growth. Moreover, over-expression of miR-199b-5p decreased the MB stem-like cells (CD133+) and also blocked expression of several cancer stem-cell genes. Further, the expression of miR-199b-5p in the non-metastatic cases was significantly higher than in the metastatic cases. The patients with high levels of miR-199b expression showed a better overall survival [30]. These results clearly suggest that miR-199 family could be very important in the regulation of multiple signaling pathways including Notch, and thus further in-depth studies are needed in order to clarify the biological significance and mechanisms on how miR-199 can regulate the Notch signaling pathway in human cancers.

3.5. miR-200

The microRNA-200 family has five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429. The miR-200c was down-regulated in benign or malignant hepatocellular tumors [76]. It has been shown that three miR-200 miRNAs (miR-200a, miR-200b and miR-429) are significantly associated with cancer recurrence and overall survival in ovarian tumors [77]. Recently many studies have shown that the miR-200 family regulates epithelial–mesenchymal transition (EMT) by targeting zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2 [78–81]. EMT is a process by which epithelial cells undergo remarkable morphological changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype. We have found that PDGF-D over-expression led to the acquisition of EMT phenotype in PC-3 prostate cells (PC3 PDGF-D cells) consistent with loss of miR-200 expression, and that the re-expression of miR-200b in PC3 PDGF-D cells led to the reversal of the EMT phenotype, which was associated with the down-regulation of ZEB1, ZEB2, and Snail2 expression [82]. Moreover, transfection of PC3 PDGF-D cells with miR-200b inhibited cell migration and invasion with concomitant repression of cell adhesion to the culture surface and cell detachment [82]. We also found that miR-200a, miR-200b, miR-200c, and many members of the tumor suppressor let-7 family were down-regulated in gemcitabine-resistant (GR) pancreatic cancer cells, which show the acquisition of EMT phenotype [83]. Furthermore, we have shown that miR-200 family regulates the expression of ZEB1, slug, E-cadherin, and vimentin, and thus the re-expression of miR-200 could be useful for the reversal of EMT phenotype to mesenchymal-to-epithelial transition [83]. We have found that the expression of both mRNA and protein levels of Notch-1 to -4, Dll-1, Dll-3, Dll-4, Jagged-2 as well as Notch downstream targets, such as Hes and Hey, were significantly higher in PC3 PDGF-D cells (unpublished data). More

importantly, we found that Notch-1 could be one of miR-200b targets because over-expression of miR-200b significantly inhibited Notch-1 expression (unpublished data). However, how the miR-200b regulates Notch gene expression will certainly require further in-depth investigations.

4. miRNA as targets by natural agents

Emerging experimental studies have shown that targeting miRNA could be a novel strategy for cancer prevention and/or treatment. There are several strategies that could be used for targeting the regulation of miRNAs, which could be useful tool for the inhibition of tumor progression and, as such, could be useful for therapy. One potential strategy could be the inactivation of oncogenic miRNAs. It has been found that 2′-O-methyl oligonucleotides or locked nucleic acid-modified oligonucleotides can block miRNA function. For example, using this anti-sense oligonucleotide, one could significantly decrease the activity of miR-21 as compared to control oligonucleotides [84]. Another strategy is to restore down-regulated miRNAs that function as tumor suppressors, such as let-7. It has been shown that over-expression of let-7 by using exogenously transfected pre-let-7 RNAs consistently showed reduction in the number of proliferating cells in lung and liver cancer cell lines [85]. This finding clearly suggests the possibility of restoration of tumor suppressor miRNAs toward cancer therapy. A third possible strategy could be the use of "natural agents" to target miRNAs that are known to contribute in the processes of tumor development and progression.

To that end, recent studies have shown that "natural agents" including curcumin, isoflavone, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), EGCG, and others could alter the expression of specific miRNAs, which may lead to increased sensitivity of cancer cells to conventional therapeutic agents, and thereby may result in the inhibition of tumor growth. We have found that alteration in the expression of miRNAs could be achieved by treating cancer cells with DIM or isoflavone. We have shown that treatment of Panc-1 or Colo-357 cells with B-DIM or genistein (isoflavone) showed decreased expression of the oncogenic miRNA such as miR-17, miR-20a, miR-106a, and increased the expression of the tumor suppressor miRNAs such as let-7, miR-16-1 [86]. Our results clearly suggest that "natural agents" may exhibit their anti-tumor effects through the regulation of miRNAs. Further support to this statement comes from findings reported by Sun et al. showing that curcumin could alter specific miRNA expression in human pancreatic cancer cells especially showing up-regulation of miR-22. They also found that up-regulation of miR-22 expression by curcumin in pancreatic cancer cells suppressed the expression of its target genes SP1 transcription factor (SP1) and estrogen receptor 1 (ESR1) [87]. Melkamu et al. reported that I3C can inhibit the expression of several oncogenic miRNAs, such as miR-21, miR-31, miR-130a, miR-146b, and miR-377 in vinyl carbamate treated animals. Further investigation showed that I3C up-regulated PTEN tumor suppressor gene though inhibition of miR-21 [49]. Tsang et al. recently reported that EGCG treatment could up-regulate the expressions of miR-16 in human hepatocellular carcinoma cells [88]. We also found that the expression of miR-200 and let-7 families could be up-regulated in gemcitabine resistant cells by DIM or isoflavone treatment as indicated above. Our results also showed that DIM treatment cause down-regulation of ZEB1, slug, and vimentin, and the morphologic reversal of EMT to epithelial morphology [83]. Considering the non-toxic characteristics of "natural agents", one could speculate that targeting miRNAs by "natural agents" could be a novel and safer approach for the prevention of tumor progression and/or treatment of human malignancies in the future.

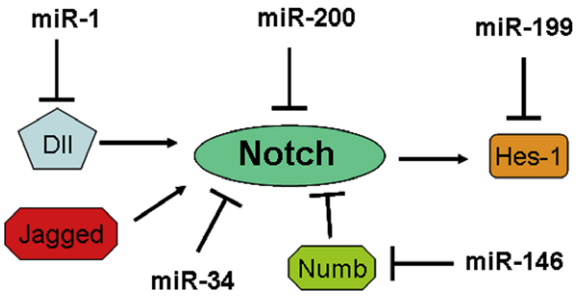

Fig. 2. Diagram of roles of miRNA in the Notch pathway.

5. Concluding remarks

In conclusion, we believe that the deregulation of miRNAs plays important roles in the development and progression of human cancers, and during the acquisition of EMT phenotype that are in part associated with the formation and maintenance of cancer stem cells (CSCs). Importantly, miRNAs have been characterized as biomarkers for diagnosis and prognosis, as well as targets for cancer therapy. Although emerging evidence suggest an interrelationship between miRNAs and Notch signaling pathway (Fig. 2), further research is warranted to ascertain the value of specific miRNA in the regulation of Notch signaling in order to exploit preventive and therapeutic strategies. Due to the non-toxic nature of "natural agents", we believe that targeting miRNAs by "natural agents" combined with conventional chemotherapeutics could be a novel and safer approach for the treatment of cancer. The findings reported in the short review article are very interesting; however, further investigations are needed in order to elucidate the roles of these and numerous other miRNAs that could be mechanistically linked with Notch and other cell signaling, and devising novel approaches on how "natural agents" could be useful in combination therapy for the prevention and/or treatment of human malignancies in the future.

Conflict of interest

None declared.

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Frequent Deregulations in the Hedgehog Signaling Network and Cross-Talks with the Epidermal Growth Factor Receptor Pathway Involved in Cancer Progression and Targeted Therapies

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***Abstract*——The hedgehog (Hh)/glioma-associated oncogene (GLI) signaling network is among the most important and fascinating signal transduction systems that provide critical functions in the regulation of many developmental and physiological processes. The coordinated spatiotemporal interplay of the Hh ligands and other growth factors is necessary for the stringent control of the behavior of diverse types of tissue-resident stem/progenitor cells and their progenies. The activation of the Hh cascade might promote the tissue regeneration and repair after severe injury in numerous organs, insulin production in pancreatic β-cells, and neovascularization. Consequently, the stimulation of the Hh pathway constitutes a potential therapeutic strategy to treat diverse human disorders, including severe tissue injuries; diabetes mellitus; and brain, skin, and cardiovascular disorders. In counterbalance, a deregulation of the Hh signaling network might lead to major tissular disorders and the development of a wide variety of aggressive and metastatic cancers. The target gene products induced through the persistent Hh activation can contribute to the self-renewal, survival, migration, and metastasis of cancer stem/progenitor cells and their progenies. Moreover, the pivotal role mediated through the Hh/GLI cascade during cancer progression also implicates the cooperation with other oncogenic products, such as mutated K-RAS and complex cross-talk with different growth factor pathways, including tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), Wnt/β-catenin, and transforming growth factor-β (TGF-β)/TGF-β receptors. Therefore, the molecular targeting of distinct deregulated gene products, including Hh and EGFR signaling components and other signaling elements that are frequently deregulated in highly tumorigenic cancer-initiating cells and their progenies, might constitute a potential therapeutic strategy to eradicate the total cancer cell mass. Of clinical interest is that these multitargeted approaches offer great promise as adjuvant treatments for improving the current antihormonal therapies, radiotherapies, and/or chemotherapies against locally advanced and metastatic cancers, thereby preventing disease relapse and the death of patients with cancer.**

I. Introduction

The hedgehog (Hh[1])/glioma-associated oncogene (GLI) developmental cascade is a highly evolutionarily conserved signaling pathway that serves critical functions in the regulation of the normal cell-fate specification, tissue polarity and patterning, and organogenesis during embryogenesis as well as the maintenance of the tissue homeostasis and repair after severe injuries in postnatal and adult life (Bak et al., 2003; McMahon et al., 2003; Cohen, 2003; Palma et al., 2005; Kasper et al., 2006a; Nielsen et al., 2008; Varjosalo and Taipale, 2008; Amankulor et al., 2009; Vaillant and Monard, 2009; Yauch et al., 2009). In particular, sonic hedgehog (SHH)/patched receptor 1 (PTCH1)/smoothened (SMO) coreceptor/GLI transcription factors are recognized as key players that provide a pivotal role in the stringent regulation of important cellular responses. The Hh signaling pathway, in conjunction with other developmental cascades, such as EGF/EGFR and Wnt/β-catenin, regulate the self-renewal ability versus differentiation; survival; intercellular and cell-matrix adhesion; and migration of diverse types of embryonic, fetal, and tissue-resident adult stem/progenitor cells and their progenies (Cohen, 2003; Beachy et al., 2004; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Katoh and Katoh, 2006; Liu et al., 2006; Sicklick et al., 2006; Zhou et al., 2006; Lin et al., 2007; Shi et al., 2008; Varjosalo and Taipale, 2008; Amankulor et al., 2009; Rittié et al., 2009). Conversely, the genetic abnormalities that belong to the Hh/GLI signaling pathway might result in an aberrant cell growth, differentiation, and migration concomitant with major tissue homeostatic imbalance and severe disorders (Bak et al., 2003; Cohen, 2003; Beachy et al., 2004; Kasper et al., 2006a; Liu et al., 2006; Varjosalo and

[1]Abbreviations: aa, amino acid(s); ABC, ATP-binding cassette; AG-1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; ALK5, activin receptor-like kinase 5; BCC, basal cell carcinoma; BM, bone marrow; BMI-1, polycomb group protein-1; CAF, cancer-associated stromal fibroblast; DHH, Desert hedgehog; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPC, endothelial progenitor cell; ERK, extracellular signal-regulated kinase; GANT58, 4-(2,4,5-tripyridin-4-ylthiophen-3-yl)pyridine; GANT61, 2,2′-[[dihydro-2-(4-pyridinyl)-1,3(2*H*,4*H*)pyrimidinediyl]bis(methylene)]bis[*N*,*N*-dimethyl]-benzenamine; GCP, granule cell precursor; GDC-0449, 2-chloro-*N*-[4-chloro-3-(2-pyridinyl)phenyl]-4-(methylsulfonyl)benzamide; GLI, glioma-associated oncogene; GLI3R, glioma-associated oncogene 3 receptor; GSK3b, glycogen synthase kinase 3b; Hh, hedgehog; Hhat, Hh acyltransferase; HHIP, hedgehog-interacting protein; IFT, intraflagellar transport; IHH, Indian hedgehog; KAAD-cyclopamine, 3-keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, extracellular-signal-regulated kinase kinase; mTOR, mammalian target of rapamycin; NBCCS, nevoid basal cell carcinoma syndrome; NF-κB, nuclear factor-κB; NVP-LDE-225, *N*-(6-((2*S*,6*R*)-2,6-dimethylmorpholino) pyridin-3-yl)-2-methyl-4′-(trifluoromethoxy)biphenyl-3-carboxamide; PD032590, *N*-[(2*R*)-2,3-dihydroxypropoxy]-3,4-difluoro-2-(2-fluoro-4-iodoanilino)benzamide; PD98059, 2′-amino-3′-methoxyflavone; PI_3K, phosphatidylinositol 3′ kinase; PTCH1, patched receptor 1; RTK, receptor tyrosine kinase; SB431542, 4-(5-benzo(1,3)dioxol-5-yl-4-pyridin-2-yl-1*H*-imidazol-2-yl)benzamide; SHH, sonic hedgehog; siRNA, small interference RNA; SMO, smoothened; SP, side population; SUFU, suppressor of fused; TGF-β, transforming growth factor-β; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; Wnt, Wingless ligand.

Taipale, 2008; Vaillant and Monard, 2009). The disorders associated with inherited or somatic alterations in the Hh signaling network include holoprosencephaly, the embryonic defect most often seem in these disorders, in which the forebrain and the face fail to develop; congenital ataxia; microcephaly; mental retardation; brain, skin, ocular and pancreatic disorders; and pediatric and adult cancer development (Ming et al., 1998; Odent et al., 1999; Bale, 2002; Bak et al., 2003; Cohen, 2003; Beachy et al., 2004; Maity et al., 2005; Lau et al., 2006; Vaillant and Monard, 2009).

Numerous studies have shown that the genetic and/or epigenetic alterations leading to the enhanced expression levels and/or activities of Hh signaling elements in stem/progenitor cells commonly occur in a wide variety of human cancers during etiopathogenesis and disease progression to locally invasive and metastatic stages (Berman et al., 2003; Cohen, 2003; Beachy et al., 2004; Rubin and de Sauvage, 2006; Taniguchi et al., 2007; Bhattacharya et al., 2008; Mimeault and Batra, 2008a; Mimeault et al., 2008; Tada et al., 2008; Varjosalo and Taipale, 2008; Schnidar et al., 2009; Yang et al., 2010; Mimeault and Batra, 2010c). Human cancer types frequently harboring a deregulation in the Hh pathway include leukemia, multiple myeloma, and brain, skin, head and neck, lung, liver, gastrointestinal, colorectal, pancreatic, prostate, mammary, ovarian, and renal carcinomas (Berman et al., 2003; Thayer et al., 2003; Karhadkar et al., 2004; Oniscu et al., 2004; Sanchez et al., 2004; Sheng et al., 2004; Ohta et al., 2005; Datta and Datta, 2006; Douard et al., 2006; Mimeault et al., 2006, 2007a; Bian et al., 2007; Chen et al., 2007b; Stecca et al., 2007; Taniguchi et al., 2007; Bhattacharya et al., 2008; Hegde et al., 2008; Tada et al., 2008; Eichenmüller et al., 2009). More importantly, accumulating lines of evidence have also revealed that the persistent activation of the Hh cascade may represent a critical step in the malignant transformation of cancer stem/progenitor cells (also designated as cancer- and metastasis-initiating cells), treatment resistance, and disease relapse (Liu et al., 2006; Bar et al., 2007; Clement et al., 2007; Ehtesham et al., 2007; Mimeault et al., 2007b, 2008; Peacock et al., 2007; Mimeault and Batra, 2008a, 2010b,c; Xu et al., 2008; Kobune et al., 2009; Ward et al., 2009). The sustained activation of the Hh signal transduction pathway might lead, in an autocrine or a paracrine manner, to the modulation of the expression levels and/or activities of numerous target gene products (Cohen, 2003; Beachy et al., 2004; Douard et al., 2006; Eichberger et al., 2006; Kasper et al., 2006a; Li et al., 2006; Clement et al., 2007; Feldmann et al., 2007; Bhattacharya et al., 2008; Mimeault and Batra, 2008a; Mimeault et al., 2008; Varjosalo and Taipale, 2008; Klarmann et al., 2009; Laner-Plamberger et al., 2009; Liao et al., 2009; Park et al., 2009). These signaling elements can contribute to the proliferation, survival, migration, invasion, and metastasis of cancer cells. Moreover, multiple cross-talks between the Hh cascade and other tumorigenic signaling components, including receptor tyrosine kinases (RTKs) such as EGFR, can cooperate during cancer initiation and progression to aggressive, invasive, and metastatic disease stages (Xie et al., 2001; Bigelow et al., 2005; Palma et al., 2005; Kasper et al., 2006b; Mimeault et al., 2006; Riobo et al., 2006a; Stecca et al., 2007; Schnidar et al., 2009; Seto et al., 2009).

In this review article, the most recent advancements on the structural and functional characterization of diverse signal transduction elements of Hh signaling network and molecular mechanisms involved in their regulation are described. The physiological functions mediated through the Hh signaling pathway during embryonic development and adult life are reviewed. The frequent deregulations in the Hh signaling network associated with diverse diseases and cancer development, and potential interactive cross-talk with other developmental cascades, including EGFR, are also discussed. The results from recent studies underlining the therapeutic interest of cotargeting Hh and EGFR pathways and other oncogenic cascades for reversing treatment resistance, eradicating the cancer stem/progenitor cells and their progenies, and improving the current clinical therapies against aggressive and metastatic cancers are also reviewed.

II. The Hedgehog Signal Transduction Pathway and Regulatory Mechanisms

It is important to understand the regulation of the Hh signaling network at the molecular level in the normal adult stem/progenitor cells and their progenies as well as how it is deregulated during carcinogenesis. This knowledge will allow us to identify new drug targets and develop novel therapeutic strategies to block this tumorigenic cascade and thus improve the current cancer treatments. Many efforts made in the last few years have led to the structural and functional characterization of diverse Hh signaling components that can contribute in a cell type- and concentration-dependent manner to the signal transduction. Important information has also been obtained about complex regulatory mechanisms that modulate the Hh-induced cellular responses in normal and pathological conditions (McMahon et al., 2003; Cohen, 2003; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Kasper et al., 2006a; Varjosalo and Taipale, 2008). In this matter, we describe the structural features and functions of Hh ligands, PTCH1 receptor, SMO coreceptor, and GLI transcription factors in mediating the Hh signal transduction and cellular responses as well as the molecular mechanisms implicated in the regulation of their functions.

A. Structures and Mechanisms of Actions of Hedgehog Ligands

The *Hh* gene has been first identified to control the segmentation pattern of fruit fly *Drosophila melano-*

gaster during embryogenesis (Nüsslein-Volhard and Wieschaus, 1980). Subsequent investigations have led to the identification of three *Hh* homologous genes in mammalian tissues encoding three different Hh proteins (Marigo et al., 1995). The Hh proteins include SHH, Indian hedgehog (IHH), and Desert hedgehog (DHH) (Marigo et al., 1995). All three mammalian Hh proteins are able to specifically bind the PTCH1 receptor and activate the Hh pathway in a time- and concentration-dependent manner (Pathi et al., 2001). The specific and redundant biological functions of Hh proteins is governed in part by their expression patterns and diverse regulatory mechanisms in a given cell type (Pathi et al., 2001). Among them, the SHH protein is the most extensively studied and characterized ligand of the Hh signaling pathway. Human SHH protein shows a similarity of 92.4% in the amino acid sequence with its murine homolog (Marigo et al., 1995). It has been shown that the SHH protein plays key roles in controlling organogenesis and morphogenesis of a variety of tissues and organs and epithelial-mesenchymal interactions during the vertebrate embryonic development as well as in the regulation of adult stem/progenitor cell behavior (Cohen, 2003; Beachy et al., 2004; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Katoh and Katoh, 2006; Liu et al., 2006; Zhou et al., 2006; Lin et al., 2007; Shi et al., 2008; Varjosalo and Taipale, 2008; Amankulor et al., 2009; Rittié et al., 2009).

1. Structural Organization, Processing and Secretion of the Sonic Hedgehog Ligand. The human *Shh* gene maps to chromosome 7 in the region 7q36 and consists of a DNA sequence of 9410 base pairs that encompass three exons (Marigo et al., 1995). Human SHH ligand is synthesized under a 462-amino acid (aa) protein precursor of approximately 45 kDa designated as preproprotein (Fig. 1) (Odent et al., 1999). The preproprotein is composed of a 23-aa signal peptide sequence, a 174-aa signaling domain, and a 265-aa autoprocessing domain endowed with an autoproteolysis activity and a cholesterol transferase activity (Fig. 1). During the post-translational processing of preproprotein in the endoplasmic reticulum, the short N-terminal hydrophobic signal peptide sequence is removed by a signal peptidase. The SHH precursor then undergoes an autocatalytic intramolecular cleavage at position 198 catalyzed by its C-terminal domain, yielding an N-terminal signaling product of approximately 19 kDa, which represents the mature and biologically active SHH form, and a C-terminal product with no known signaling function (Fig. 1) (Porter et al., 1995; Cohen, 2003; Varjosalo and Taipale, 2008). During this reaction, a cholesterol moiety is covalently attached at the C-terminal residue of the cleaved N-terminal signaling fragment of SHH (Cohen, 2003; Varjosalo and Taipale, 2008). Moreover, the N-terminal cysteine residue of the cleaved N-terminal signaling fragment of SHH is also modified by palmitoylation (Fig. 1) (Buglino and Resh, 2008). It has been reported that a stable attachment of a fatty acid [a palmitate molecule catalyzed by a palmitoylacyltransferase, Hh acyltransferase (Hhat)] might occur on both the SHH precursor and SHH protein along the secretory pathway (Fig. 1) (Buglino and Resh, 2008). These hydrophobic lipid modifications of mature SHH protein might promote its interaction with caveolin, tethering at the plasma membrane within caveolin- and cholesterol-enriched lipid raft microdomains, designated as caveolea, and thereby increase its local concentration and the efficiency of signal transduction (Karpen et al., 2001; Cohen, 2003; Mao et al., 2009). Hence, the mature and lipid-modified SHH protein resulting from intracellular processing may be secreted from cells into the extracellular compartment and mediated its biological effects on responsive cells (Fig. 1).

2. Autocrine and Paracrine Mechanisms of Actions of Hedgehog Ligands. The secreted SHH ligand can act, in autocrine and paracrine manners, under the form of monomers and/or oligomers on producing cells and responsive cells localized near or at a distant localization of the secreting cells (Fig. 1). In fact, the secreted SHH protein and other Hh ligands, IHH and DHH, can diffuse and act as morphogens by forming a concentration gradient for short- and long-range actions (Fig. 1) (Cohen, 2003; Varjosalo and Taipale, 2008; Vyas et al., 2008). In this regard, the results from a recent study have also indicated that the full-length unprocessed SHH protein can traffic to the plasma membrane and thereby participate in a localized manner to certain short-range effects (Tokhunts et al., 2010). More specifically, the paracrine signals mediated through the Hh cascade require the release of the membrane-tethered Hh ligands from producing cells and their transport to the surrounding-responsive cells or more distant cells, including the stromal cells (Fig. 1) (Cohen, 2003; Vyas et al., 2008; Dierker et al., 2009a,b). This diffusion process, which is accomplished through the formation of large nanoscale oligomers by ligand molecules, might be modulated through different molecular mechanisms. In particular, the release of Hh ligand oligomers from producing cells may be promoted via their interaction with a 12-pass transmembrane protein known as dispatched, and cell-surface heparan sulfate proteoglycans (Fig. 1) (Cohen, 2003; Dierker et al., 2009a,b). The formation of large Hh ligand oligomers may permit their release into the extracellular compartment and transport via the lipoprotein carriers over a long distance, where they can act in a paracrine fashion on surrounding cells (Fig. 1) (Vyas et al., 2008; Dierker et al., 2009a,b). Conversely, a negative regulatory feedback loop might also be induced through the enhanced expression of an endogenous Hh inhibitor, hedgehog-interacting protein (HHIP), found at the plasma membrane, that can interact with a high affinity with the three Hh ligands (Chuang and McMahon, 1999; Cohen, 2003; Varjosalo and Taipale, 2008; Bosanac et al., 2009). This molecular event would im-

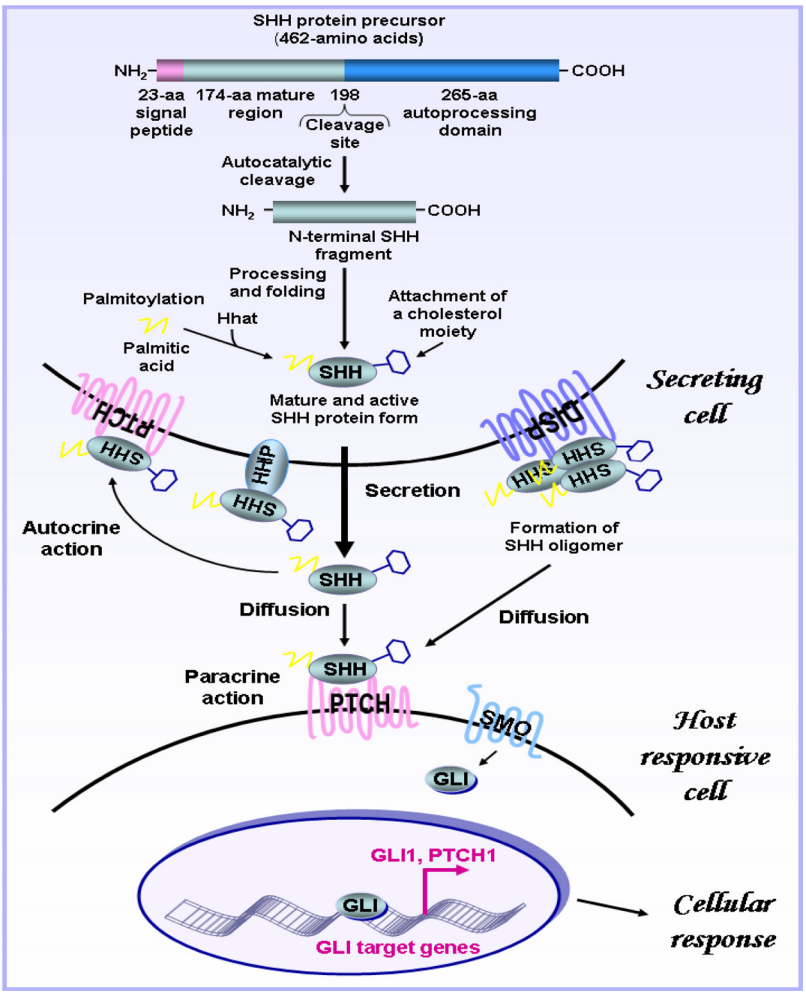

FIG. 1. Schematic representation of the molecular events associated with cellular processing, lipid modification, and secretion of the SHH protein and the autocrine and paracrine actions of mature and secreted SHH protein. The scheme shows the molecular mechanisms associated with the cellular processing of the SHH protein precursor, including the cleavage of its N-terminal signal peptide fragment. The autocatalytic cleavage at the position 198 of SHH protein precursor catalyzed by its C-terminal fragment, which results in the release of cleaved N-terminal and C-terminal products, is also shown. Moreover, the lipid modifications of cleaved N-terminal SHH fragment via the attachment of a cholesterol moiety at the C-terminal position and a palmitate molecule at the N-terminal position catalyzed by the palmitoylacyltransferase Hhat are also indicated. The secretion of the mature and lipid-modified SHH protein into extracellular space as well as its diffusion and potential autocrine or paracrine action on secreting and neighboring responsive cells are also illustrated. In addition, the function of dispatched (DISP) transmembrane protein in the formation of large SHH oligomers and their secretion in extracellular compartment is illustrated. Moreover, the inhibitory effect on the SHH actions induced through the sequestration of cell surface-associated SHH molecules by HHIP is shown.

pede the binding of Hh ligands to the PTCH1 receptor and inhibit the signal transduction (Fig. 1).

B. Hedgehog Signal Transduction

A simplified view of the stimulation of the Hh signaling network implicates the binding of a secreted Hh protein, including SHH, IHH, or DHH ligand to its cognate 12-pass transmembrane PTCH1 or PTCH2 receptor on responsive cells (Fig. 1) (Stone et al., 1996; Carpenter et al., 1998; Fuse et al., 1999; Kalderon, 2000; Taipale et al., 2002; Cohen, 2003). The PTCH1, which is the better characterized receptor of Hh ligands, displayed 54% sequence homology with PTCH2 protein. Despite the fact that all three Hh ligands can bind both PTCH1 and PTCH2, the specific functions of these receptors depend in part of their expression pattern (Carpenter et al., 1998; Cohen, 2003). In general, the binding of a Hh ligand to PTCH1 relieves the repressive effect induced by this receptor on the activity of its signaling partner, a seven-pass transmembrane coreceptor, SMO protein (Kalderon, 2000; Taipale et al., 2002; Cohen, 2003; Rubin and de Sauvage, 2006; Varjosalo and Taipale, 2008). The stimulation of the SMO signaling transduction element results in the activation of cytoplasmic GLIs and their translocation to the nucleus, where they participate with other transcription factors in the stringent regulation of the expression of numerous Hh target gene products (Fig. 1) (Cohen, 2003; Kasper et al., 2006a; Kasai et al., 2008; Varjosalo and Taipale, 2008; Jia et al., 2009; Yue et al., 2009).

Although work has been done to characterize the different signaling elements of the canonical Hh cascade, the molecular events and signaling molecules involved in the repressive effect induced through the PTCH1

receptor on SMO activity in the absence of the Hh ligand and stimulation of the SMO protein in the presence of the Hh ligand remain not precisely established. Different models of the molecular mechanisms of Hh signal transduction have been proposed to explain the repressive effect induced by PTCH1 in the absence of Hh ligand on SMO activity and the activation of SMO coreceptor after the formation of Hh ligand-PTCH1 complexes (Fig. 2) (Taipale et al., 2002; Rubin and de Sauvage, 2006; Rohatgi and Scott, 2007). In general, it has been proposed that the binding of the Hh ligand, including SHH protein, to PTCH1 might result in a SMO conformational change from inactive to active state (Taipale et al., 2002; Rubin and de Sauvage, 2006; Rohatgi and Scott, 2007). More specifically, the formation of the Hh ligand-PTCH1 receptor complexes might indirectly stimulate the SMO activity, possibly through the induction of membrane changes, activation of intracellular positive modulators, and/or stimulation of an endogenous SMO agonist (Fig. 2a and b) (Rosenbaum and Witman, 2002; Taipale et al., 2002; Bijlsma et al., 2006; Corcoran and Scott, 2006; Rubin and de Sauvage, 2006; Rohatgi and Scott, 2007; Rohatgi et al., 2007).

In view of the fact that the PTCH1 receptor contains a sterol-sensing domain and shows a structural homology with diverse family members of membrane transporters, such as Niemann-Pick C1 protein and bacterial proton-driven transmembrane molecular transporters, it has been proposed that the PTCH1 receptor can act as a transmembrane transporter of small molecules (Davies et al., 2000; Strutt et al., 2001; Taipale et al., 2002; Corcoran and Scott, 2006; Rubin and de Sauvage, 2006). Then, the PTCH1 transporter, unbound by Hh ligand, could pump the endogenous molecules, such as cholesterol derivatives, including oxysterols, out of cells (Fig. 2b). The binding of Hh ligand to PTCH1, however, could lead to the intracellular accumulation of endogenous molecules, including oxysterols, that, in turn, can positively modulate the SMO activity (Fig. 2b) (Corcoran and Scott, 2006; Dwyer et al., 2007).

Consistent with these models, it has been observed that the activating mutations in the SMO protein or inactivating mutations in the PTCH1 receptor might lead to the adoption of a constitutively active conformation by the SMO protein (Fig. 2, c and d) (Johnson et al., 1996; Raffel et al., 1997; Rubin and de Sauvage, 2006). Moreover, it has been observed that the sterol synthesis inhibitors reduced SHH induced-target gene transcription and blocked SHH pathway-dependent proliferation of medulloblastoma cells (Corcoran and Scott, 2006). The inhibitory effect induced by the sterol inhibitors, however, could be reversed by a treatment of medulloblastoma cells with exogenous cholesterol or specific oxysterols (Corcoran and Scott, 2006). In addition, different SMO full agonists (such as the synthetic chlorobenzothiophene-containing SMO agonist termed SAG) and antagonists (including a plant-derived steroidal alkaloid, cyclopamine) have been shown to specifically interact with the heptahelical bundle of the SMO protein, and thereby modulate its activity and cellular response (Fig. 2e and f) (Chen et al., 2002; Frank-Kamenetsky et al., 2002; Corcoran and Scott, 2006; Rubin and de Sauvage, 2006). It is noteworthy that recent accumulating lines of experimental evidence have also indicated that the primary cilium found in Hh responsive cells might play a critical role in the activation of Hh signal transduction in certain normal and cancer cell types.

1. Roles of the Primary Cilium in the Hedgehog Signal Transduction Mechanism. Recent studies have revealed that an extracellular projection found at the cell surface, designated as primary cilium, which is a microtubule-based organelle, constitutes a key specialized structure that is required to concentrate the Hh signaling components and trigger the SMO-mediated canonical pathway in certain types of SHH-responsive cells (Fig. 3) (Rosenbaum and Witman, 2002; Corbit et al., 2005; Haycraft et al., 2005; Rohatgi and Scott, 2007; Rohatgi et al., 2007; Han et al., 2008; Spassky et al., 2008; Bailey et al., 2009; Han et al., 2009; Veland et al., 2009; Wong et al., 2009). More specifically, it has been shown that the PTCH1 receptor unbound by the SHH ligand is localized at the base of the primary cilium and can prevent the SMO ciliary localization (Fig. 3) (Rohatgi et al., 2007). Moreover, all three full-length GLI proteins as well as the negative regulator of GLI activities, suppressor of fused (SUFU) are also colocalized at the distal tip of cilium in the absence of the Hh ligand (Fig. 3) (Haycraft et al., 2005; Rohatgi et al., 2007). The binding of secreted SHH protein to the PTCH1 receptor found at the primary cilium, however, might result in a decreased number of PTCH1 molecules in the primary cilium because of its re-localization at the cell surface out of the ciliary structure, its internalization in intracellular vesicles, and/or degradation (Rohatgi et al., 2007). Hence, the exclusion of PTCH1 molecules from the ciliary structure may allow SMO molecules to translocate to the plasma membrane in the primary cilium, and thereby lead to the activation of downstream GLI transcriptional factors and Hh target gene expression (Fig. 3) (Corbit et al., 2005; Rohatgi et al., 2007).

Although the molecular mechanisms by which the formation of Hh ligand-PTCH1 complexes results in an increase of SMO levels into the primary cilium are not precisely established, it has been reported that the C-terminal sequence motif of the SMO protein, which is constituted of hydrophobic and basic residues, is required for its transport to the primary cilium and activation in certain types of cultured cells (Corbit et al., 2005). In support with this, the occurrence of mutations in this C-terminal domain of SMO protein has been observed to prevent its ciliary translocation and subsequent GLI activation (Corbit et al., 2005). Moreover, it has been observed that the SMO full agonist, SAG, or small regulatory molecules, including oxysterols, can

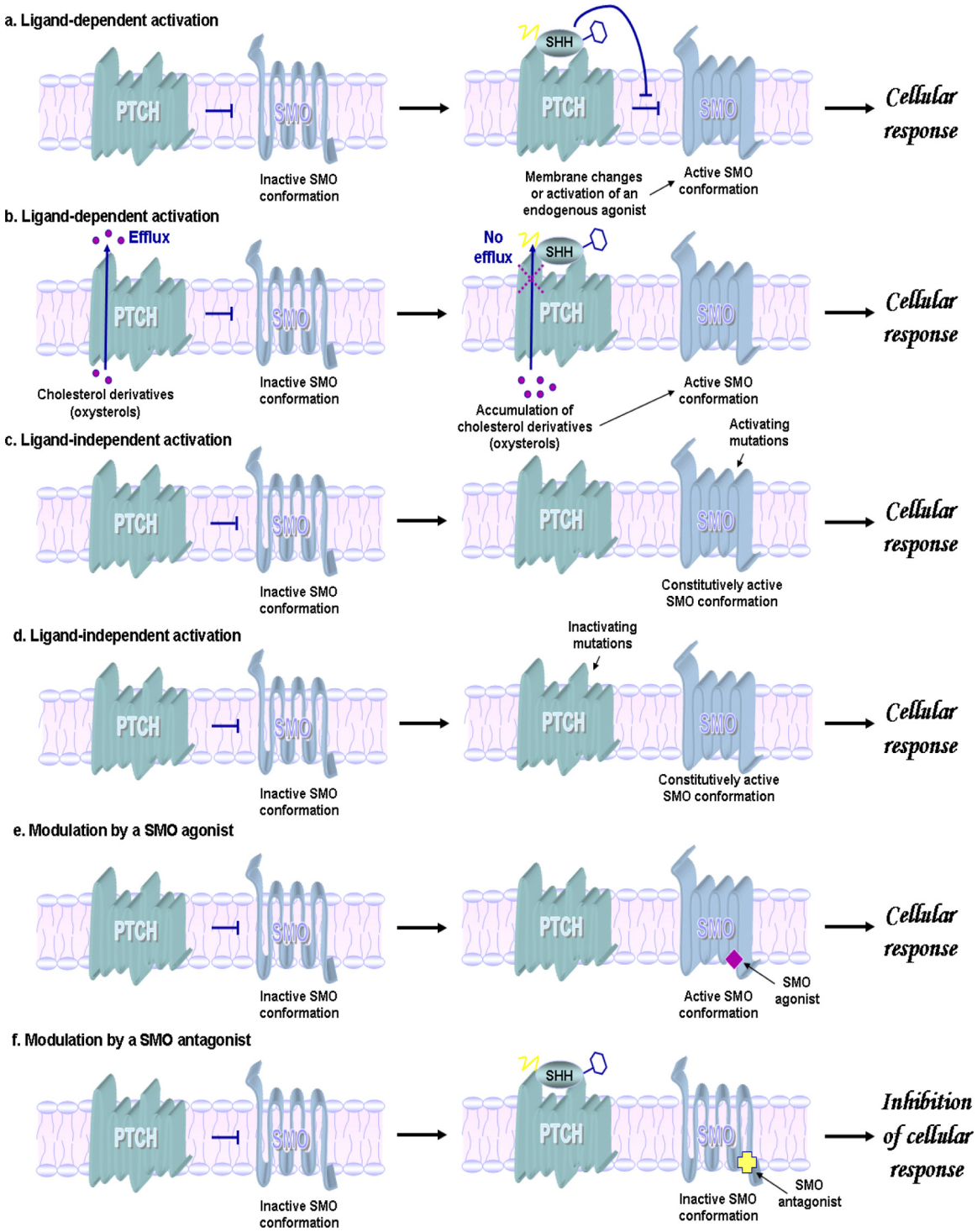

FIG. 2. Proposed models of the molecular mechanisms involved in the regulation of ligand-dependent and independent-SMO activation and modulation by the pharmacological agents. a, the binding of SHH protein to the PTCH1 transmembrane receptor might lead to either membrane changes or activation of an endogenous SMO agonist. These molecular events, in turn, may result in the adoption of an active conformation by the SMO transmembrane protein and the stimulation of SMO-mediated cellular response. b, in the absence of SHH ligand, the PTCH1 receptor can act as a transporter and pump the endogenous cholesterol derivatives, such as oxysterols, out of the cells. The binding of the SHH protein to PTCH1 receptor, however, might inhibit the efflux of cholesterol derivatives such as oxysterols, and thereby promote the adoption of an active conformation by the SMO protein and SMO-induced cellular response. The occurrence of activating mutations in the SMO oncoprotein (c) or inactivating mutations in the PTCH1 tumor suppressor protein (d) might result in the adoption of an active conformation by SMO protein in the absence of SHH ligand and a sustained induction of a cellular response. In the same way, the exposure of cells to a pharmacological agent acting as a SMO agonist (e) also can induce the adoption of an active conformation by the SMO protein and a cellular response. In contrast, the exposure of cells to a chemical compound acting as a SMO antagonist (f), such as cyclopamine, KAAD-cyclopamine, IPI-269609, or GDC-0449, can inhibit the SHH protein-induced SMO activation and cellular response.

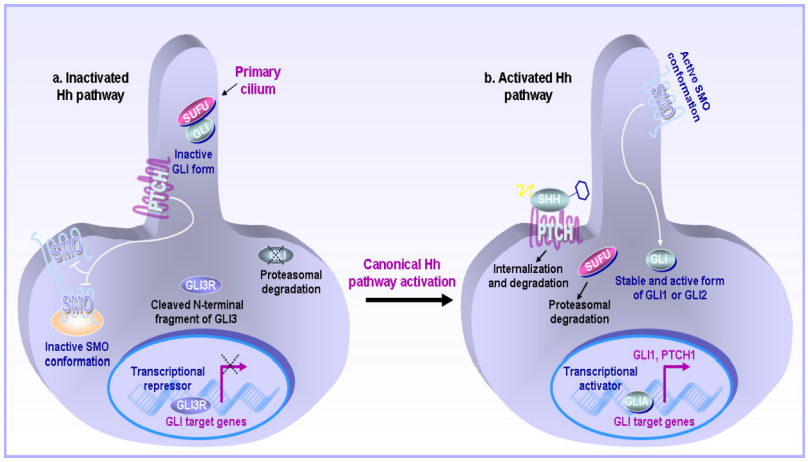

FIG. 3. Schematic representation of molecular events associated with the repressive effect induced by PTCH1 receptor on the SMO activity and the activation of the Hh signaling pathway mediated by the SHH protein in the primary cilium. a, in the absence of the SHH ligand, PTCH1 is localized at the base of the ciliary structure and inhibits SMO protein translocation to the primary cilium and its activation. In the absence of activated SMO protein, the negative modulator of Hh cascade SUFU sequesters full-length GLI proteins in the cytoplasm and prevents their nuclear translocation, Hh target gene expression, and induction of a cellular response. Moreover, the cytoplasmic GLI proteins also may be degraded through the proteasomal pathway, and the GLI3 protein cleaved into a C-terminal fragment (GLI3R) that acts as a nuclear transcriptional repressor of Hh gene expression. b, the binding of the mature and lipid-modified SHH protein to the PTCH1 receptor in the primary cilium leads to its translocation out of the ciliary structure and retrieves its repressive effect on the SMO protein localized in intracellular vesicles or plasma membrane out of the primary cilium. These molecular events culminate in SMO translocation into the primary cilium and activation of downstream signaling elements, GLI proteins localized in the primary cilium. The negative modulator of GLI proteins, SUFU protein, is then degraded by the proteasomal pathway, and the activated GLI zinc-finger transcriptional activators, GLI1 or GLI2 molecules, are translocated to nucleus and participate to the up-regulation of Hh target gene expression, including *GLI1* and *PTCH1*, and induction of a cellular response.

induce the SMO translocation to the primary cilium and activate the Hh signaling cascade (Dwyer et al., 2007; Rohatgi et al., 2007). Hence, the endogenous molecules, such as oxysterols (which are cholesterol derivatives), might represent important factors that indirectly regulate the Hh signaling transduction by modulating the ciliary translocation and activation of the SMO protein. Therefore, the use of specific pharmacological agents that are able to interfere with the sterol synthesis constitutes then a potential therapeutic approach to modulate the Hh pathway in pathophysiological conditions, including cancers (Corcoran and Scott, 2006).

Consistent with the critical role of primary cilium in the Hh signal transduction, it has been reported that the conditional ablation of primary cilium on the surface of granule cell progenitors in mice disrupted SHH-mediated expansion of granule cell precursors (GCPs) and cerebellar development (Han et al., 2008; Spassky et al., 2008). Moreover, the mutations in intraflagellar transport (IFT) proteins, which are essential for the primary cilium assembly, formation, and maintenance, also resulted in major defects in mouse neural tube patterning and reduced the expression of the Hh downstream genes *GLI-1* and *PTCH1* reminiscent of deregulated Hh signaling (Huangfu et al., 2003; Liu et al., 2005a). The loss of IFT proteins was also associated with alterations in the proteolytic processing of the GLI3 protein that abrogated its repressor function in mice (Haycraft et al., 2005; Liu et al., 2005a). Thus, IFT proteins seem to be able to provide different functions, including the maintenance of the ciliary structure as well as the regulation of processing, activator, and repressor activities of the GLI proteins in the Hh pathway.

It has also been observed that high levels of SMO and GLI2 and low levels of PTCH are detected in the primary cilium of PANC-1 and CFPAC-1 pancreatic cancer cells, whereas the nuclear level of the GLI3R repressor form is low, suggesting that an autonomous activation of Hh cascade might be prevalent in these cancer cells (Nielsen et al., 2008). Moreover, it has also been observed that the occurrence of activating mutations in the SMO protein might promote its translocation to the ciliary structure and GLI activation in the absence of the Hh ligand (Corbit et al., 2005). The SMO antagonist cyclopamine has also been shown to inhibit the translocation of the SMO protein from the intracellular compartment to the primary cilium, and thereby prevent the biological effects induced by downstream effectors, GLI proteins (Corbit et al., 2005). Hence, the relocalization of the SMO protein in appropriate subcellular compartments, such as the primary cilium, might be a determinant factor that governs the dynamic process of Hh pathway activation and GLI induced-target gene expression in certain Hh-responsive cells. In particular, the localization of the SMO protein in primary cilium may be an important factor that contributes to the aberrant activation of the Hh cascade in certain cancer cell types.

2. Functions of Glioma-Associated Oncogene Transcription Factors in Modulating Hedgehog Target Gene Expression. The GLI family comprises three nuclear zing-finger transcription factors, GLI1, GLI2, and GLI3, that contain conserved C2-H2 zing finger domains and can specifically interact with the DNA sequences encompassing a GACCACCCA motif found in target gene promoters (Kinzler et al., 1987; Kinzler and Vogelstein, 1990; Ruppert et al., 1990; Matise and Joyner, 1999;

Park et al., 2000; Cohen, 2003; Kasper et al., 2006a; Ruiz i Altaba et al., 2007; Varjosalo and Taipale, 2008; Tsanev et al., 2009). The GLI proteins can cooperate with distinct nuclear cofactors in the regulation of the expression levels of Hh target gene products. More particularly, the full-length GLI proteins can act as transcriptional activators and induce the expression of target genes, whereas the N-terminal fragment of GLI proteins, generated after processing and intracellular proteolytic cleavage, can act as a transcriptional repressor (Figs. 3 and 4) (Matise and Joyner, 1999; Sasaki et al., 1999; Cohen, 2003; Kasper et al., 2006a; Ruiz i Altaba et al., 2007; Varjosalo and Taipale, 2008; Tsanev et al., 2009). Because the full-length GLI1 transcription factor does not contain a repressor domain, it consequently acts as a strong transcriptional activator (Matise and Joyner, 1999; Kasper et al., 2006a; Ruiz i Altaba et al., 2007). Although GLI2 and GLI3 proteins contain both activator and repressor domains, GLI2 has been observed to act principally as a transcriptional activator, whereas GLI3 acts mainly as a repressor of the target gene expression (Matise and Joyner, 1999; Sasaki et al., 1999; Cohen, 2003; Kasper et al., 2006a; Ruiz i Altaba et al., 2007; Varjosalo and Taipale, 2008; Tsanev et al., 2009). The cellular processing of full-length 190-kDa GLI3 protein involves its phosphorylation by protein kinase A followed by an intracellular proteolytic cleavage that generates an 83-kDa N-terminal fragment of GLI3 that acts as a potent transcriptional repressor, GLI3R (Fig. 4) (Wang and Li, 2006). It has been observed that the processing of full-length GLI3 protein into its repressor form is promoted in the absence or at low levels of the SHH protein (Fig. 3) (Wang et al., 2000; Litingtung et al., 2002; Huangfu et al., 2003). Hence, the balance between the cellular levels of GLI3R repressor form versus GLI1 and GLI2 transactivators determines the final outcome on Hh target gene expression in a given cell type.

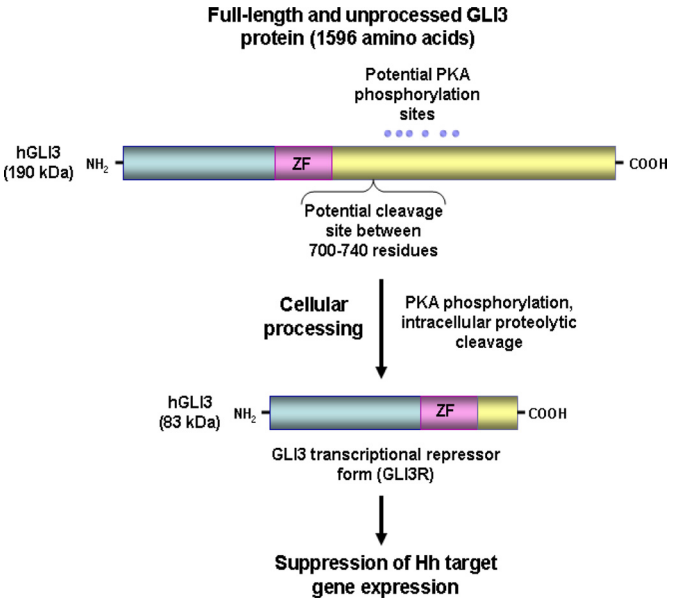

FIG. 4. Schematic representation of structural features of human GLI3 protein and molecular events associated with its processing into a transcriptional repressor. The scheme shows the positions of the zinc finger DNA-binding domain (ZF), the six potential sites of the phosphorylation by protein kinase A (PKA) (asterisks) identified by mutagenesis analyses, and the intracellular cleavage site of the full-length GLI3 protein. The processing of the full-length 190-kDa GLI3 protein, which implicates its phosphorylation by PKA followed by its intracellular proteolytic cleavage, yielding an N-terminal fragment of GLI3, is also illustrated. The cleaved N-terminal fragment of GLI3 of approximately 83 kDa (GLI3R) can act as a transcriptional repressor and inhibit the Hh target gene expression.

In general, the Hh ligand-dependent activation of the Hh cascade leads to the inhibition of SUFU protein by SMO and the nuclear translocation of GLI1 and/or GLI2 transcriptional activators that, in turn, up-regulate the expression levels of numerous Hh target genes in a cell-type and context-dependent manner (Fig. 3) (Kinzler et al., 1987; Kinzler and Vogelstein, 1990; Ruppert et al., 1990; Cohen, 2003; Kasper et al., 2006a; Rahnama et al., 2006; Yue et al., 2009). The Hh target gene products include GLI1 as well as PTCH1 and HHIP, which constitute the positive and negative feedback mechanisms involved in the regulation of Hh cascade, respectively (Rahnama et al., 2006). Other up-regulated Hh gene products also comprise bone morphogenic protein-1, cyclins D1 and D2, JUN transcription factor, and polycomb ring finger oncogene, BMI-1, that can act by down-regulating $p16^{INK4A}$ and forkhead box M1 transcription factor, which in turn can contribute to the up-regulation of c-Myc and BMI-1 expression (Douard et al., 2006; Eichberger et al., 2006; Kasper et al., 2006a; Li et al., 2006; Clement et al., 2007; Feldmann et al., 2007; Bhattacharya et al., 2008; Varjosalo and Taipale, 2008; Laner-Plamberger et al., 2009; Park et al., 2009). Moreover, the Hh activation also results in an up-regulated expression of Wingless ligands (Wnts), Notch ligand JAG2, interleukin-1 receptor type 2, snail, antiapoptotic factors such as Bcl-2, ATP-binding cassette (ABC) multidrug transporters, and CXC chemokine receptor 4 (Eichberger et al., 2006; Kasper et al., 2006a; Clement et al., 2007; Sims-Mourtada et al., 2007; Katoh and Katoh, 2010). The regulation of the Hh cascade is also influenced by diverse external and internal stimuli and interactive cross-talk with other signaling pathways initiated by diverse growth factors.

C. Regulatory Mechanisms of the Hedgehog Ligand Expression, Glioma-Associated Oncogene Activities and Hedgehog Ligand-Dependent and -Independent Activation of the Hedgehog Pathway

The stimulation of different growth factor and cytokine pathways and genetic and epigenetic alterations during embryonic development; tissue regeneration; and repair after severe injury, chronic inflammation, and cancer development may activate different intracellular signaling elements (Hingorani et al., 2005; Koga et al., 2008). These signaling components include nuclear factor-κB (NF-κB), phosphatidylinositol 3′ kinase (PI_3K)/

Akt and/or K-RAS that can contribute to increase the cellular expression level of Hh ligands, including SHH protein, GLI activities and Hh signaling activation (Fig. 5) (Schmidt-Ullrich et al., 2006; Amankulor et al., 2009; Kasperczyk et al., 2009; Cui et al., 2010). More specifically, it has been shown that EGF can up-regulate SHH protein expression and secretion through the activation of PI_3K/Akt signaling components in gastric parietal cells and thereby stimulate gastric acid secretion (Stepan et al., 2005). Moreover, the activation of macrophages caused by severe tissue injury and inflammation might result in the production of diverse pro-inflammatory cytokines that, in turn, can up-regulate SHH transcriptional expression (Amankulor et al., 2009). As a matter of fact, it has been shown that the activation of NF-κB might occur after inflammatory stimuli (such as tumor necrosis factor-α, interleukin-1α, and lipopolysaccharide) and during cancer progression (Nakashima et al., 2006; Schmidt-Ullrich et al., 2006; Kasperczyk et al., 2009; Cui et al., 2010). Thereby, the activated NF-κB can specifically interact with human *SHH* promoter, up-regulate the SHH expression level in a variety of normal and malignant cells, and contribute to their proliferation and survival in vitro and in animal models in vivo (Nakashima et al., 2006; Schmidt-Ullrich et al., 2006; Kasperczyk et al., 2009; Cui et al., 2010). A hypomethylation status of the *SHH* region promoter has also been associated with an up-regulation of its transcriptional expression during breast cancer progression suggesting a potential epigenetic regulation of the *SHH* expression under specific physiological and pathological conditions (Yakushiji et al., 2007; Cui et al., 2010).

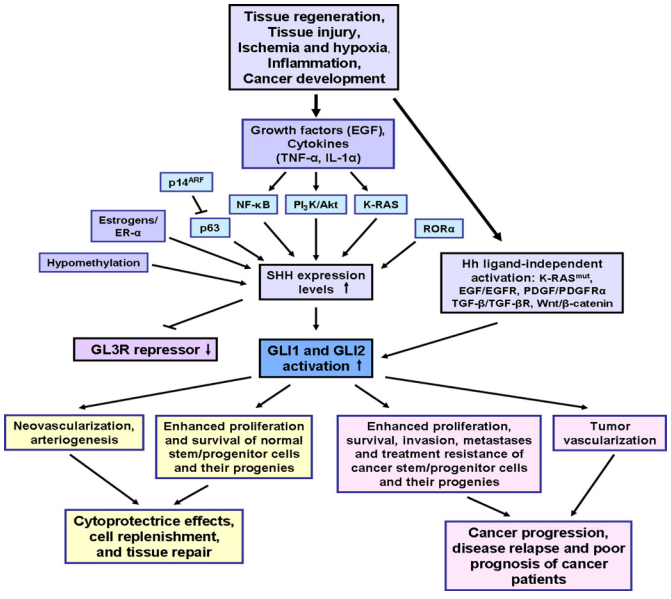

FIG. 5. Cellular events and signaling elements involved in the regulation of SHH expression, GLI activation, and mediation of the Hh activation-induced cellular response. The increase of the SHH expression, which might be induced during tissue regeneration in homeostatic conditions, and after an adaptive response to tissue injury, ischemia and hypoxia, chronic inflammation, and cancer progression, is indicated. The potential cellular signaling elements involved in the regulation of the SHH expression are also indicated. These intracellular signaling components include NF-κB, PI_3K/Akt, and K-RAS, which might be induced through the stimulation of different growth factor and cytokine signaling pathways in normal and cancer cells. The possibility of SHH-dependent and -independent activation of GLI1 and GLI2 transcriptional activators by different growth factor pathways is also indicated. In addition, the potential biological effects induced through stimulation of GLI protein-induced Hh target gene expression in normal and cancer cells are also indicated. ER-α, estrogen receptor α; PDGF, platelet-derived growth factor; RORα, retinoid-related orphan receptor α; TNF-α, tumor necrosis factor-α.

Among other potential regulators of the SHH expression, the overexpression of the p63 protein, which is a homolog of the p53 tumor suppressor protein and is known to provide a regulatory role in the maintenance of epithelial stem cells and tumorigenesis, has been shown to interact with the *SHH* promoter and up-regulate its expression (Fig. 5) (Caserta et al., 2006). It has also been noticed that the transactivation of the *SHH* gene by p63 protein could be inhibited by $p14^{ARF}$ tumor suppressor protein (Caserta et al., 2006). Moreover, the orphan nuclear receptor RORα has been shown to be able to interact with the *SHH* promoter and to promote the recruitment of other coactivators, including β-catenin and p300 (Gold et al., 2003). Thereby, these nuclear factors can cooperate to up-regulate the *SHH* expression in GCPs and contribute to their proliferation during cerebellar development (Gold et al., 2003). The activation of estrogen/estrogen receptor-α axis has also been reported to up-regulate the *SHH* expression and proliferation of breast cancer cells (Koga et al., 2008).

In addition, a growing body of experimental evidence has revealed that different signaling elements might negatively or positively modulate the expression level, stability, and activity of GLI proteins and influence the cellular responses mediated through the canonical Hh cascade (Cohen, 2003; Haycraft et al., 2005; Kasper et al., 2006a; Ruiz i Altaba et al., 2007; Varjosalo and Taipale, 2008). These regulatory mechanisms include changes at the transcriptional and post-transcriptional levels, subcellular localization, phosphorylation status, and stability versus degradation of GLI proteins. Among the important negative regulators of the Hh pathway, SUFU protein can bind, stabilize, and retain the three GLI proteins in the cytoplasm in the absence of Hh ligands and thereby prevent their activation and nuclear translocation (Kogerman et al., 1999; Kasper et al., 2006a; Ruiz i Altaba et al., 2007). It has consistently been shown that the inactivation of SUFU by mutations, gene targeting, or small interference RNA (siRNA) is sufficient to up-regulate the GLI-induced Hh gene expression in normal or cancer cells (Taylor et al., 2002; Varjosalo et al., 2006). Moreover, the activation of the SHH signaling cascade also might promote the ubiquitination and proteasomal degradation of SUFU molecules in normal and cancer cells and thereby contribute to the Hh ligand-mediated cell growth (Yue et al., 2009). The negative regulation of GLI proteins is also

regulated by protein kinase A and glycogen synthase kinase 3b (GSK3b) that can phosphorylate, destabilize, and inactivate GLI proteins (Mizuarai et al., 2009).

On the other hand, the positive regulatory mechanisms might be induced in the presence or absence of the Hh ligand. As mentioned previously, the activation of canonical Hh cascade might lead to a feedback loop in which the nuclear GLI2 or GLI3 transactivators can directly interact with the *GLI1* promoters and up-regulate its expression (Dai et al., 1999; Cohen, 2003; Ikram et al., 2004; Haycraft et al., 2005; Kasper et al., 2006a; Varjosalo and Taipale, 2008). Likewise, the increase of GLI1 expression level may result in an up-regulation of the GLI2 level through an indirect mechanism that does not involve the transactivation of the *GLI2* promoter by the GLI1 protein (Regl et al., 2002). In this regard, the results from recent studies have also revealed that the SUFU function and/or GLI expression, stability, and/or transcriptional activity in normal and cancer cells may be positively modulated via the persistent stimulation of different growth factor cascades. These signaling pathways include EGF/EGFR, Wnt/β-catenin, and the TGF-β1/TGF-βR system, which can cooperate with the canonical Hh ligand-induced signaling to activate GLI proteins and Hh target gene expression (Figs. 5 and 6) (Xie et al., 2001; Bigelow et al., 2005; Palma et al., 2005; Kasper et al., 2006b; Riobo et al., 2006a; Dennler et al., 2007, 2009; Stecca et al., 2007; Schnidar et al., 2009; Seto et al., 2009). For instance, it has been shown that TGF-β protein can up-regulate *GLI1* and *GLI2* expression and thereby contribute to the acquisition of a more malignant behavior by cancer cells (Dennler et al., 2007, 2009). More specifically, the activation of TGF-β/TGF-βR1-ALK5 system might result in the nuclear translocation of Smad3-Smad4 complexes that directly interact with the *GLI2* promoter and promote the recruitment of β-catenin. Then, these nuclear factors can cooperate to up-regulate the *GLI2* expression, which in turn can induce the transactivation of Hh target genes, including *GLI1* (Fig. 6) (Dennler et al., 2009). Hence, the integration of these diverse mechanisms of negative and positive regulation of the Hh cascade determines the biological effect induced in a given cell type.

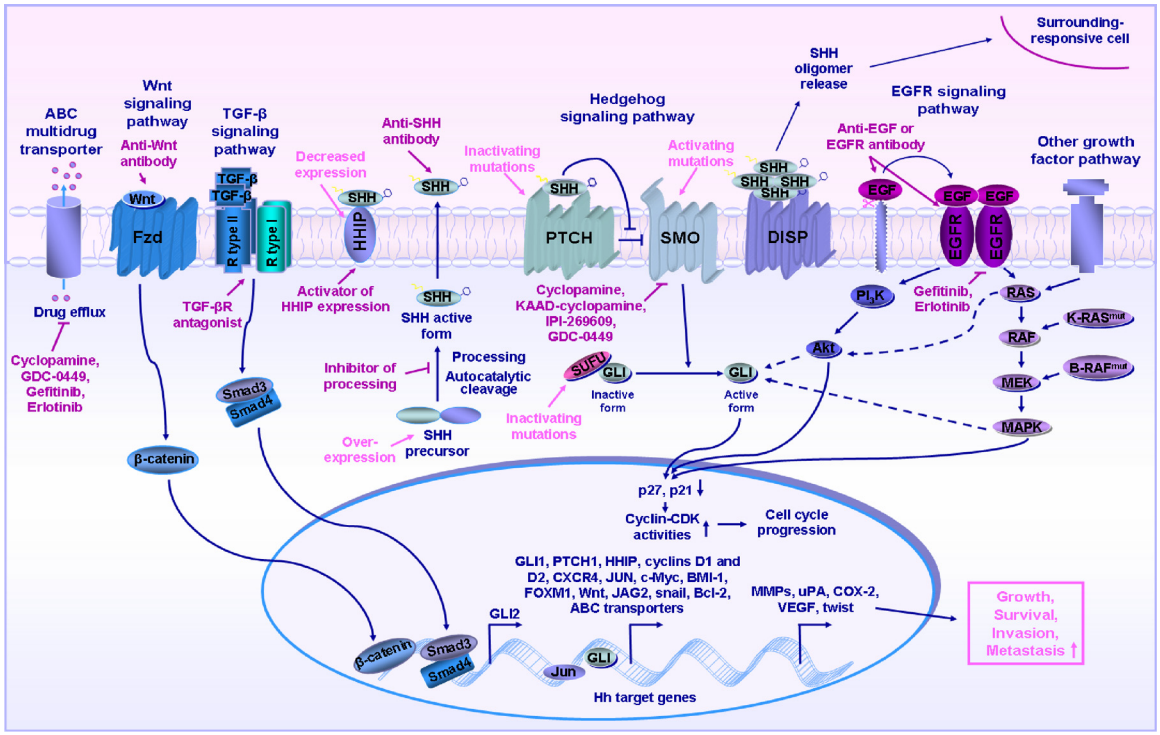

FIG. 6. Scheme showing the signaling elements and frequent deregulations in the Hh signaling network and potential cross-talk with the EGFR signaling pathway involved in the malignant behavior of cancer cells. The molecular events associated with the cellular processing of the SHH precursor into a biologically active form via autocatalytic cleavage and lipid modifications are illustrated. Autocrine and paracrine stimulation of the cancer cells by the monomeric and multimeric SHH molecules is also illustrated. The repressive effect of SUFU on the GLI activity is shown. Moreover, the frequent deregulations, including overexpression of the SHH ligand; inactivating mutations in HHIP, PTCH, or SUFU; or activating mutations in SMO coreceptor, which may contribute to cancer development, are also indicated. The potential stimulatory effect induced by the activation of EGFR pathway and oncogenic mutations in K-RAS^{mut} and B-RAF^{mut} on the GLI transcriptional activity is indicated. The target gene products induced through the activation of Hh and EGFR signaling pathways are also described. Moreover, the stimulatory effect induced through the activation of TGF-β/TGF-βR/Smad3-Smad4 and Wnt/β-catenin on the *GLI2* expression is illustrated. In addition, the potential inhibitory effect induced by diverse pharmacological agents, such as a mAb directed against SHH ligand, EGF, EGFR, and Wnt, selective inhibitors of SMO (cyclopamine, KAAD-cyclopamine, IPI-269609, or GDC-0449), EGFR tyrosine kinase activity (gefitinib and erlotinib), TGF-β type I activin receptor-like kinase, and ALK5 (SB431542) are also indicated. COX2, cyclooxygenase 2; CXCR4, CXC chemokine receptor 4; FOXM1, forkhead box M1 transcription factor; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

III. Critical Functions of the Hedgehog Signaling Pathway during Embryonic and Postnatal Development and Adult Life and Their Therapeutic Implications

In mammals, the interplay of diverse growth factor pathways, including Hh/GLI, EGF/EGFR, Wnt/β-catenin, and TGF-β/TGF-β receptors, is involved in the stringent control of the tissue patterning and organogenesis during embryogenesis and fetal development as well as the tissue homeostasis and repair after severe injuries and inflammation in the postnatal period or adulthood (Cohen, 2003; Beachy et al., 2004; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Liu et al., 2006; Zhou et al., 2006; Shi et al., 2008; Varjosalo and Taipale, 2008; Amankulor et al., 2009). Among them, Hh proteins play critical roles by acting as potent morphogens, mitogens, and survival factors for a variety of cell types (including pluripotent embryonic stem/progenitor cells and multipotent tissue-resident adult stem/progenitor cells) in a time- and concentration-dependent manner (Cohen, 2003; Beachy et al., 2004; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Liu et al., 2006; Zhou et al., 2006; Shi et al., 2008; Varjosalo and Taipale, 2008; Amankulor et al., 2009). Despite the fact that complex molecular mechanisms are involved in the regulation of the Hh signaling network in normal physiological conditions, the inactivation or hyperactivation of the Hh cascade might lead to severe congenital diseases and hyperproliferative disorders in postnatal life, including cancer development (Ming et al., 1998; Odent et al., 1999; Bale, 2002; Bak et al., 2003; Cohen, 2003; Beachy et al., 2004; Maity et al., 2005; Vaillant and Monard, 2009).

Numerous loss- and gain-function studies of Hh signaling elements carried out with transgenic mice, animal models of diseases and cell cultures have provided important insights into the critical roles of Hh pathway during embryonic and postnatal development and along life span in adulthood. Analyses of *SHH*-null mice have particularly indicated that numerous defects occur in diverse structures during embryonic development and result in a rapid perinatal lethality of *SHH* mutants compared with wild-type mice (Chiang et al., 1996; Lu et al., 2000; Ishibashi and McMahon, 2002; Rallu et al., 2002). These defects consist of the absence of distal limb structures and the spinal column; ventral cell types within the neural tube; reduced size of dorsoventral structures of telencephalon and diencephalon; abnormalities in skin development, including hair follicle morphogenesis; and cyclopia, which refers to the presence of a single eye in the center of the face. In contrast, the conditional null alleles of the *SHH* or *SMO* genes resulted in only minor brain patterning abnormalities, whereas the number of neural progenitors in both the postnatal subventricular zone and the dentate gyrus of hippocampus was dramatically reduced and was associated with a marked increase in programmed cell death (Machold et al., 2003).

In addition, the gain-of-function approaches, in vitro and in vivo up-regulation, or exogenous application of the SHH protein have also given complementary information about SHH functions in the tissue patterning and the early and later stages of neurogenesis during embryogenesis until postnatal development and adult brain maturity (Gaiano et al., 1999; Lu et al., 2000; Charytoniuk et al., 2002b; Machold et al., 2003; Vaillant and Monard, 2009). Specifically, it has been observed that an up-regulation of SHH expression in mice promoted the proliferation of GCPs and oligodendrocyte specification in telencephalon and resulted in an enhanced number of differentiated oligodendrocytes in the embryonic and postnatal cerebellar region (Lu et al., 2000; Nery et al., 2001; Machold et al., 2003). In same way, the loss-of function mutations in *PTCH* tumor suppressor gene or activating mutations in *SMO* oncogene also led to phenotypic changes as observed for mice overexpressing SHH protein (Hahn et al., 1999, 2000). It has been observed that SHH target genes are aberrantly activated in heterozygous *PTCH*(−/+) knockout mice, and these mice have a higher tendency to develop nevoid basal cell carcinoma syndrome (NBCCS), and a variety of cancers including cerebellar and skin tumors compared with wild-type mice (Goodrich et al., 1997). In fact, the NBCCS, also known as Gorlin syndrome or basal cell nevus syndrome, is an autosomal-dominant disorder associated with inherited inactivating mutations in the PTCH receptor. Patients with NBCCS exhibit a variety of developmental defects accompanied by a predisposition to develop a variety of postnatal disorders and cancer types, such as basal cell carcinomas (BCCs), medulloblastoma, ovarian dermoid and fibroma, meningioma, fibrosarcoma, rhabdomyosarcoma, and cardiac fibroma (Hahn et al., 1996, 1999; Goodrich et al., 1997; Zurawel et al., 2000). Hence, these observations suggest that the SHH cascade supplies important roles for patterning of a variety tissues during embryonic development and maintenance of cellular functions and organ integrity after the birth, and more particularly in the embryonic and postnatal development of brain. Therefore, the alterations in the Hh cascade might cause severe human disorders and cancers in numerous tissues and organs.

A. Functions of the Hedgehog Cascade in Adult Tissues and Their Therapeutic Implications

The analyses of the expression patterns of human Hh proteins have revealed that SHH, IHH, and DHH are differently expressed in different adult tissues and organs, in homeostatic conditions, and during tissue regeneration and repair after severe injury (Pathi et al., 2001; Bak et al., 2003; Nielsen et al., 2004; Sicklick et al., 2005, 2006; Spicer et al., 2009; Vaillant and Monard, 2009). Consequently, they can contribute to the mediation of specific functions dependent of their expression

levels in a given cell type. Among them, the SHH protein is required for the regulation of multiple key cellular events in a wide range of adult tissue and organ types including bone marrow (BM), central nervous system and peripheral nerves, cardiovascular system, and epithelial tissues such as skin, lung, liver, gastrointestinal tract, pancreas, prostate, breast, and ovary (McMahon et al., 2003; Nielsen et al., 2004; Paladini et al., 2005; Katoh and Katoh, 2006; Sicklick et al., 2006; Vaillant and Monard, 2009). It has been shown that Hh proteins can promote the proliferation of diverse multipotent tissue-resident adult stem/progenitor cells, including hematopoietic, neural, skin, cochlear, gastrointestinal, hepatic, pancreatic, and mammary stem/progenitor cells, and thereby participate in cell replenishment and tissue regeneration and repair after severe injuries (Bhardwaj et al., 2001; Machold et al., 2003; McMahon et al., 2003; Nielsen et al., 2004; Ahn and Joyner, 2005; Paladini et al., 2005; Sicklick et al., 2005; Katoh and Katoh, 2006; Lau et al., 2006; Liu et al., 2006; Sicklick et al., 2006; Lin et al., 2007; Mimeault and Batra, 2008c). Hence, the SHH protein plays important roles in the replenishment of cells that are lost during tissue turnover and injuries. For instance, the activation of the SHH pathway is required for the maintenance of hair follicle stem/progenitor cells found in bulge areas. The SHH protein can cooperate with EGF in controlling the follicular growth and cycling, including the transition from the resting phase (telogen) to the growth phase (anagen), and participate in the skin regeneration after injury (Paladini et al., 2005; Kasper et al., 2006b; Rittié et al., 2009). The stimulation of the SHH pathway might also contribute to the long-term repopulating of epidermal progenitors after severe wounding (Levy et al., 2007). Therefore, the activation of Hh cascade by topical application of exogenous SHH protein or its synthetic Hh agonist might represent a potential therapeutic strategy to treat diverse skin disorder associated with a decreased proliferation of epidermal and epithelial cells and hair cycle defects (Paladini et al., 2005).

On the other hand, the analyses of the expression levels of Hh signaling components in mice, rat, and bovine ovaries have also revealed that IHH and DHH RNAs were detected in granulosa cells (Spicer et al., 2009). In contrast, PTCH1 and SMO transcripts were detected in thecal-interstitial cells of small versus large follicles of cattle and PTCH1 and GLI expression levels enhanced after ligand stimulation, suggesting a paracrine mechanism of the Hh system in follicular development (Spicer et al., 2009). Furthermore, the activation of the Hh signaling pathway was also associated with an enhanced proliferation and steroidogenesis, including androgen production in mammalian ovarian cells (Spicer et al., 2009). In this matter, we are reporting in a more detailed manner the specific functions provided by the Hh proteins in other adult tissues, including the maintenance of the adult pancreas, BM, brain, and cardiovascular system and their therapeutic implications.

1. Functions of Hedgehog Proteins in the Pancreas and Their Therapeutic Implications. The Hh proteins provide important functions in the regulation of pancreatic morphogenesis during embryonic development as well as the ductal epithelial cell regeneration and maintenance of the pancreatic β-cell mass and regulation of insulin production in adult pancreas (Thomas et al., 2000; Kim and Hebrok, 2001; Lau et al., 2006; Mimeault and Batra, 2008a; Parkin and Ingham, 2008). In particular, IHH and DHH, SMO, and PTCH1 are expressed in adult pancreatic islets of Langerhans, and the activation of Hh cascade in adult pancreatic β-cells may result in a transcriptional activation of islet duodenum homeobox-1, *IDX-1*, also designated as *PDX-1*, that in turn, can interact with the insulin promoter and up-regulate its expression (Thomas et al., 2000; Kim and Hebrok, 2001). Hence, the stimulation of the Hh cascade may represent a potential therapeutic strategy to up-regulate the IDX-1-induced insulin expression and maintain normal glucose homeostasis, and thereby treat diverse disorders, including diabetes mellitus. In counterbalance, the enhanced expression of SHH and IHH in pancreatic ductal epithelial cells in the exocrine compartment, which can be induced through the NF-κB activation during inflammation, however, might result in development of chronic pancreatitis and cancer (Kayed et al., 2003, 2004; Lau et al., 2006; Nakashima et al., 2006; Morton et al., 2007; Mimeault and Batra, 2008a; Parkin and Ingham, 2008). These observations support, then, the therapeutic interest of targeting the NF-κB or SHH pathway to treat inflammatory disorders of the pancreas such as pancreatitis and pancreatic cancer.

2. Functions of the Sonic Hedgehog Protein in the Bone Marrow and Their Therapeutic Implications. Numerous external and internal stimuli are involved in the stringent control of BM-resident hematopoietic stem/progenitor cells and hematopoiesis in homeostatic conditions and after injury (Trowbridge et al., 2006; Kiuru et al., 2009; Merchant et al., 2010). In particular, it has been proposed that the activation of the Hh signaling pathway might induce the expansion of primitive BM-resident hematopoietic cells under homeostatic conditions and during acute regeneration (Trowbridge et al., 2006). Moreover, the SHH protein can cause changes in endosteal hematopoietic stem cell niche, including osteoblasts, and thereby alter early lymphoid differentiation (Kiuru et al., 2009). It has also been shown that SHH protein or oxysterols, certain naturally occurring oxygenated derivatives of cholesterol, including 20(*S*)-hydroxycholesterol, can induce the antiadipogenic and osteogenic effects on multipotent BM-stromal cells by activating the canonical Hh pathway through SMO signaling element (Kim et al., 2007a, 2010; Amantea et al., 2008). In fact, the osteogenic effect induced through the stimulation of the Hh cascade seems to be mediated, at

least in part, via the enhanced expression of gene products associated with the Notch (HES-1, HEY-1, and HEY-2) and Wingless (Dkk-1) pathways (Amantea et al., 2008; Kim et al., 2010). Hence, the stimulation of the Hh cascade by the SHH protein or oxysterols in BM-resident stromal cells may constitute a potential approach to induce their osteogenic differentiation and bone-forming properties. This strategy could be used to treat diverse osteopenic disorders, such as the osteoporosis resulting from an increase of the adipocyte differentiation concomitant with a decrease of bone formation by osteoblasts occurring during chronological aging, which constitute the major causes of morbidity and mortality in elderly persons.

3. Functions of the Sonic Hedgehog Cascade in the Postnatal Developing and Adult Brain and Their Therapeutic Implications. In postnatal development and adult brain, the SHH protein has been shown to provide critical roles in neurogenesis occurring in the subventricular zone and hippocampal dentate gyrus, the zones known as the niches of adult neural stem/progenitor cells in mice and human in homeostatic conditions and after brain injuries (Pola et al., 2001; Calcutt et al., 2003; Palma and Ruiz i Altaba, 2004; Palma et al., 2005; Sicklick et al., 2005, 2006; Alvarez-Medina et al., 2009; Bambakidis et al., 2009; Gulino et al., 2009; Sims et al., 2009; Wang et al., 2009a). More specifically, it has been shown that the SHH protein can display neuroprotective effects and cooperate with EGF to induce the proliferation of neural stem/progenitor cells in the subventricular zone and generate new olfactory interneurons in mice in vivo (Palma and Ruiz i Altaba, 2004; Palma et al., 2005). Furthermore, the SHH protein, which is synthesized and secreted by the Purkinje cells in the postnatal developing cerebellum and adult brain, can also promote, in a paracrine manner, the activation of the GLI-mediated canonical pathway and proliferation of GCPs (Wechsler-Reya and Scott, 1999; Charytoniuk et al., 2002a). It is noteworthy that reactive astrocytes in an injured cerebral cortex can also produce and secrete the SHH protein, which can stimulate $Oligo^{2+}$ expressing progenitor cells (Amankulor et al., 2009). Hence, $Oligo^{2+}$ GCPs can give rise to mature oligodendrocytes that contribute to re-myelination of injured axons. These observations support the therapeutic interest of stimulating the SHH pathway for treating diverse brain defects and injuries, neurodegenerative disorders, and cerebral cortical injuries such as multiple sclerosis (Bambakidis et al., 2009; Gulino et al., 2009; Zhang et al., 2009).

4. Functions of the Hedgehog Cascade in the Cardiovascular System and Their Therapeutic Implications. Numerous accumulating lines of evidence have also indicated that the activation of Hh signaling cascade may promote the neovascularization process after severe ischemic injuries (Pola et al., 2001; Kusano et al., 2005; Asai et al., 2006; Lee et al., 2007; Lavine et al., 2008; Renault et al., 2009; Sims et al., 2009). More specifically, it has been shown that the SHH protein plays a critical role in coronary development and can promote the formation of coronary vessels in the embryonic and adult heart. Moreover, it has been shown that Hh signaling molecules are expressed in human peripheral monocytes, and the SHH protein induces the migration of monocytes in blood samples from control patients, but it does not induce a chemotactic effect on monocytes from diabetic patients with coronary artery disease (Dunaeva et al., 2010). The impaired response of diabetic patients to the SHH protein has been associated with a strong transcriptional up-regulation of the PTCH1 receptor, which can negatively regulate the SMO transducer activity. In addition, the SHH protein can also contribute to the neoangiogenesis process by promoting the proliferation, migration, and vascular endothelial growth factor production via the PI_3K/Akt signaling pathway in BM-derived endothelial progenitor cells (EPCs), which might be recruited to injured tissues (Fu et al., 2006).

It is of therapeutic interest that the exogenous administration of the SHH protein or *SHH* gene transfer has been shown to induce angiogenesis and accelerate the repair of ischemic brain injury, acute and chronic myocardial ischemia, and skeletal muscle ischemia in animal models in vivo (Pola et al., 2001; Kusano et al., 2005). Moreover, a strategy consisting of topically applied *SHH* gene therapy also accelerated the cutaneous wound healing in a diabetic mouse model in vivo, in least in part, via the stimulation of dermal fibroblasts and indirectly by enhancing the recruitment of BM-derived EPCs at damaged skin, which in turn promoted microvasculature remodeling and wound repair (Asai et al., 2006). Hence, the stimulation of the Hh signaling pathway might represent a potential strategy to promote neoangiogenesis and arteriogenesis and thereby prevent diverse cardiovascular disorders such as ischemic injury and heart failure.

Despite great clinical interest to stimulate the Hh cascade to treat diverse human disorders, it is noteworthy that additional investigations are required to confirm the therapeutic benefit of this strategy versus its detrimental effect on normal adult stem/progenitor cells, including the potential induction of their malignant transformation and cancer development.

IV. Critical Functions of the Hedgehog Signaling Pathway in the Malignant Transformation of Cancer- and Metastasis-Initiating Cells and Their Progenies

The sustained activation of the Hh signaling pathway in tissue-resident adult stem/progenitor cells and their progenies has been proposed to represent a potential event that may contribute to their malignant transformation during cancer initiation and progression (Thayer et al., 2003; Cohen, 2003; Beachy et al., 2004; Karhadkar et al., 2004; Oniscu et al., 2004; Sanchez et al., 2004;

Ohta et al., 2005; Datta and Datta, 2006; Mimeault et al., 2006, 2007a,b, 2008; Bian et al., 2007; Chen et al., 2007b; Clement et al., 2007; Ehtesham et al., 2007; Peacock et al., 2007; Stecca et al., 2007; Bhattacharya et al., 2008; Mimeault and Batra, 2008a; Schnidar et al., 2009). More specifically, inherited or somatic inactivating mutations in the *PTCH1* or *SUFU* tumor suppressor gene leading to a loss-of-function and/or activating mutation in *SMO* oncogene that aberrantly activates Hh signal transduction may result in the development of diverse cancers (Fig. 6) (Dahmane et al., 1997; Reifenberger et al., 1998; Taylor et al., 2002; Berman et al., 2003; Beachy et al., 2004; Sheng et al., 2004; Douard et al., 2006). These cancer types include BCCs and common pediatric tumors such as medulloblastoma, a highly malignant tumor derived from cerebellar granule neuron progenitor cells, and rhabdomyosarcoma, a tumor that originates in the soft tissues of the body, including the skeletal muscles, tendons, and connective tissues (Dahmane et al., 1997; Reifenberger et al., 1998; Taylor et al., 2002; Berman et al., 2003; Ruiz i Altaba et al., 2004; Beachy et al., 2004). It has also been reported that the gene encoding HHIP, an endogenous Hh inhibitor, may be transcriptionally silenced by hypermethylation and chromatin remodeling in diverse cancers, such as gastrointestinal and hepatocellular carcinomas, and thereby contribute to the persistent activation of the Hh cascade (Taniguchi et al., 2007; Tada et al., 2008; Eichenmüller et al., 2009). In addition, an up-regulation of expression levels and/or activities of Hh signaling elements, including Hh ligands, SMO coreceptor, and GLI proteins often occurs during cancer initiation, and progression to locally invasive and metastatic disease stages. The overexpression of Hh signaling elements might result in the sustained growth and enhanced invasive properties of malignant cells in multiple myeloma, melanoma, glioma, gastrointestinal tract, pancreatic, hepatic, small-cell lung, prostate, mammary, and ovarian cancers (Thayer et al., 2003; Beachy et al., 2004; Karhadkar et al., 2004; Oniscu et al., 2004; Sanchez et al., 2004; Sheng et al., 2004; Ohta et al., 2005; Douard et al., 2006; Mimeault et al., 2006, 2007a,b; Bar et al., 2007; Bian et al., 2007; Chen et al., 2007b; Ehtesham et al., 2007; Peacock et al., 2007; Stecca et al., 2007; Bhattacharya et al., 2008; Liao et al., 2009; Mizuarai et al., 2009; Schnidar et al., 2009; Yang et al., 2010). More particularly, the reactivation of the Hh pathway and other developmental cascades, including EGFR and Wnt/β-catenin, in tissue-resident adult stem/progenitor cells during severe tissue injury, chronic inflammation, and intense stress along chronological aging might promote the cancer initiation and development (Beachy et al., 2004; Nielsen et al., 2008; Mimeault and Batra, 2009, 2010a,c; Strobel et al., 2010). Moreover, the up-regulation of Hh signaling components along the epithelial-mesenchymal transition process, might play a pivotal role in the proliferation, survival, invasion, and metastases of cancer stem/progenitor cells and their progenies at distant tissues (Mimeault and Batra, 2007, 2010a,c; Klarmann et al., 2009).

In support of the critical implication of the Hh signaling network in cancer initiation, it has been shown that PTCH knockout mice or SMOA1;Bmi(+/+) and SMOA1; Bmi(+/−) mice expressing SMO and BMI-1 spontaneously developed typical medulloblastoma arising from the expansion of the cerebellar granule neuron precursors (Michael et al., 2008; Yang et al., 2008; Ward et al., 2009). Furthermore, it has been shown that the activation of the Hh cascade by the SHH ligand may induce a transitory differentiation of prostate stem/progenitor cells into $CD44^+$/p63(+/−) hyperplasia basal cells with a intermediate phenotype (CK8/14) (Chen et al., 2006a, 2007a). This early transforming event culminated toward tumorigenesis by giving rise to CD44, PTCH1, and GLI-expressing prostate cancer cells (Chen et al., 2006a, 2007a). In the same way, it has been reported that the Hh cascade is activated in human breast $CD44^+CD24^{-/low}Lin^-$ cancer stem cells, and the overexpression of GLI2 transcriptional activator in mammosphere-initiating cells resulted in the formation of ductal hyperplasia in a humanized nonobese diabetic-severe combined immunodeficient (NOD/SCID) mouse model in vivo (Liu et al., 2006; Tanaka et al., 2009). The overexpression of GLI1 in the mouse mammary gland also resulted in tumor development arising from the expansion of epithelial cells expressing the progenitor cell markers keratin 6 and BMI-1 (Fiaschi et al., 2009). Moreover, the activated Hh-GLI signaling pathway might regulate the expression levels of stemness genes, self-renewal ability, and survival of $CD133^+$ glioma cancer stem cells and may contribute to sustained glioma growth and tumor cell survival in vivo (Clement et al., 2007).

In addition to the oncogenic effects induced through the activation of the Hh pathway in cancer cells, it has also been reported that Hh ligands can contribute to the pathogenesis of diverse human epithelial cancers, including pancreatic, colon, prostate, breast, and ovarian cancers by acting on the surrounding stromal cells and promoting the tumor neovascularization process (Yauch et al., 2008; Kasper et al., 2009; Olive et al., 2009; Shaw et al., 2009; Theunissen and de Sauvage, 2009; Nakamura et al., 2010; Walter et al., 2010). Moreover, different molecular cross-talk between the Hh cascade and other oncogenic signaling elements might cooperate for the tumor development and transition to invasive and metastatic disease stages.

A. Cross-Talks between the Hedgehog Cascade and Other Oncogenic Signaling Elements

A growing body of evidence has indicated that the aberrant activation of the Hh pathway combined with the occurrence of other oncogenic events, including the activating mutations in oncogenes such as K-RAS or inactivation of tumor suppressor gene products (p53,

$p16^{INK4A}$, and/or phosphatase and tensin homolog deleted chromosome 10), may cooperate in the malignant transformation of diverse epithelial cells and tumor development (Thayer et al., 2003; Hingorani et al., 2005; Pasca di Magliano et al., 2006; Carrière et al., 2007; Ji et al., 2007; Morton et al., 2007; Reinisch et al., 2007; Abe et al., 2008; Kasai et al., 2008; Frappart et al., 2009; Seto et al., 2009; Stecca and Ruiz I Altaba, 2009). For instance, it has been reported that the endogenous expression of mutated K-RAS (G12D) in a population of pancreatic exocrine progenitors characterized by the expression of nestin resulted in the formation of pancreatic intraepithelial neoplasias in a mouse model in vivo (Carrière et al., 2007). Moreover, the activation of the SHH signaling pathway cooperated with oncogenic K-RAS to promote pancreatic ductal adenocarcinoma development (Thayer et al., 2003; Pasca di Magliano et al., 2006; Ji et al., 2007; Kasai et al., 2008). More specifically, the malignant transformation of human pancreatic ductal epithelial cells induced by mutated K-RAS through the stimulation of RAF/extracellular-signal-regulated kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) signaling elements was accompanied by an increase of the GLI transcriptional activity leading to enhanced GLI1 expression (Ji et al., 2007). It has also been reported that the oncogenic K-RAS-induced cell transformation in pancreatic epithelium may be mediated, at least in part, through an enhanced expression of SHH ligand (Thayer et al., 2003; Hingorani et al., 2005; Pasca di Magliano et al., 2006). In this regard, it is noteworthy that the oncogenic K-RAS has been observed to enhance the association of the SCL/TAL1 interrupting locus product, a cytoplasmic protein overexpressed in pancreatic ductal adenocarcinoma, with SUFU protein in pancreatic cancer cell lines (Kasai et al., 2008). Thus, the formation of the SCL/TAL1 interrupting locus-SUFU complexes may inhibit the repressive effect induced by SUFU protein on the GLI activity and result in an upregulation of GLI target gene expression (Kasai et al., 2008). On the other hand, it has also been shown that the stimulation of the Hh signaling cascade may activate human double minute 2 and thereby increase the p53 degradation by ubiquitination and inhibit the p53-mediated tumor suppressive effect in human breast cancer cell lines (Abe et al., 2008).

In addition, the persistent activation of RTKs such as EGFR and platelet-derived growth factor receptor α as well as TGF-β/TGF-βR and Wnt/β-catenin also can cooperate with the canonical Hh-GLI pathway (Xie et al., 2001; Bigelow et al., 2005; Palma et al., 2005; Kasper et al., 2006; Maeda et al., 2006; Riobo et al., 2006; Riobó et al., 2006; Stecca et al., 2007; Nolan-Stevaux et al., 2009; Schnidar et al., 2009; Seto et al., 2009; Stecca and Ruiz, 2010). The integration of these signaling cascades may promote the acquisition of a more malignant behavior by cancer cells and the development of diverse aggressive cancers (Xie et al., 2001; Bigelow et al., 2005; Palma et al., 2005; Kasper et al., 2006b; Riobo et al., 2006a; Dennler et al., 2007, 2009; Stecca et al., 2007; Schnidar et al., 2009; Seto et al., 2009). The signaling cross-talk between Hh and other growth factor cascades may be mediated through different molecular mechanisms (Kasper et al., 2006b; Riobo et al., 2006a; Schnidar et al., 2009; Seto et al., 2009). In particular, an increase of GLI1 and GLI2 transcriptional expression may be induced through the activation of TGF-β/TGF-βR/Smads and Wnt/β-catenin during cancer progression (Dennler et al., 2007, 2009). Moreover, the stability and activities of the GLI1 and GLI2 transcriptional effectors of the Hh pathway may be modulated through the integration of distinct intracellular transduction signals induced through RTK activation. These transforming events consist of a sustained activation of RAS/RAF/MEK/extracellular signal-regulated kinase (ERK)/MAPK, PI_3K/Akt/mammalian target of rapamycin (mTOR)/$p70^{S6K2}$, and/or protein kinase C-δ (Kasper et al., 2006b; Riobo et al., 2006; Riobó et al., 2006; Stecca et al., 2007; Mizuarai et al., 2009; Schnidar et al., 2009; Seto et al., 2009). In fact, the stimulation of these distinct signaling elements can cooperate with GLI proteins in the regulation of specific target gene expression, including *PTCH1* and *GLI1*, in a cancer cell type-dependent manner (Kasper et al., 2006b; Riobo et al., 2006a; Stecca et al., 2007; Schnidar et al., 2009; Seto et al., 2009). Numerous investigations have revealed that the overexpression of EGFR signaling elements frequently occurs in numerous aggressive and metastatic cancers, and can cooperate with the Hh pathway for the malignant transformation and survival of cancer cells.

B. Cross-Talks between the Hedgehog and Epidermal Growth Factor Receptor Signaling Cascades

The enhanced expression and/or activity of EGFR and its ligands, EGF, TGF-α, heparin-binding EGF, amphiregulin, and epiregulin has been associated with the development of diverse aggressive cancers, such as brain, skin, cervical, head and neck, renal, non–small-cell lung, liver, gastrointestinal, colorectal, pancreas, prostate, breast, and ovarian cancers and sarcomas (Ohsaki et al., 2000; Yarden and Sliwkowski, 2001; Di Lorenzo et al., 2002; Hernes et al., 2004; Hynes and Lane, 2005; Shepherd et al., 2005; Citri and Yarden, 2006; Shah et al., 2006; Cerciello et al., 2007; Mimeault and Batra, 2007, 2008a; Mimeault et al., 2008; Yonesaka et al., 2008; Schnidar et al., 2009; Seto et al., 2009). The EGFR signaling network can contribute to the tumor growth, invasiveness, and angiogenic process through autocrine and paracrine loops by activating diverse intracellular cascades. Among these signaling elements, there are MAPKs, PI_3K/Akt, NF-κB, phospholipase Cγ, and the transcriptional repressor of E-cadherin expression, snail and twist (Fig. 6) (Yarden and Sliwkowski, 2001; Hynes and Lane, 2005; Angelucci et al., 2006; Citri

and Yarden, 2006; Mimeault et al., 2006, 2008; Lo et al., 2007; Mimeault and Batra, 2007, 2008a).

Accumulating lines of evidence have indicated that different bidirectional cross-talk between Hh and EGFR signaling cascades may contribute to the malignant transformation of cancer cells. For instance, it has been shown that the stimulation of EGFR/RAS/RAF/MEK/ERK might lead to an activation of the GLI transcription factor and selective transcriptional modulation of GLI target gene expression in HaCaT keratinocytes and BCC, gastric, and pancreatic cancer cell lines (Bigelow et al., 2005; Kasper et al., 2006b; Schnidar et al., 2009; Seto et al., 2009). More specifically, the activation of the EGFR pathway resulted in the stimulation of transcription factors such as activator protein 1 family member JUN that cooperated with GLI1 and/or GLI2 activators for triggering selective expression of target gene products involved in the oncogenic transformation of BCC cell lines (Schnidar et al., 2009). Likewise, the stimulation of the Hh pathway can also modulate the expression and activities of EGFR signaling elements (Bigelow et al., 2005; Mimeault et al., 2006; Pasca di Magliano et al., 2006; Heo et al., 2007). For instance, it has been observed that the constitutive SHH expression was associated with an enhanced phosphorylation of EGFR in mouse embryonic stem cells and human HaCaT keratinocytes as well as an increase of collagen matrix invasion of HaCaT keratinocytes (Bigelow et al., 2005; Heo et al., 2007). It has also been reported that the EGFR signaling was activated in undifferentiated tumors formed in the GLI2-overexpressing *Pdx-Cre*;*CLEG2* mouse model, and up-regulation of EGFR/Akt signaling contributed to the proliferation and survival of pancreatic cancer cells (Pasca di Magliano et al., 2006). The inhibition of the Hh cascade using cyclopamine was also accompanied by a down-regulation of EGFR expression level in prostate and pancreatic cancer cells (Mimeault et al., 2006; Hu et al., 2007).

Together, these observations have revealed that an up-regulation of Hh and RTKs, including EGFR signaling elements, during cancer progression and multiple cross-talks between these tumorigenic cascades can cooperate for the sustained growth, survival, and treatment resistance of cancer stem/progenitor cells and their progenies. Thus, the targeting of these tumorigenic signaling elements might constitute a potential therapeutic strategy of great clinical interest for overcoming treatment resistance and developing novel combination therapies against aggressive, metastatic, and recurrent cancers.

V. Targeted Therapies

The molecular targeting of different oncogenic products in cancer cells represents a potential strategy for improving the current therapeutic treatments by antihormonal therapies, radiotherapies, and chemotherapies against locally advanced, invasive, metastatic, and recurrent cancers. Because the resistance of cancer- and metastasis-initiating cells to current therapies can provide critical roles in tumor regrowth, metastases, and disease relapse, the molecular targeting of these immature cells endowed with high self-renewal and aberrant differentiation abilities constitutes a promising therapeutic strategy to prevent disease recurrence. The potential molecular targets include Hh/GLI, EGFR family members, Wnt/β-catenin, Notch, hyaluronan/CD44, TGF-β/TGF-βR, and stromal cell-derived factor-1/CXC chemokine receptor 4 signaling elements (Mimeault and Batra, 2007, 2008a,b, 2010a,b; Mimeault et al., 2008). Based on a growing body of experimental evidence indicating that the persistent activation of Hh and/or EGFR pathways represents a critical step in cancer progression to invasive and metastatic stages and disease recurrence, many efforts have been made to develop specific inhibitory agents targeting these tumorigenic cascades. We review the results from recent investigations supporting the clinical interest of targeting the Hh and/or EGFR cascades to eradicate the total tumor cell mass, improve current treatment, and develop new effective combination therapies against aggressive and recurrent cancers.

A. Targeting of the Canonical Hedgehog Tumorigenic Signaling Pathway

One of the therapeutic approaches to block the Hh signaling cascade is the use of a specific inhibitor of the SMO coreceptor. These chemical compounds include the natural plant-derived steroidal alkaloid cyclopamine and its derivatives, such as 3-keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine (KAAD-cyclopamine), and semisynthetic D-homo-ring analogs IPI-269609 (Feldmann et al., 2008) and IPI-926 (Olive et al., 2009) as well as small synthetic molecules such as 2-chloro-*N*-[4-chloro-3-(*2-pyridinyl*)phenyl]-4-(methylsulfonyl)benzamide (GDC-0049; HhAntag691), a proline derivative Cur61414 (Rubin and de Sauvage, 2006), and *N*-(6-((2*S*,6*R*)-2,6-dimethylmorpholino)pyridin-3-yl)-2-methyl-4′-(trifluoromethoxy)biphenyl-3-carboxamide (NVP-LDE-225) (Table 1) (Williams et al., 2003; Romer et al., 2004; Mimeault et al., 2006; Riobo et al., 2006a; Rubin and de Sauvage, 2006; Bar et al., 2007; Chen et al., 2007b; Clement et al., 2007; Lauth et al., 2007; Peacock et al., 2007; Stecca et al., 2007; Feldmann et al., 2008; Büttner et al., 2009; Eichenmüller et al., 2009; Jimeno et al., 2009; Olive et al., 2009; Robarge et al., 2009; Tremblay et al., 2009; Mimeault and Batra, 2010b; Pan et al., 2010; Stanton and Peng, 2010; Yang et al., 2010). Moreover, small synthetic structural analogs of the second and third intracellular loops of the SMO proteins, such as small palmitoylated peptides as short as 10 residues, have also been designed (Remsberg et al., 2007). In addition, other therapeutic strategies include the use of anti-PTCH1 or SHH monoclonal antibody

TABLE 1
Potential inhibitory agents of hedgehog and EGFR tumorigenic signaling elements

Targeted Signaling Element	Name of Inhibitory Agent
Growth factor signaling element	
SHH ligand	Anti-SHH antibody, HHIP
SMO coreceptor	Cyclopamine, KAAD-cyclopamine, IPI-926, IPI-269609, GDC-0449, NVP-LDE-225 small palmitoylated peptides
GLI-1 or GLI-2	siRNA, shRNA
EGFR (erbB1)	Anti-EGFR-antibody (mAb-C225, ICM-C225)
EGFR-TKI	Gefitinib, erlotinib, AG1478, EKB-569
Wnt/β-catenin	Anti-Wnt antibody, WIF-1
Notch	γ-Secretase inhibitor DAPT, Compound E, DBZ
VEGFR	Anti-VEGFR antibody (DC101)
VEGFR/EGFR	ZD6474
Signal transduction element	
BMI-1	Vorinostat, azacitidine decitabine
Bcl-2	Antisense-Bcl-2 (oblimersen sodium), ABT-737
Farnesyl transferase	FTI (BMS-214662, R115577)
RAF	Sorafenib, RAF265
MEK	PD0325901, SL 327, U012
PI_3K/Akt	ZSTK474
mTOR	Rapamycin, CCI-779
NF-κB	IκBα inhibitor, sulfasalazine, bortezomib (PS-341), salinosporamide A (NPI-0052), parthenolide, DHMEQ
COX-2	NS-396 (aspirine), etodolac, celecoxib, rofecoxib
ABC transporters	Cyclopamine, gefitinib, erlotinib

ABT-737, 4-[4-[[2-(4-chlorophenyl)phenyl]methyl]piperazin-1-yl]-*N*-[4-[[(2*R*)-4-(dimethylamino)-1-phenylsulfanylbutan-2-yl]amino]-3-nitrophenyl]sulfonylbenzamide; BMS-214662, (3*R*)-3-benzyl-1-(1*H*-imidazol-5-ylmethyl)-4-thiophen-2-ylsulfonyl-3,5-dihydro-2*H*-1,4-benzodiazepine-7-carbonitrile; CCI-779, rapamycin 42-[3-hydroxy-2 (hydroxymethyl)-2-methylpropanoate]; Compound E, [(2*S*)-2-[[(3,5-difluorophenyl)-acetylamino]-*N*-[(3*S*)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl] propanamide]; DAPT, *tert*-butyl (2S)-2-[[(2*S*)-2-[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate; DBZ, (2S)-2-[2-(3,5-difluorophenyl)-acetylamino]-*N*-(5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl)-propionamide; DHMEQ, dehydroxymethylepoxyquinomicin ; EKB-569, (*E*)-*N*-[4-(3-chloro-4-fluoroanilino)-3-cyano-7-ethoxyquinolin-6-yl]-4-(dimethylamino)but-2-enamide; FTI, farnesyl transferase inhibitor; NS-396, 2-acetyloxybenzoic acid; PD0325901, *N*-[(2*R*)-2,3-dihydroxypropoxy]-3,4-difluoro-2-(2-fluoro-4-iodoanilino)benzamide; RAF265, 1-methyl-5-[2-[5-(trifluoromethyl)-1*H*-imidazol-2-yl]pyridin-4-yl]oxy-*N*-[4-(trifluoromethyl)phenyl] benzimidazol-2-amine; R115577, 6-[(*R*)-amino-(4-chlorophenyl)-(3-methylimidazol-4-yl)methyl]-4-(3-chlorophenyl)-1-methylquinolin-2-one; salinosporamide A, (1*S*,2*R*,5*R*)-2-(2-chloroethyl)-5-[[(1*S*)-cyclohex-2-en-1-yl]-hydroxymethyl]-1-methyl-7-oxa-4-azabicyclo[3.2.0]heptane-3,6-dione; shRNA, short hairpin RNA; SL 327, α-[amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile; TKI, tyrosine kinase activity inhibitor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; ZD6474, N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine; ZSTK474, 2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine.

(mAb), endogenous Hh inhibitor, HHIP, small-molecule inhibitors of GLI1/GLI2 transcriptional activity, and silencing of GLI1 or GLI2 by siRNA or short hairpin RNAs (Table 1) (Karhadkar et al., 2004; Sanchez et al., 2004; Ohta et al., 2005; Rubin and de Sauvage, 2006; Bar et al., 2007; Clement et al., 2007; Ji et al., 2007, 2008; Kim et al., 2007b; Lauth et al., 2007; Nakamura et al., 2007; Peacock et al., 2007; Stecca et al., 2007; Thiyagarajan et al., 2007; Narita et al., 2008; Shaw and Prowse, 2008; Xu et al., 2008; Hyman et al., 2009; Jimeno et al., 2009; Tanaka et al., 2009). The targeting of GLI proteins in cancer cells is of particular interest for overcoming the development of resistance to the SMO inhibitors (Yauch et al., 2009) and Hh ligand-independent GLI activation.

The data from numerous in vitro and in vivo studies have revealed that the blockade of the Hh pathway with these agent types results in an inhibition of growth and invasiveness, metastatic spread, and/or the apoptotic death of the cancer- and metastasis-initiating cells and their progenies, whereas the normal cells were insensitive to these cytotoxic effects (Berman et al., 2003; Williams et al., 2003; Beachy et al., 2004; Karhadkar et al., 2004; Kubo et al., 2004; Sheng et al., 2004; Romer and Curran, 2005; Douard et al., 2006; Mimeault et al., 2006; Rubin and de Sauvage, 2006; Clement et al., 2007; Ji et al., 2007; Lauth et al., 2007; Peacock et al., 2007; Remsberg et al., 2007; Stecca et al., 2007; Eichenmüller et al., 2009; Jimeno et al., 2009; Kobune et al., 2009; Liao et al., 2009; Robarge et al., 2009; Tanaka et al., 2009; Tremblay et al., 2009). It has been observed that the inhibition of the SMO signaling effector with cyclopamine inhibited tumor growth, invasion, and metastases of cancer cells with stem cell-like properties in human leukemia, multiple myeloma, and glioma as well as pancreatic, breast, and liver cancers (Liu et al., 2005b, 2006; Douard et al., 2006; Bar et al., 2007; Clement et al., 2007; Feldmann et al., 2007, 2008; Peacock et al., 2007; Stecca et al., 2007; Xu et al., 2008; Eichenmüller et al., 2009; Kobune et al., 2009; Tanaka et al., 2009). In particular, it has been observed that the systemic delivery of cyclopamine or a novel, orally bioavailable small-molecule SMO inhibitor, IPI-269609, prevented the metastatic spread of pancreatic cells and reduced the number of tumor cells expressing the stem cell-like marker aldehyde dehydrogenase in the animal model in vivo (Feldmann et al., 2007, 2008). Moreover, cyclopamine treatment or GLI-1 knockdown by siRNA was also effective at eradicating the clonogenic glioma cells expressing the stem cell-like markers in vitro and inhibiting intracranial growth of glioma stem cell-derived tumors in vivo (Bar et al., 2007; Clement et al., 2007; Xu et al., 2008).

In addition, recent investigations have revealed that the primary cilium might supply key functions for the activation of the SMO protein in certain types of cancer cells (Fig. 3) (Rosenbaum and Witman, 2002; Corbit et

al., 2005; Haycraft et al., 2005; Rohatgi and Scott, 2007; Rohatgi et al., 2007; Han et al., 2008, 2009; Spassky et al., 2008; Bailey et al., 2009; Veland et al., 2009; Wong et al., 2009). Therefore, the interference with the IFT proteins and/or the inhibition of the translocation of SMO molecules to the ciliary structure may also represent an alternative therapeutic strategy. It has been shown that specific products of the sterol biosynthesis, including cholesterol and oxysterols, can provide critical roles for the SMO translocation to the primary cilium and activation of the Hh pathway in medulloblastoma cells, which are derived from the malignant transformation of cerebellar GCPs (Corcoran and Scott, 2006). It is noteworthy that the blockade of the sterol synthesis using specific inhibitors reduced the Hh pathway-mediated cell proliferation in medulloblastoma cells (Corcoran and Scott, 2006). In this regard, it has also been shown that SMO antagonists, including SANT-1 and SANT-2, can inhibit the translocation of the SMO molecule to the primary cilium (Wang et al., 2009b). Moreover, it has been reported that four Hh pathway inhibitors designated as HPIs that do not target SMO protein can act downstream of SUFU-modulated GLI activation by decreasing the extent of the SMO accumulation in the primary cilium induced in response to SHH ligand (Hyman et al., 2009). Therefore, the use of these Hh signaling inhibitors might constitute another potential therapeutic strategy to reduce the SMO accumulation in the ciliary structure and SMO-mediated Hh cascade activation in cancer cells.

B. Antitumoral Effects Induced by the Canonical Hedgehog Cascade Blockade in Tumor Stromal Cells

Accumulating evidence has revealed that the secreted SHH ligand can also contribute in a paracrine manner to tumor-stromal cell interactions by stimulating neighboring stromal cells, including fibroblasts, and enhancing the recruitment of BM-derived EPCs to tumor, thereby promoting tumor angiogenesis and disease progression (Bailey et al., 2007, 2009; Yamazaki et al., 2008; Yauch et al., 2008; Kasper et al., 2009; Olive et al., 2009; Shaw et al., 2009; Theunissen and de Sauvage, 2009; Nakamura et al., 2010; Walter et al., 2010). More specifically, the SHH protein has been shown to act through a paracrine mechanism by inducing the GLI1 expression in primary pancreatic cancer-associated stromal fibroblasts (CAF) established from human pancreatic adenocarcinoma that overexpress SMO coreceptor compared with normal pancreatic fibroblasts (Walter et al., 2010). It has also been noticed that the siRNA knockdown of SMO in primary CAFs was accompanied by a downregulation of GLI1 expression level, suggesting that the targeting of the Hh pathway in CAFs might represent another therapeutic strategy to counteract the pancreatic cancer progression (Walter et al., 2010). In the same way, blockade of Hh signaling using the SMO inhibitor IPI-926 also induced antiangiogenic effects on a pancreatic cancer cell model in vivo (Olive et al., 2009). Moreover, the results from another recent study have revealed that the inhibition of the Hh cascade by using cyclopamine reduced the expression levels of GLI1 and GLI2 in stroma and inhibited the tumor neoangiogenic process and growth of pancreatic cancer cell-derived xenografts (Nakamura et al., 2010). This antiangiogenic effect of cyclopamine was mediated in part by an inhibition of the SHH release from pancreatic cancer cells and recruitment of BM-derived pro-angiogenic cells at primary pancreatic tumor (Nakamura et al., 2010). More specifically, it has been observed that a decrease of the angiotensin-1 and insulin-like growth factor-1 expression occurred in BM-derived proangiogenic cells after the cyclopamine treatment and resulted in a reduction of tumor neovascularization (Nakamura et al., 2010). In the same pathway, the *GLI2* gene silencing by specific short hairpin RNAs also induced the apoptosis in a mouse BCC-like tumor cell line and markedly inhibited the tumor vascularization in an in vivo mouse tumor model (Ji et al., 2008).

C. Clinical Trials with the Canonical Hedgehog Cascade Inhibitors

Among the chemical compounds acting as the specific inhibitors of the canonical Hh pathway, only the SMO antagonists have been tested in humans. A preliminary study performed with the natural steroidal alkaloid cyclopamine, consisting of its topical application in a cream formulation to four patients with BCC, has revealed that the tumors rapidly regressed in all cases without adverse effects, and the normal skin and putative stem cells exposed to cyclopamine were preserved (Taş and Avci, 2004). Histological and immunohistochemical analyses have also indicated that the topical cyclopamine application resulted in an inhibition of the proliferation and induced the apoptotic death of tumor cells (Taş and Avci, 2004). The potential teratogenic effect of cyclopamine at high doses may limit its use. Other potent SMO inhibitors that have also reached the clinical trials include the orally active IPI-926 semisynthetic derivative of cyclopamine and different synthetic compounds such as GDC-0449, Cur61414, and NVP-LDE-225 (LoRusso et al., 2008; Molckovsky and Siu, 2008; Rudin et al., 2009; Von Hoff et al., 2009; Stanton and Peng, 2010).

The selective SMO antagonist GDC-0449 is among the more potent chemical compounds that has recently suspired great interest based on promising results from a phase I clinical trial. This small synthetic molecule was discovered through a high-throughput screening of a library of chemical compounds to analyze their potential inhibitory effect on the GLI1 expression by luciferase reporter gene assays followed by an optimization of the pharmacological properties of the most active SMO antagonists by medicinal chemistry (Robarge et al., 2009). The data from a phase I multicenter clinical trial con-

sisting of the administration of orally active GDC-0449 in patients with advanced and metastatic cancers revealed that this pharmacological agent showed antitumoral activity and was well tolerated, with no grade 5 adverse events and dose-limiting toxicity, in a subset of cancer patients (LoRusso et al., 2008; Molckovsky and Siu, 2008; Von Hoff et al., 2009). More specifically, the results from this phase I clinical trial, obtained with 33 patients diagnosed with locally advanced or metastatic BCCs that were refractory to standard therapies, treated daily with oral GDC-0449 during a median time of 9.8 months, revealed that two patients showed a complete tumor response and 16 had a partial response to this treatment, whereas other patients had stable or progressive disease (Von Hoff et al., 2009). Moreover, a case report about the enrollment in a phase I clinical trial of a 26-year-old patient diagnosed with a systemic metastatic medulloblastoma associated with inactivating mutation in *PTCH1* that was refractory to multiple prior clinical treatments indicated that rapid tumor regression and reduction of symptoms occurred in this patient after treatment with GDC-0449 (Rudin et al., 2009). Unfortunately, the development of resistance to GDC-0449 treatment associated with the occurrence of a mutation in the SMO protein impairing the binding of GDC-0449 to the SMO molecules led to tumor regrowth evident approximately 3 months after GDC-0449 treatment initiation at some sites (Rudin et al., 2009; Yauch et al., 2009). Consequently, this patient was removed from the clinical trial because of disease progression and died after approximately 2 months, despite a series of subsequent therapies (Rudin et al., 2009).

Hence, these data from a phase I clinical trial suggest that the GDC-0449 might induce the antitumoral effects in a subset of patients with locally advanced or metastatic BCCs or medulloblastomas characterized by a sustained activation of the Hh signaling cascade despite the intrinsic or acquired resistance to this treatment type, which might be prevalent in certain patients may counteract the efficacy of this therapeutic strategy. Phase I and II clinical trials are also ongoing to investigate long-term antitumoral activity and safety of GDC-0449, alone or in combination with current chemotherapeutic treatments, in patients diagnosed with a variety of cancers. These cancers include localized, metastatic, or recurrent BCC, medulloblastoma, glioblastoma multiforme, and small-cell lung, pancreatic, stomach, colorectal, breast, and ovarian cancers. Thus, the results from these additional clinical trials with GDC-0449 and other specific Hh inhibitors should confirm the therapeutic benefit and safety of inhibiting the Hh signaling pathway in treating patients with a wide range of solid tumors.

D. Targeting of the Noncanonical Hedgehog Tumorigenic Signaling Pathways

Emerging lines of experimental evidence have revealed that the activation of noncanonical Hh pathways may contribute in cooperation with the canonical Hh pathways mediated through Hh ligand/PTCH1/SMO/GLIs to the acquisition of more malignant phenotypes by cancer cells during disease progression (Lauth and Toftgård, 2007; Lauth et al., 2007; Jenkins, 2009; Stecca and Ruiz, 2010). More particularly, it has been reported that the oncogenic EWS-FLI1 fusion protein detected in the majority of Ewing's sarcomas, oncogenic K-RAS, and the activation of RTKs such as EGFR and platelet-derived growth factor receptor α as well as Wnt/β-catenin and the TGF-β1/TGF-βR system, can stimulate *GLI* expression and/or activities and Hh target gene expression during cancer development (Figs. 5 and 6) (Xie et al., 2001; Bigelow et al., 2005; Palma et al., 2005; Kasper et al., 2006; Maeda et al., 2006; Riobo et al., 2006; Riobó et al., 2006; Dennler et al., 2007, 2009; Ji et al., 2007; Stecca et al., 2007; Nolan-Stevaux et al., 2009; Schnidar et al., 2009; Seto et al., 2009; Stecca and Ruiz, 2010). Hence, these oncogenic signaling elements can cooperate with the canonical Hh ligand-induced signaling cascade in certain types of cancer cells. In fact, the bidirectional signaling cross-talk between these developmental pathways can cooperate to induce the epithelial-mesenchymal transition process in cancer cells and progression to invasive, metastatic, and recurrent cancers (Mimeault and Batra, 2007, 2010c; Yoo et al., 2008; Nolan-Stevaux et al., 2009; Stecca and Ruiz, 2010). Therefore, the targeting of these growth factor cascades and/or their intracellular signaling effectors, including GLI proteins, might constitute a promising therapeutic approach, alone or in combination with the specific inhibitor of canonical Hh pathway, to eradicate the cancer-initiating cells and their progenies (Dennler et al., 2007; Mimeault and Batra, 2007; Hyman et al., 2009). In support of this, it has been observed that the oncogenic K-RAS transformation of human pancreatic ductal epithelial cells, which resulted in an increase of GLI transcriptional activity, was inhibited by siRNA targeting *K-RAS* or a MEK-specific inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126) or 2′-amino-3′-methoxyflavone (PD98059) (Ji et al., 2007). In the same way, treatment of mouse pancreatic cell lines with siRNA constructs targeting *K-RAS* or *GLI1* also inhibited expression of GLI1 and PTCH1 and induced the apoptotic death of these cancer cells in vitro (Nolan-Stevaux et al., 2009). Moreover, it has been observed that the TGF-β type I activin receptor-like kinase 5 (ALK5) inhibitor 4-(5-benzo(1,3)dioxol-5-yl-4-pyridin-2-yl-1*H*-imidazol-2-yl)benzamide (SB431542) completely abrogated the stimulatory effect induced by TGF-β on *GLI2* expression and inhibited the growth of pancreatic cancer cells in vitro and in vivo (Dennler et al., 2007). It has also been shown that the small molecules termed 4-(2,4,5-tripyridin-4-ylthiophen-3-yl)pyridine (GANT58) and 2,2′-[[dihydro-2-(4-pyridinyl)-1,3(2*H*,4*H*)-pyrimidinediyl]bis(methylene)]bis[*N*,*N*-dimethyl]-benzenamine (GANT61), acting as inhibitors of GLI1- and GLI2-mediated Hh

gene expression, inhibited the growth of the PANC-1 pancreatic and recurrent 22Rv1 prostate cancer cell lines in vitro and tumor growth of 22Rv1 xenografts in nude mice in vivo more potently than the SMO antagonist cyclopamine (Lauth et al., 2007). On the other hand, it has been reported that the activation of GSK3b, which may contribute to the inactivation of GLI proteins, might be inhibited through the stimulation of PI_3K/Akt/mTOR/$p70^{S6K2}$ intracellular signaling (Yuan et al., 2007; Mizuarai et al., 2009). In contrast, the silencing of $p70^{S6K2}$ activated GSK3b, enhanced GLI degradation, and inhibited the viability of non–small-cell lung cancer cells (Yuan et al., 2007; Mizuarai et al., 2009). These data suggest that $p70^{S6K2}$ might also represent a potential therapeutic target to inhibit Hh ligand-independent activation of the Hh cascade mediated through high GLI expression levels in certain types of cancer cells. In this regard, we reviewed in a more detailed manner the therapeutic strategies consisting of targeting EGFR, alone or in combination with a Hh inhibitor, for improving the current clinical cancer therapies.

E. Targeting of the Epidermal Growth Factor Receptor Tumorigenic Signaling Pathway

Numerous preclinical and clinical studies have indicated that the selective blockade of the EGFR signaling pathway may represent a potent strategy, alone or in combination with the other conventional treatments, to counteract cancer progression and prevent disease relapse (Yarden and Sliwkowski, 2001; Budiyanto et al., 2003; Chung and Saltz, 2005; Haruki et al., 2005; Hynes and Lane, 2005; Shelton et al., 2005; del Carmen et al., 2005; Angelucci et al., 2006; Citri and Yarden, 2006; Mimeault et al., 2006, 2010; Cerciello et al., 2007; Tortora et al., 2007; Shaw and Prowse, 2008; Griffero et al., 2009). Among the selective agents targeting the EGFR cascade are the anti-EGFR antibodies (mAb-C225 and ICM-C225, also designated cetuximab and erbitux, respectively), antisense oligonucleotide directed against EGFR or its ligands EGF and TGF-α, and the selective inhibitors of EGFR tyrosine kinase activity such as 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline (AG1478), *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine (gefitinib; Iressa; ZD1839) N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine (erlotinib; Tarceva; OSI-774) (Table 1) (Chung and Saltz, 2005; Haruki et al., 2005; Hynes and Lane, 2005; Shelton et al., 2005; Shepherd et al., 2005; del Carmen et al., 2005; Bonner et al., 2006; Citri and Yarden, 2006; Hanna et al., 2006; Khambata-Ford et al., 2007; Nogueira-Rodrigues et al., 2008; Shaw and Prowse, 2008; Yonesaka et al., 2008; Griffero et al., 2009; Montagut and Settleman, 2009). Alternatively, the molecular targeting of EGFR downstream signaling elements, including RAS/RAF/MEK, PI_3K/Akt/mTOR, NF-κB, cyclooxygnase-2, and vascular endothelial growth factor/vascular endothelial growth factor receptor might represent another effective therapeutic approach (Table 1) (Chung and Saltz, 2005; Haruki et al., 2005; Hynes and Lane, 2005; Shelton et al., 2005; del Carmen et al., 2005; Citri and Yarden, 2006; Xu et al., 2008; Montagut and Settleman, 2009). Many in vitro and in vivo studies have revealed that these agents can induce an inhibition of the growth, invasiveness and apoptotic death of diverse cancer cell types and counteract the angiogenic process (Haruki et al., 2005; Hynes and Lane, 2005; Citri and Yarden, 2006; Mimeault et al., 2010). For instance, it has been reported that a specific inhibitor of EGFR tyrosine kinase activity, gefitinib or erlotinib, induced the antiproliferative and cytotoxic effects on $EGFR^+/CD133^+$ tumor-initiating cells from five patients with glioma blastomas (Griffero et al., 2009). Certain mAbs directed against EGFR (cetuximab) and EGFR tyrosine kinase activity inhibitors (gefitinib and erlotinib) have also reached clinical trials and are now used in the chemotherapeutic arsenals for treating diverse common solid tumors, including non–small-cell lung, breast, colorectal, head, and neck squamous cell and pancreatic cancers (Hynes and Lane, 2005; Shepherd et al., 2005; Bonner et al., 2006; Hanna et al., 2006; Khambata-Ford et al., 2007; Nogueira-Rodrigues et al., 2008; Yonesaka et al., 2008). The response of cancer patients to the blockade of the EGFR signaling cascade may be influenced by different factors, including the activating mutations in the EGFR and expression levels of EGFR ligands in cancer cells (Khambata-Ford et al., 2007; Yonesaka et al., 2008).

F. New Combination Therapies against Aggressive and Recurrent Cancers

In view of the fact that aggressive, metastatic, and recurrent cancers are typically characterized by activation of numerous oncogenic signaling elements, combination therapies may represent more effective treatments than monotherapies for improving the current therapeutic regimens and remedy the potential toxicity associated with the inclusion of high doses of individual drugs. In this regard, the molecular targeting of Hh and/or EGFR cascades has notably been shown to be a potential strategy for reversing the treatment resistance and improving the efficacy of the current antihormonal therapy, chemotherapy, and/or radiotherapy (Haruki et al., 2005; Hynes and Lane, 2005; del Carmen et al., 2005; Cerciello et al., 2007; Clement et al., 2007; Feldmann et al., 2007; Mimeault and Batra, 2007 2008a; Morton et al., 2007; Tortora et al., 2007; Mimeault et al., 2008, 2010; Shaw and Prowse, 2008; Jimeno et al., 2009; Kobune et al., 2009; Schnidar et al., 2009). For instance, the combined pharmacological inhibition of Hh/GLI and EGFR pathways by cyclopamine and gefitinib resulted in enhanced antiproliferative and apoptotic effects on prostate and pancreatic cell lines in vitro (Mimeault et al., 2006, 2010; Hu et al., 2007). Moreover, the combined use of cyclopamine and gefitinib also resulted in suppression of the growth of BCC cell lines derived from mice in vitro (Schnidar et al., 2009). We have also shown

that a combination of gefitinib and/or cyclopamine with current chemotherapeutic drug docetaxel or mitoxantrone led to a more massive rate of apoptotic death on diverse metastatic prostate cancer cells, including side-population (SP) and non-SP cell fractions from invasive prostate cancer cells, relative to individual drugs or two-drug combination treatments (Mimeault et al., 2007a,b, 2010). Of particular interest is that the combination of cyclopamine with the current chemotherapeutic drug temozolomide also induced additive and synergistic anticarcinogenic effects on glioma stem cell culture cells in vitro (Clement et al., 2007). Likewise, a combination of cyclopamine and gemcitabine has been observed to inhibit tumor growth and metastatic spread and decrease the expression levels of stem cell-like markers, including aldehyde dehydrogenase detected by immunohistochemistry in a pancreatic cancer xenograft model in vivo (Feldmann et al., 2007; Jimeno et al., 2009). Moreover, the inhibition of Hh signaling with cyclopamine, HHIP, or anti-SHH mAb also induced apoptosis in $CD34^+$ acute myeloid leukemic cells and significantly improved the cytotoxic effect induced by cytarabine on these leukemic cells (Kobune et al., 2009).

Of particular interest is that the Hh/GLI and EGF-EGFR system may contribute to the regulation of the expression and/or cellular localization of ABC transporters in certain cancer cells, including the SP cell fraction with stem cell-like properties (Fig. 6) (Chen et al., 2006; Meyer zu Schwabedissen et al., 2006; Sims-Mourtada et al., 2007; Mimeault et al., 2008; Zhang et al., 2009). Therefore, the inhibition of Hh and/or EGFR cascades may represent a potential strategy for reversing multidrug resistance phenotypes mediated through ABC multidrug efflux pumps. In support of this, cyclopamine has been observed to reduce the expression levels of ABCG2/breast cancer resistance and ABCB1/multidrug resistance protein-1/P-glycoprotein in the metastatic and tumorigenic PC3 prostate cancer cell line and to improve the cytotoxic effects induced by different chemotherapeutic drugs on diverse cancer cell lines (Sims-Mourtada et al., 2007). The SMO antagonist GDC-0449 (HhAntag691) was also effective in inhibiting the drug efflux pump activity of ABCG2 and multidrug resistance protein-1 (Zhang et al., 2009). Likewise, it has been shown that EGF can increase the expression of ABC transporters at the cell surface and in the SP fraction, whereas the blockade of the EGFR cascade can inhibit this effect (Takada et al., 2005; Chen et al., 2006b; Meyer zu Schwabedissen et al., 2006).

On the other hand, because the secreted ligands of Hh and EGFR cascades can act through a paracrine mode on surrounding tumor cells and stromal cells, the interference with their secretion and transport or SMO activity inhibition in host stromal cells could also constitute a potential adjuvant therapeutic strategy to counteract tumor development (Fig. 6). In this regard, it has been reported that blockade of the Hh pathway by using the SMO inhibitor IPI-926 improved tumor delivery and antitumoral efficacy of gemcitabine, at least in part, by disrupting the desmoplastic stroma in a pancreatic cancer cell model in vivo (Olive et al., 2009).

VI. Conclusions and Future Directions

Together, these recent studies have underlined the critical physiological roles played by the Hh signaling network during the developmental process and adult life in the maintenance of stem/progenitor cells and their progenies and complex molecular mechanisms involved in its regulation. Future studies, however, are necessary to more precisely delineate the signaling elements implicated in the positive and negative regulation of the transcriptional expression of Hh ligands and GLI proteins, SMO and GLI activation, and specific functions of the primary cilium in physiological and pathological conditions. In particular, it will be important to further determine the factors involved in the up-regulated expression of SHH and GLI activities and potential interactive cross-talk with other oncogenic pathways during cancer progression. The establishment of the precise molecular mechanisms of Hh signal transduction, including signaling elements involved in SMO translocation to the primary cilium and precise functions of IFT proteins in cellular responses induced in human normal and malignant cell types, is also of great interest. This additional work should help to develop novel potential pharmacological agents to modulate these cellular processes, and thereby counteract the activation of the Hh pathway and cancer development.

In addition, accumulating lines of experimental evidence have revealed that an aberrant activation of Hh/GLI and RTKs, such as the EGFR signaling cascade, 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. Future studies are required to more precisely establish the molecular mechanisms and specific downstream signaling elements that may contribute to the cooperative or synergistic interactions of the Hh/GLIs and RTK signaling pathways, including EGFR, in cancer- and metastasis-initiating cells versus their differentiated progenies. 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. Hence, these additional investigations should lead to the identification of new potential targets to eradicate the total cancer cell mass and improve current therapies.

In view of the promising results from preclinical studies and a recent phase I clinical trial performed with specific SMO antagonists, and more particularly the

successful tumor regression observed with GDC-0449 in certain patients with cancer, the targeting of the Hh cascade seems to represent a therapeutic strategy of great clinical interest. The data obtained with a broad spectrum of patients diagnosed with different cancer types treated with a Hh inhibitor over the long-term should confirm the therapeutic benefit and safety to selectively target the Hh cascade, alone or in combination with the current conventional therapies. In this regard, the establishment of accurate screening tests of diagnosis and molecular mechanisms associated with the intrinsic and acquired resistance of cancer patients to the treatment with a Hh or EGFR inhibitor or other therapeutic targets is also of great clinical interest. These additional studies should lead to an optimization of the choice of therapeutic regimens and personalized medicine as well as the development of novel effective multitarget strategies that could be used for improving the current cancer therapies against locally invasive and metastatic cancers, which are generally associated with a high rate of disease relapse and the death of cancer patients.

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Cross-Talk Between Notch and Hedgehog Regulates Hepatic Stellate Cell Fate in Mice

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Liver repair involves phenotypic changes in hepatic stellate cells (HSCs) and reactivation of morphogenic signaling pathways that modulate epithelial-to-mesenchymal/mesenchymal-to-epithelial transitions, such as Notch and Hedgehog (Hh). Hh stimulates HSCs to become myofibroblasts (MFs). Recent lineage tracing studies in adult mice with injured livers showed that some MFs became multipotent progenitors to regenerate hepatocytes, cholangiocytes, and HSCs. We studied primary HSC cultures and two different animal models of fibrosis to evaluate the hypothesis that activating the Notch pathway in HSCs stimulates them to become (and remain) MFs through a mechanism that involves an epithelial-to-mesenchymal–like transition and requires cross-talk with the canonical Hh pathway. We found that when cultured HSCs transitioned into MFs, they activated Hh signaling, underwent an epithelial-to-mesenchymal–like transition, and increased Notch signaling. Blocking Notch signaling in MFs/HSCs suppressed Hh activity and caused a mesenchymal-to-epithelial–like transition. Inhibiting the Hh pathway suppressed Notch signaling and also induced a mesenchymal-to-epithelial–like transition. Manipulating Hh and Notch signaling in a mouse multipotent progenitor cell line evoked similar responses. In mice, liver injury increased Notch activity in MFs and Hh-responsive MF progeny (i.e., HSCs and ductular cells). Conditionally disrupting Hh signaling in MFs of bile-duct–ligated mice inhibited Notch signaling and blocked accumulation of both MF and ductular cells. *Conclusions:* The Notch and Hedgehog pathways interact to control the fate of key cell types involved in adult liver repair by modulating epithelial-to-mesenchymal–like/mesenchymal-to-epithelial–like transitions. (HEPATOLOGY 2013;58:1801-1813)

The outcome of liver injury is dictated by the efficiency of repair responses that replace damaged liver tissue with healthy hepatic parenchyma. Defective repair of chronic liver injury can result in cirrhosis, a scarring condition characterized by dramatic changes in the cellular composition of the liver. Outgrowth of progenitors and myofibroblasts (MFs) is particularly prominent during scarring.[1] Because these cell types are critical for successful regeneration of damaged livers,[1,2] their accumulation in cirrhotic liver suggests that scarring may occur because regenerative mechanisms become stalled prematurely. Therefore, to restore healthy wound healing, it is necessary to characterize and prioritize the key signals that regulate the fate of cells that are required for liver repair.

Reconstruction of damaged adult liver utilizes several highly conserved signaling pathways that orchestrate organogenesis during fetal development, including Wnt, Hedgehog (Hh), and Notch.[3] During embryogenesis,

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; BDL, bile duct ligation; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; DTG, double transgenic; FACS, fluorescence-activated cell sorting; GFAP, glial fibrillary acidic protein; Gli, glioblastoma; Hes, hairy and enhancer of split; Hey, hairy/enhancer-of-split related with YRPW; HFD, high-fat diet; Hh, Hedgehog; HNF, hepatocyte nuclear factor; HSCs, hepatic stellate cells; IHC, immunohistochemistry; Krt, keratin; MF, myofibroblast; mRNAs, messenger RNAs; Oct-4, octamer-binding transcription factor 4; PPAR-γ, peroxisome proliferator-activated receptor gamma; Ptc, Patched; Q, quiescent; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; shh, Sonic Hedgehog; Smo, smoothened; α-SMA, alpha smooth muscle actin; Sox9, SRY (sex determining region Y)-box 9; TGF-β, transforming growth factor beta; TMX, tamoxifen; WT, wild type.

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these pathways interact to modulate survival, proliferation, and differentiation of their target cells so that developing organs become appropriately populated with all of the cell types necessary for tissue-specific functions. For example, cross-talk between Hh and Notch controls the fate of embryonic stem cells,[4] zebrafish neural progenitors,[5] and *Drosophila* eye precursors.[6] In cancer biology, the importance of cell-autonomous cross-talk between Hh and Notch is also emerging. Overexpression of both the Notch- and Hh-signaling pathways occurs in a subpopulation of chemotherapy-resistant cancer stem cells, and targeting Notch and Hh depleted this population.[7] However, whether similar cross-talk occurs when damaged adult livers are regenerated, which cell types are involved, and whether or not such signaling becomes deregulated during defective repair, is not well understood. Also uncertain is if and how these newly uncovered pathways in the damaged adult liver fit into the classical paradigms for cirrhosis pathogenesis, and whether they are more or less important for that process than well-established regulators of adult liver growth, such as transforming growth factor beta (TGF-β), which is generally credited for driving defective liver repair in adults.[1]

Therfore, the aims of this study were to investigate if and how Notch signaling regulates damage-related outgrowth of liver MFs. We focused on MF derived from HSCs because adult HSCs are TGF-β-responsive cells that are also influenced by developmental morphogenic pathways, such as Wnt and Hh, which reactivate during adult liver repair. Adult HSCs require Hh signaling to become and remain MFs.[8] Recent lineage tracing studies in adult mice with injured livers demonstrated that some MFs became multipotent progenitors that regenerated hepatocytes, cholangiocytes, and HSCs. In parallel experiments, Cre recombinase-mediated knockdown of canonical Hh signaling in cells expressing the MF gene, alpha smooth muscle actin (α-SMA), both blocked MF accumulation and inhibited outgrowth of ductular cells during cholestatic liver injury.[9] Both autocrine and paracrine signaling regulated by the Hh pathway might be involved. For example, Sonic hedgehog ligand is known to promote the transcription of Jagged-1,[10] and MF-derived Jagged-1 is thought to work in a paracrine fashion to promote ductular differentiation of Notch-responsive liver progenitors.[2] Previous work suggested that HSCs themselves may also be capable of Notch signaling.[11] Most recently, Chen et al. reported that N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor that blocks Notch signaling, decreased expression of various MF genes in a rat HSC line (HSC-T6).[12] They also found that DAPT inhibited CCl_4-related fibrosis in rats and showed that this was accompanied by reduced hepatic expression of TGF-β, Snail, and various mesenchymal genes, but up-regulation of E-cadherin, suggesting that blocking Notch promoted mesenchymal-to-epithelial transitions.[13] However, an earlier study of cultured HSCs correlated induction of Notch-1 and Hes1 with suppression of α-SMA expression and proliferation, and showed that knocking down expression of Notch-1 enhanced HSC growth.[14]

Indeed, the effects of Notch on MF differentiation and growth are complex and appear to vary according to the type of MF precursor. Notch signaling inhibits myofibroblastic differentiation of myoblast precursors and some types of fibroblasts.[15,16] In contrast, it enhances MF differentiation of lung MF precursors,[17] airway epithelial cells,[18] and dermal fibroblasts.[19] Activating Notch also promotes epithelial-to-mesenchymal transition in kidney cells,[20] stimulates expansion of cardiac progenitors at the expense of MFs,[21] and promotes an epithelial-to-mesenchymal transition process that enhances the stem-like properties of cancer stem cells.[22]

Notch signaling is critical for biliary morphogenesis during development.[23-25] As mentioned earlier, the fate of adult liver progenitors is also directed by Notch: Increasing Notch signaling promotes differentiation along the biliary lineage, whereas suppressing the Notch pathway shifts progenitors toward an hepatocytic fate.[2] Deregulated Notch signaling has been implicated in the pathogenesis of hepatocellular carcinoma and cholangiocarcinoma.[26,27] Despite growing evidence for Notch pathway involvement in liver cancer and fibrosis, it is unclear how Notch interfaces with other key signaling pathways that have been implicated in those disorders, or how Notch signaling in one type of liver cell (e.g., MFs) might influence the accumulation of other types

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of liver cells (e.g., epithelial progenitors) that are required for adult liver repair.

In this study, we evaluated the hypothesis that Notch pathway activation in HSCs stimulates them to become (and remain) MFs through a mechanism that involves an epithelial-to-mesenchymal–like transition requiring cross-talk with canonical (i.e., TGF-β-independent) Hedgehog signaling.

Materials and Methods

Full methods are available in the Supporting Information.

Animals. Male C57BL/6 mice and *Smo*^{tm2Amc}/J (Smoothened [Smo]/flox) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).[28] Smo/flox mice were crossed with α-SMA-Cre-ER^{T2} transgenic mice[29] to generate double-transgenic (DTG) mice in which treatment with tamoxifen induces conditional deletion of Smo in α-SMA-positive cells.[9] Mice (8-12 weeks old) were subjected to bile duct ligation (BDL) or sham surgery for 14 days. Other 8-10-week-old wild-type (WT) mice were fed with a high-fat diet (HFD) and given intraperitoneal injection of either vehicle (olive oil) or CCl_4 (1 μL/g body weight, prediluted 1:3 in olive oil) twice per week for 2 weeks and sacrificed 72 hours after last CCl_4 injection.[30] Animal experiments fulfilled National Institutes of Health (Bethesda, MD) and Duke University Institutional Animal Care and Use Committee (Durham, NC) requirements for humane animal care.

Immunohistochemistry. Formalin-fixed, paraffin-embedded livers were prepared for immunohistochemistry (IHC).[9] Protocols and antibodies used are listed in the Supporting Information.

Molecular Techniques. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and immunoblottings were performed as previously described.[31]

Cell Isolation. Primary HSCs were isolated from C57BL/6 mice using standard approaches. Purity of the preparations was rigorously analyzed as previously described.[9]

Pharmacological Manipulation of Notch and Hh Signaling. Day 4 primary HSC cultures were treated with the γ-secretase inhibitor, DAPT (10 μM; Sigma-Aldrich, St Louis, MO), or the Smoothened agonist, GDC-0449 (1 μM; Selleck Chemicals, Houston, TX), for 3 days. Controls were treated with dimethyl sulfoxide (DMSO). 603B cells were treated the same way for 2 days.

Statistical Analysis. Results are expressed as mean ± standard error of the mean. Analyses were performed using the Student t test. $P < 0.05$ was considered significant.

Results

Activation of Notch Signaling in Desmin-Expressing Cells During Hepatic Injury. We found up-regulation of messenger RNAs (mRNAs) for Notch-2, Jagged-1, and several Notch-target genes (Hes1, Hey1, Hey2, and HeyL) in a mouse BDL model (Fig. 1A), consistent with previous reports that adult liver injury activates Notch signaling.[2,23] In addition to ductal cells (known Notch targets),[23] stromal cells expressed Notch-2, Jagged-1, and Hey2 post-BDL (Fig. 1B and Supporting Fig. 1A). Some of these stromal cells costained with the HSC marker, Desmin, suggesting that activated Notch signaling occurs in MFs/HSCs during liver injury. Quantitative IHC indicated that approximately 60% of the Desmin(+) cells coexpressed Notch-2 and/or Jagged-1 and 30% coexpressed Hey2. These findings were confirmed with fluorescence-activated cell sorting (FACS) analysis of HSCs isolated from BDL mice, which showed increased Notch-2, Jagged-1, and Hey2, compared to HSCs harvested from sham controls (Fig. 1C and Supporting Fig. 1B).

We also examined mice treated with HFD ± CCl_4 for 2 weeks to provoke liver sinusoidal fibrosis. Compared to HFD-fed controls, mice treated with HFD/CCl_4 demonstrated increased mRNA expression of Notch-2, Jagged-1, Hes1, Hey1, and Hey2, as well a ductular marker, keratin (Krt)19 (Fig. 1D). As noted in BDL mice with portal-based fibrosis (Fig. 1B,C), quantitative IHC also demonstrated increased Notch-2, Jagged-1, and Hey2 expression in Desmin-positive cells of mice with CCl_4-induced sinusoidal fibrosis (Fig. 1E and Supporting Fig. 1C).

***Up-Regulation of Notch Signaling During HSC Activation* In Vitro.** Although it is established that cholangiocytes and their precursors are capable of Notch signaling,[24,25,27] it is uncertain whether primary HSCs and/or their progeny (e.g., MFs/HSCs) respond to Notch. Because IHC and FACS revealed Notch signaling components in Desmin-expressing cells that accumulate in fibrotic livers (Fig. 1B,C,E), we evaluated the expression of Notch-pathway genes in primary mouse HSCs (both freshly isolated HSCs and 7-day, culture-activated MFs/HSCs; Fig. 2A,B). Results in HSCs were compared to those in a mouse ductular cell line (603B), which served as a positive control for Notch signaling (Fig. 3). FACS showed that 603B cells express the cholangiocyte marker, Krt19, progenitor markers (SRY [sex determining region Y]-box 9

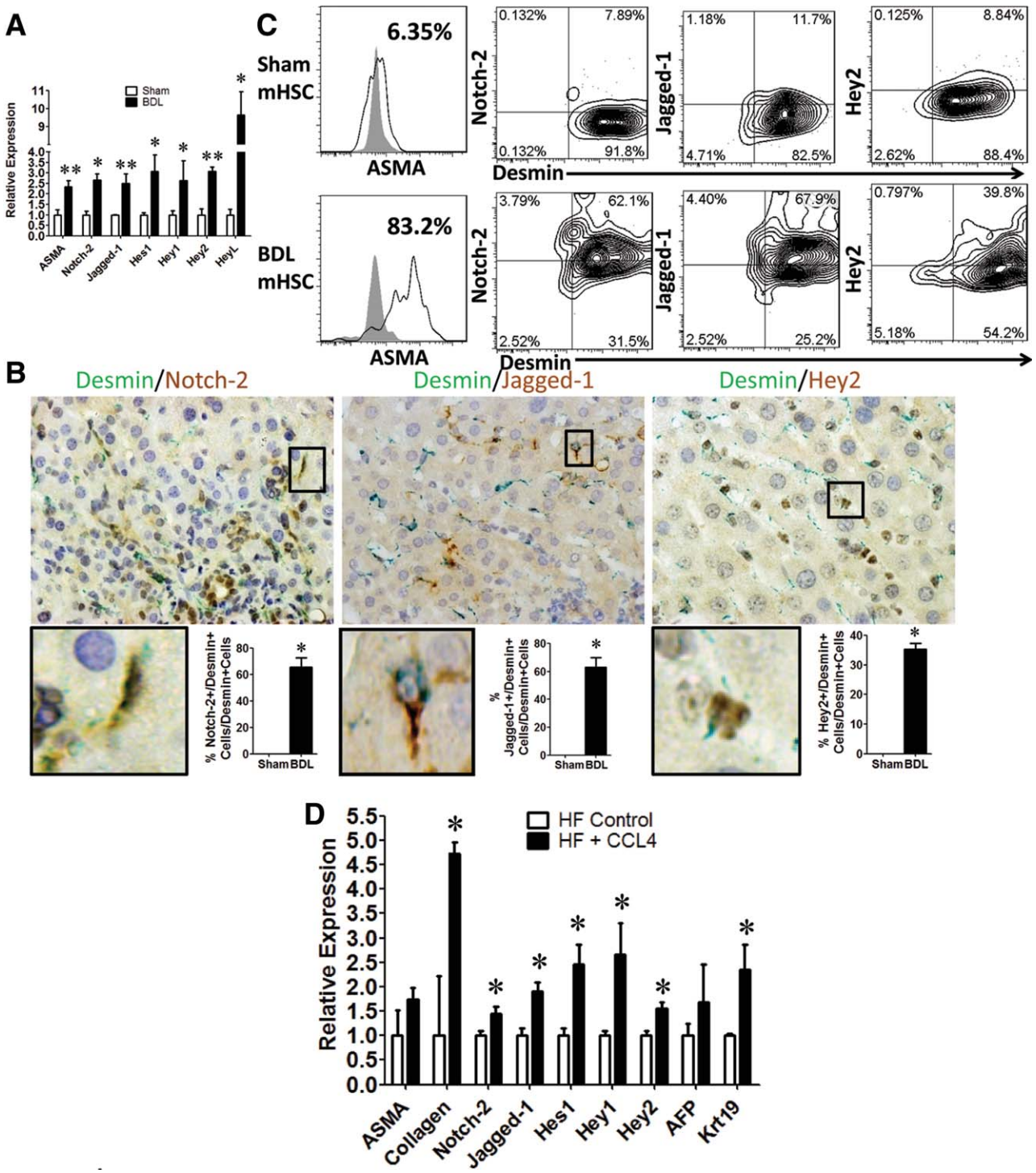

Fig. 1. Liver injuries increase Notch signaling in Desmin-expressing stromal cells. (A) qRT-PCR analysis of total liver mRNA from WT mice 14 days after sham or BDL surgery for expression of Notch-pathway genes. $^*P < 0.05$ versus sham; n = 4. (B) Double IHC for Notch-2, Jagged-1, or Hey2 (brown) with Desmin (green) in BDL mouse livers demonstrates colocalization (inset) of these markers. Percentages of double-positive cells among Desmin(+) cells were also quantified in 10 randomly selected fields. $^*P < 0.001$ versus sham; n = 3 mice/group. (C) FACS analysis of HSCs isolated from WT mice 14 days after sham or BDL surgery for expression of α-SMA, Notch-2, Jagged-1, or Hey2. Desmin was used as a marker for HSCs. (D) qRT-PCR analysis of total mRNA from livers of WT mice treated for 14 days with HFD/CCl_4. $^*P < 0.05$; $^{**}P < 0.01$ versus HFD controls; n = 3. (E) Double IHC for Notch-2, Jagged-1, or Hey2 (brown) and Desmin (green) in HFD/CCl_4 mouse livers demonstrates colocalization (inset) of these markers. Magnification, ×40. Percentages of double-positive cells among Desmin(+) cells were also quantified in 10 randomly selected fields. $^*P < 0.001$ versus HF Ctrl; n = 3 mice/group.

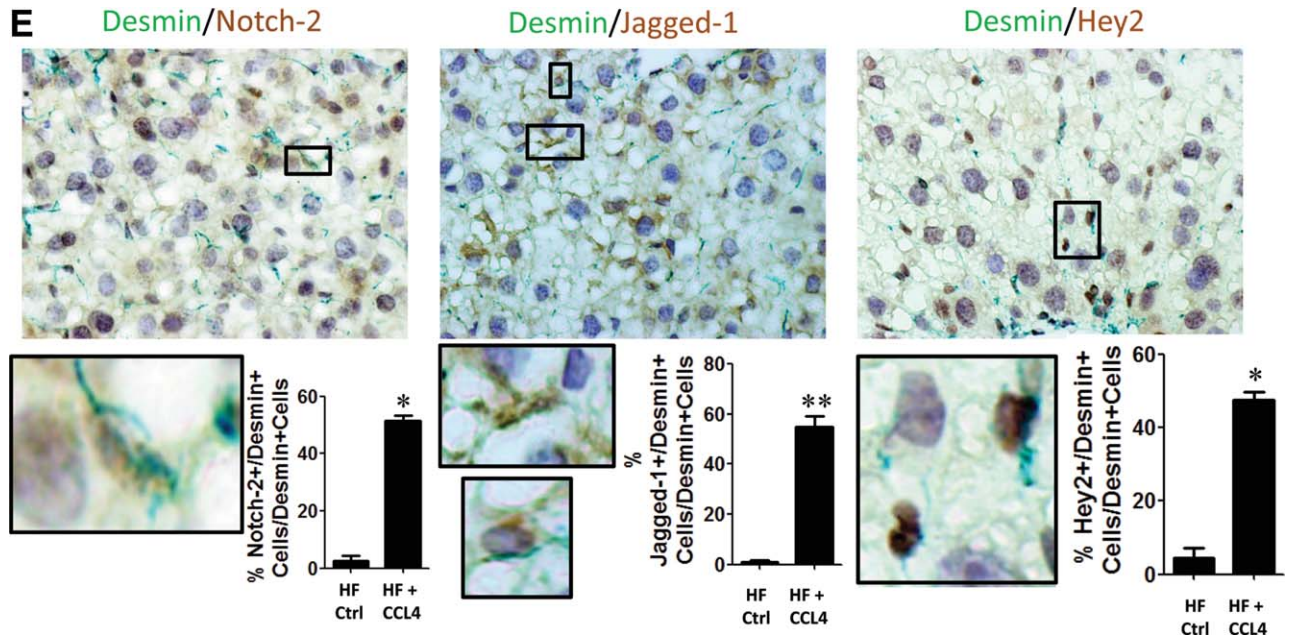

Fig. 1. (Continued)

[Sox9], FN14, and CD24), and Notch pathway components (Notch-2 and Jagged-1) at very high levels, confirming that such cells are immature ductular-type cells with Notch-signaling capability (Fig. 3A). FACS similarly revealed that HSCs express proteins that regulate Notch signaling, including the Notch ligand, Jagged-1, Notch-1, and Notch-2 receptors, and Numb, a Notch-signaling repressor (Fig. 2A and Supporting Fig. 2A). qRT-PCR analysis readily demonstrated mRNA for these factors (Fig. 2B), whereas expression of another Notch ligand (Jagged-2) and other Notch receptors (Notch-3 and Notch-4) was detected at much lower levels (Supporting Fig. 2B).

Compared to freshly isolated (day 0) HSCs, which were relatively enriched with cells expressing Notch-1 and Numb proteins, MFs/HSCs demonstrated much lower expression of Notch-1 and Numb, but much higher expression of Jagged-1 and Notch-2 (Fig. 2A and Supporting Fig. 2A), consistent with a previous report showing decreased Notch-1 expression during rat HSC culture activation.[11] Thus, expression of proteins regulating Notch signaling changed substantially during MF transdifferentiation. To determine whether pathway activity also changed as quiescent (Q)-HSCs transitioned into MFs/HSCs, qRT-PCR analysis was performed to assess the expression of various Notch target genes (Hes1, Hey1, Hey2, and c-Myc; Fig. 2B). Hey2 and c-Myc mRNA expression increased significantly during HSC activation. This induction of Notch target genes occurred in conjunction with up-regulation of Jagged-1 and Notch-2 mRNAs and coincided with down-regulation of mRNAs for Notch-1 and Numb. The results suggest that HSCs activate Notch signaling as they become MFs. This possibility is supported by evidence that several Notch target gene (Hes1, Hey1, and Hey2) mRNA levels in HSCs are generally equal to or higher than their levels in ductular-type cells with acknowledged Notch-signaling capability (Fig. 2B).

Phenotypic and Genotypic Similarities in Notch-Responsive Liver Cells. Notch regulates the fate of bipotent liver epithelial progenitors,[2,25] and lineage-tracing evidence in adult mice indicates that bipotent liver epithelial progenitors and HSCs derive from a common multipotent progenitor that is controlled by the Hh pathway.[9,32] Thus, it is conceivable that Notch interacts with Hh to direct the differentiation of adult progenitors during liver injury. We began to examine this issue by further characterizing 603B cells by FACS (Fig. 3A,B) and using qRT-PCR to compare gene expression in 603B cells, mature liver cells (primary mouse hepatocytes), and freshly isolated or culture-activated primary HSCs (Fig. 3C).

FACS showed that although 97%-99% of 603B cells express well-accepted markers of ductular progenitors (Krt19, Krt7, and Sox9), only approximately one third express the biliary-associated transcription factor, HNF6. Hepatocyte nuclear factor (HNF)−4α, a hepatocyte-associated transcription factor, is evident in ∼50%, suggesting that 603B cells are capable of

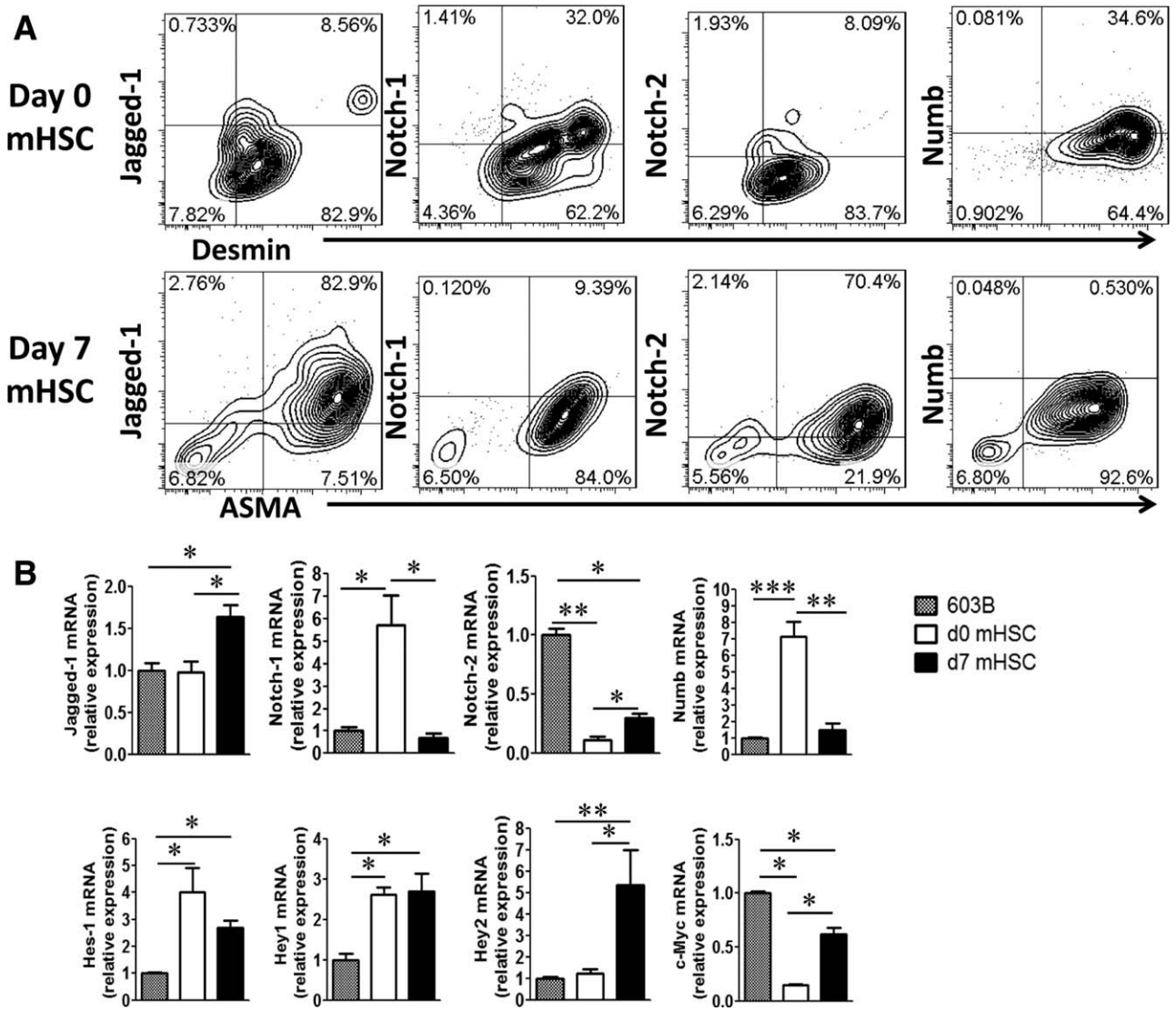

Fig. 2. Notch signaling is activated during transdifferentiation of primary HSCs. (A) FACS analysis of quiescent (freshly isolated, day 0) and myofibroblastic (culture day 7) HSCs. Desmin and α-SMA were used as markers for quiescent or myofibroblastic HSCs, respectively. (B) qRT-PCR analysis of Notch inhibitor (Numb), receptors (Notch-1 and Notch-2), ligand (Jagged-1), and target genes (Hes1, Hey1, Hey2, and c-Myc) in quiescent and myofibroblastic HSCs. Results were compared to gene expression in ductular progenitor cells (603B). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n = 3.

differentiating along both biliary and hepatocytic lineages. Consistent with that concept, virtually all of the cells (97%-99%) express established markers of hepatoblasts (a.k.a. oval cells), such as CD24, FN14, and albumin (ALB). More than 80% of 603B cells also express a putative HSC marker, glial fibrillary acidic protein (GFAP), suggesting that 603B cells may be multipotent (i.e., capable of differentiating into hepatocytes, cholangioctyes, and HSCs). Indeed, approximately one third of 603B cells express Desmin and approximately 25% are α-SMA positive. Coexpression of ductular, hepatocytic, and HSC markers occurs in Hh-responsive multipotent liver progenitors that are undergoing epithelial-mesenchymal transitions.[9] Ninety-nine percent of 603B cells coexpress Krt7 (epithelial marker), vimentin (mesenchymal marker), and one or more Hh target genes (Patched [Ptc], glioblastoma [Gli]1, and Gli2), exhibiting the phenotype of multipotent liver progenitors that are in the midst of epithelial-mesenchymal transitions (Fig. 3A,B).

qRT-PCR analysis provided additional evidence that 603B cells are transitioning multipotent liver progenitors. Compared to freshly isolated primary hepatocytes from healthy adult mice, 603B cells express significantly higher mRNA levels of Hh target genes (Ptc and Gli2), cholangiocyte-associated genes (e.g., Krt19 and HNF-6), and HSC-associated genes (e.g., Desmin and GFAP), but significantly lower mRNA levels of HNF-

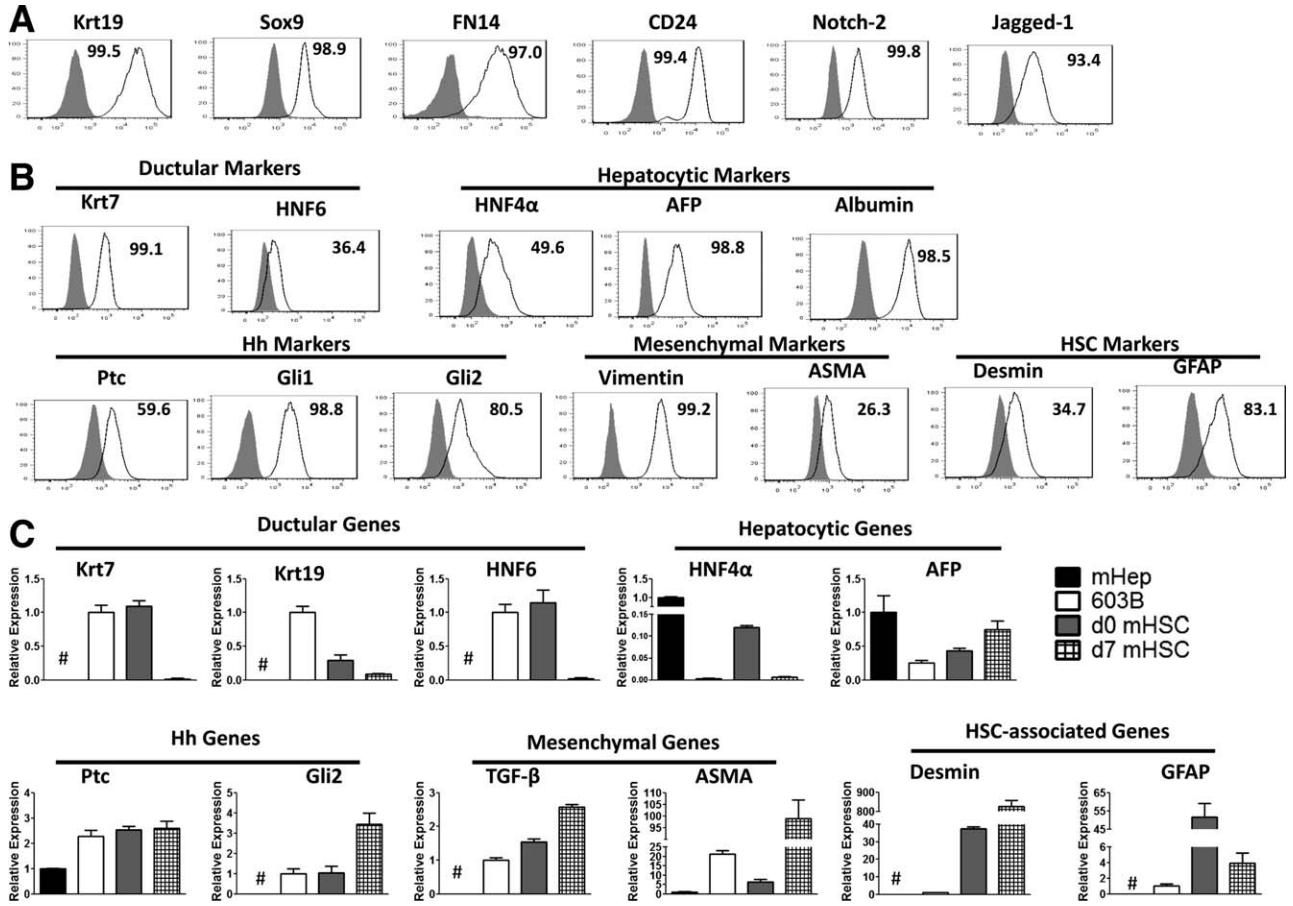

Fig. 3. Notch-responsive liver progenitors (603B) coexpress ductular, hepatocytic, HSC, and mesenchymal markers. (A) FACS analysis confirmed that 603B cells are mouse ductular progenitors with active Notch signaling. Gray lines indicate isotype controls. (B) FACS analysis of 603B cells demonstrated expression of other ductular markers (Krt7 and HNF-6), but also hepatocytic markers (HNF-4α, AFP, and ALB), Hh-signaling factors/target genes (Ptc, Gli1, and Gli2), mesenchymal markers (Vimentin and α-SMA), and HSC-associated markers (Desmin and GFAP). (C) Comparison of gene expression in 603B cells with primary mouse hepatocytes (mHep) and freshly isolated or culture-activated primary mouse HSCs (d0 mHSC and d7 mHSC, respectively) by qRT-PCR analysis; n = 3/group. #Nondetectable signal.

4α, a transcription factor that is strongly expressed by mature hepatocytes. As reported for transitional multipotent progenitors,[9] gene expression in 603B cells is more similar to HSCs than hepatocytes. For example, primary HSCs and 603B cells express comparable mRNA levels of Krt7, HNF-6, alpha-fetoprotein (AFP), Ptc, and Gli2. However, mRNA levels of Desmin and GFAP are significantly lower in 603B cells than freshly isolated HSCs, and this discrepancy is magnified when HSCs undergo culture activation to become MFs (Fig. 3C). Nevertheless, the aggregate data demonstrate genotypic and phenotypic similarities in Notch-responsive liver cells, and indicate that such cells are Hh responsive and inherently plastic (i.e., capable of undergoing epithelial-mesenchymal transitions).

***DAPT Inhibits Notch Signaling in Both Progenitors and HSCs* In Vitro.** To investigate the functional significance of Notch signaling in HSCs, the Notch pathway was suppressed by treating cultured primary MFs/HSCs with a γ-secretase inhibitor (DAPT). Results in HSCs were compared to those in multipotent progenitor cells (603B), which served as a positive control for Notch signaling. As expected, studies in 603B cells showed that DAPT treatment significantly reduced expression of Jagged-1, Notch-2, and Notch target genes (Hes1, Hey1, and Hey2; Fig. 4). Inhibiting Notch signaling in 603B cells suppressed the expression of cholangiocyte-associated genes (Krt7, Krt19, HNF-1β, and HNF-6) and permitted induction of hepatocyte lineage markers (AFP, HNF-1α, and HNF-4α), consistent with previous reports that activation of Notch signaling drives liver progenitors toward the biliary lineage, whereas its suppression promotes differentiation along the hepatocytic lineage.[2,24,25] Blocking Notch signaling in 603B enhanced expression of GFAP, a Q-HSC marker, but reduced α-SMA, an MF/HSC marker, and TGF-β, a profibrogenic cytokine that promotes ductular differentiation of liver

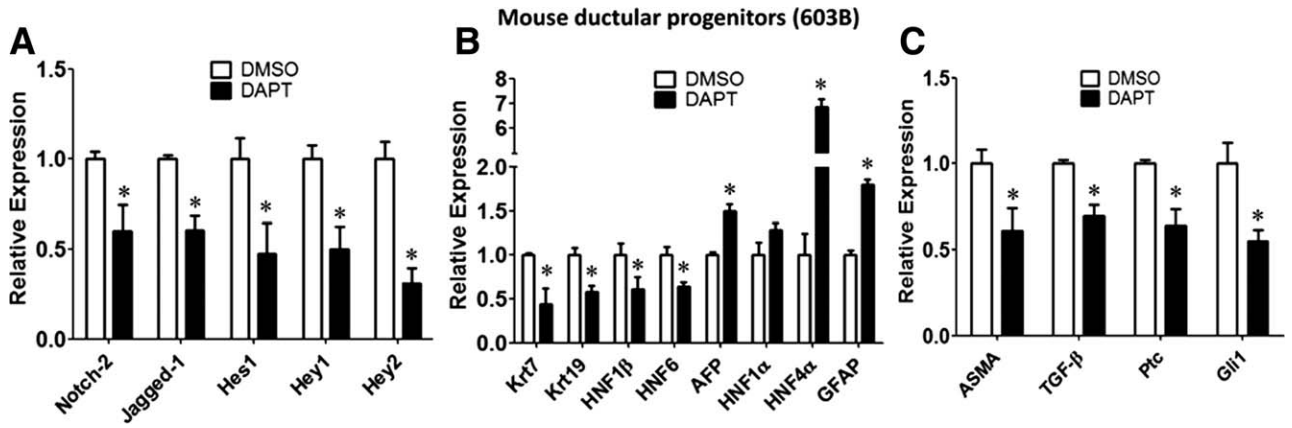

Fig. 4. Inhibiting Notch signaling suppresses Hedgehog signaling and promotes a mesenchymal-to-epithelial–like transition and hepatocytic differentiation in ductular-type progenitor cells. qRT-PCR analysis of 603B cells treated with DAPT (a γ-secretase inhibitor) for 48 hours for changes in (A) Notch-pathway genes, (B) epithelial/quiescence genes, and (C) MF/Hh genes. $^*P < 0.05$ versus DMSO control; n = 3.

progenitors in developing embryos.[33] Blocking Notch also down-regulated key Hedgehog target genes (Gli1 and Ptc) in 603B cells. The aggregate findings suggest that Notch signaling interfaces with fibrogenic signals that are transduced by TGF-β and the Hh pathway in multipotent liver progenitor cells. This is particularly intriguing because both TGF-β and Hh signaling promote epithelial-to-mesenchymal transitions in developing embryos,[34] and Hh has been proven to stimulate epithelial-to-mesenchymal–like transitions in both adult HSCs and progenitor cells.[8,35]

Having confirmed that DAPT performed as anticipated in Notch-responsive liver progenitor cells, we evaluated its actions in HSCs. For these studies, primary murine HSCs were cultured for 4 days to induce MF transdifferentiation and then treated with DAPT for an additional 3 days. As in 603B cells (Fig. 4), MFs/HSCs showed DAPT-inhibited expression of Notch-2, Jagged-1, and several Notch target gene (Hey1, Hey2, and HeyL) mRNAs (Fig. 5A). IHC confirmed that mRNA suppression was accompanied by decreased protein expression (Fig. 5E). Blocking Notch signaling in MFs/HSCs also repressed typical MF-associated genes (α-SMA, collagen, and TGF-β) and Hh target genes that are known to be expressed by MFs/HSCs (Gli2, Ptc, and Sonic Hedgehog [Shh]; Fig. 5B). In contrast, mRNA levels of various epithelial genes (bone morphogenic protein-7, desmoplakin, E-cadherin, AFP, HNF-4α, and Krt19) and Q-HSC markers (peroxisome proliferator-activated receptor gamma [PPAR-γ] and GFAP) were up-regulated (Fig. 5C). Immunocytochemistry confirmed the DAPT-induced reversion of MFs/HSCs to a more quiescent phenotype, showing decreased staining for α-SMA and Ki67 (proliferation marker) and increased Oil Red O staining, indicative of neutral lipid accumulation (Fig. 5F). Interestingly, when Notch signaling was inhibited and MFs/HSCs reverted to a more quiescent phenotype, mRNA expression of delta-like 1 homolog, a Notch-related gene that marks liver progenitors,[36] and mRNAs encoding other progenitor cell markers (e.g., Nanog, octamer-binding transcription factor 4 [Oct4], and FN14) were down-regulated (Fig. 5D). Thus, Notch signaling is activated during culture-induced primary MF/HSC transdifferentiation, and this permits the cells to acquire a more mesenchymal phenotype with progenitor-like features. This process parallels activation-associated induction of Hh signaling and might be regulated by cross-talk between the Notch and Hh pathways, because HSCs require Hh signaling to become MFs.[8,31]

***Inhibiting Hedgehog Signaling Blocks Notch Signaling* In Vitro.** To further examine possible cross-talk between Notch and Hh signaling, the two Notch-responsive cell types (603B and primary MFs/HSCs) were treated with an Hh-signaling antagonist (GDC-0449). GDC-0449 directly interacts with and inhibits the Hh coreceptor, Smoothened.[37] Earlier work has proven that GDC-0449 recapitulates the effect of Smoothened gene knockdown in MFs/HSCs, with both approaches inhibiting canonical Hh signaling, thereby blocking the nuclear localization and transcriptional activation of Gli DNA-binding proteins.[31] In both cell types, antagonizing Smoothened caused suppression of Notch-2, Jagged-1, and Notch target genes (Fig. 6A,B), demonstrating that canonical Hh-pathway activity promotes the expression of Notch-signaling pathway genes. Given that DAPT, a γ-secretase inhibitor that specifically blocks Notch signaling, suppressed expression of Shh ligand, Gli2 (Hh-regulated

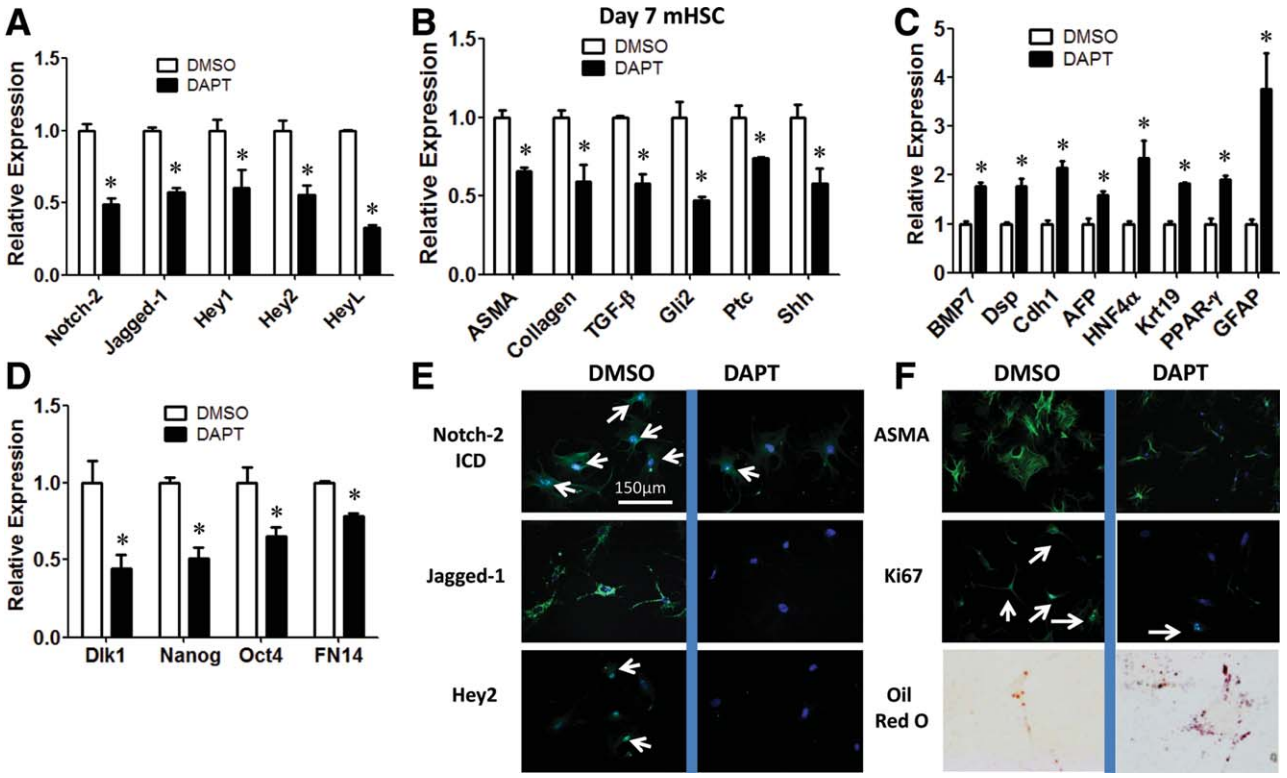

Fig. 5. Notch inhibition suppresses Hedgehog signaling and promotes a mesenchymal-to-epithelial–like transition in primary HSCs. qRT-PCR analysis of primary MFs/HSCs treated with DAPT for 3 days for changes in (A) Notch genes, (B) MF/Hh target genes, (C) epithelial/quiescence genes, and (D) progenitor genes. $*P < 0.05$ versus DMSO control; $n = 3$. (E) DAPT-treated MFs/HSCs were stained for cleaved Notch-2, Jagged-1, and Hey2 protein. Scale bar: 150 μM. (F) Effect of DAPT on HSC expression of α-SMA, proliferation (Ki67), and lipid content (Oil Red O) was examined.

transcription factor), and Ptc (a direct transcriptional target of Gli) (Fig. 5), the Notch pathway seems to stimulate Hh-pathway activity. Hence, the results identify a previously unsuspected Hh-Notch-positive feedback loop that regulates cell-fate decisions in immature ductular-type cells and MFs/HSCs. In certain types of adult liver injury, these two cell types accumulate and intermingle within fibrotic septae that extend outward from portal tracts to cause bridging fibrosis, an antecedent to cirrhosis.[38] This suggests that Notch-Hh interactions might regulate cirrhosis pathogenesis by controlling the fate of two key cell types that are involved in liver repair.

***Blocking Hh Signaling in MF Inhibits Notch Signaling* In Vivo.** To verify that Hh signaling regulates Notch signaling *in vivo*, as observed *in vitro*, and to evaluate the functional implications of this interaction for liver repair, we used a genetic approach to conditionally delete Smoothened in MFs/HSCs. DTG mice were created by crossing $Smo^{flox/flox}$ mice with α-SMA/Cre-ER^{T2} mice. Treating such DTG mice with tamoxifen (TMX) induced selective deletion of the floxed Smo gene, but only in α-SMA-expressing cells,[31] providing a useful tool for examining the effects of Hh signaling in MFs/HSCs and their progeny.[9] DTG mice underwent BDL to provoke liver injury and compensatory repair responses. Four days later, treatment with either vehicle or TMX was initiated and given every other day through day 10; mice were sacrificed on day 14 post-BDL for liver tissue analysis. In an earlier study, we showed that this approach knocked down expression of Smo in the liver, reduced the hepatic content of α-SMA(+) cells by >85%, and significantly decreased collagen gene expression, hepatic hydroxyproline content, and Sirius Red staining, as well as accumulation of Krt19(+) ductular cells.[9] In this study, we confirmed that TMX reduced both Smo and α-SMA expression (Fig. 6C), and showed that decreasing Hh-responsive MFs dramatically decreased numbers of Notch-2(+) and Hey2(+) cells, both along liver sinusoids (colocalized with Desmin(+) cells) and in residual ductular structures (Fig. 6D). qRT-PCR analysis of whole-liver RNA demonstrated that loss of Notch-2-expressing cells in TMX-treated DTG mice was accompanied by significantly reduced whole-liver expression of Notch target genes, compared

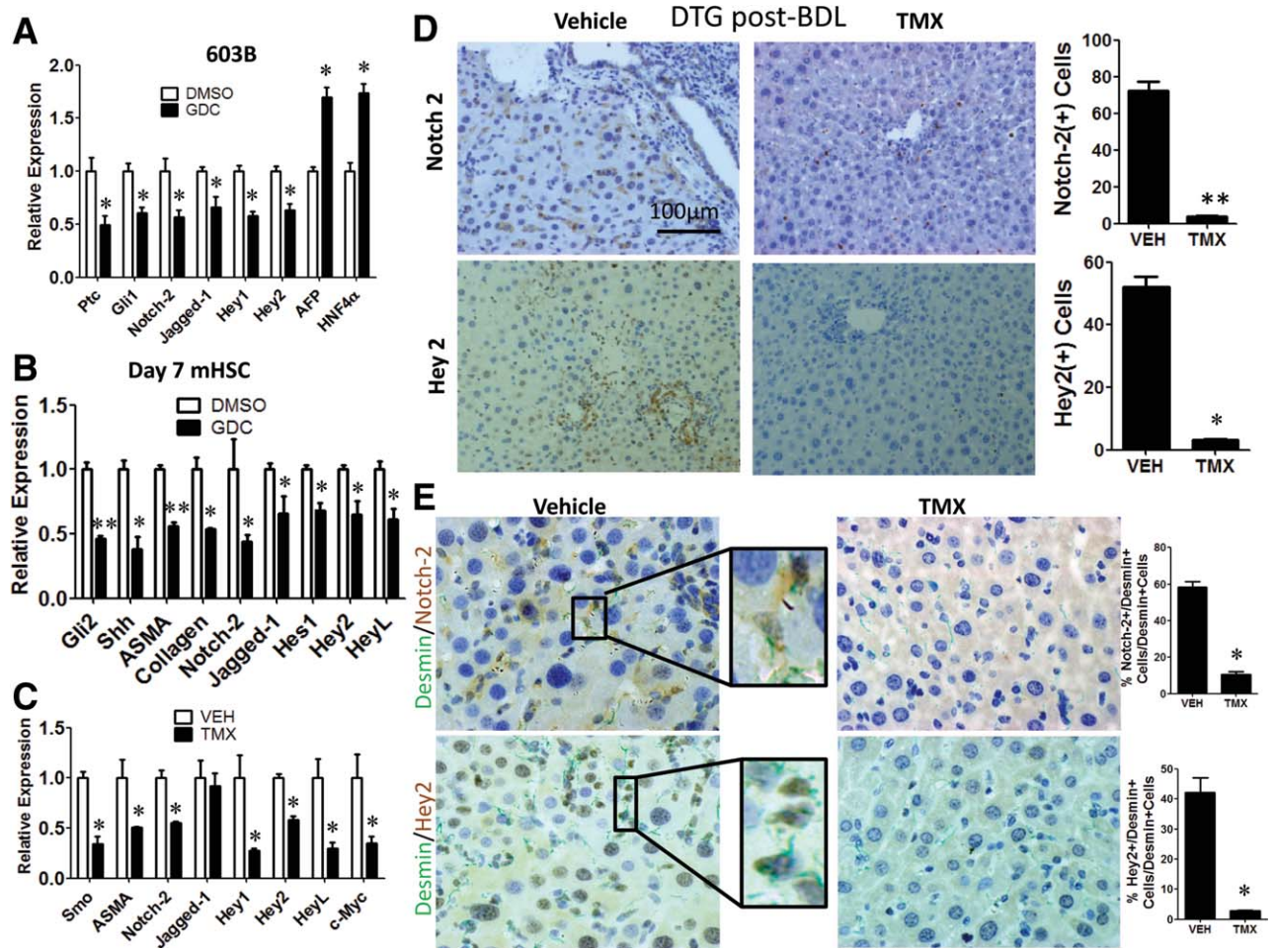

Fig. 6. Blocking Hedgehog signaling in myofibroblastic liver cells inhibits Notch signaling. (A) qRT-PCR analysis of 603B cells treated with an Hh inhibitor (GDC-0449) or DMSO for 48 hours for changes in Hh target genes (Ptc and Gli1), Notch genes (Notch-2, Jagged-1, Hey1, and Hey2), and epithelial genes (AFP and HNF-4α). $*P < 0.05$ versus DMSO control; n = 3. (B) qRT-PCR analysis of primary MFs/HSCs treated with GDC-0449 for 3 days. $*P < 0.05$; $**P < 0.01$ versus DMSO control. (C-E) α-SMA/Cre-ER^{T2}–Smo-flox (DTG) mice were subjected to BDL and treated with vehicle (VEH, olive oil; n = 3) or TMX (n = 4) every other day from days 4 to 10 post-BDL. (C) qRT-PCR analysis of total liver mRNA; $*P < 0.05$. (D) Representative IHC and quantification for Notch-2 and Hey2. Scale bar: 100 μm. $*P < 0.05$; $**P < 0.01$. (E) Double staining of Notch-2 or Hey2 (brown) with Desmin (green) in liver sections described in the legend to Fig. 5D. Percentages of Notch-2/Desmin or Hey2/Desmin double-positive cells among Desmin(+) cells were also quantified. At least 10 fields were counted per mouse. $*P < 0.05$; n = 3.

to vehicle-treated controls (Fig. 6C). Immunoblotting analysis of whole-liver lysates confirmed that suppression of Notch signaling was accompanied by the expected loss of proteins that mark ductular-type cells and their progenitors (e.g., Krt19 and HNF-6), with concomitant induction of the hepatocyte-enriched transcription factor, HNF-4α (Supporting Fig. 3C). Interestingly, however, we were unable to detect differences in expression of Jagged-1 mRNA (Fig. 6C) or protein (Supporting Fig. 3A) in our BDL mice, despite significant reductions in α-SMA-expressing cells at the time point we examined. IHC demonstrated colocalization of Jagged-1 in Desmin(+) stromal cells that persisted after Smo deletion, suggesting that unlike culture-activated MFs/HSCs (Fig. 5A), *in vivo*–activated HSCs maintain Jagged-1 expression for at least a while after they revert from a myofibroblastic state to a more quiescent HSC phenotype. To determine whether or not Jagged-1 is able to activate Notch signaling after Smo knockdown, we tested responses to recombinant Jagged-1 ligand in primary HSCs from $Smo^{flox/flox}$ mice after HSCs were culture activated to MFs and treated with Cre-recombinase adenoviral vectors to delete Smo. Results were compared to $Smo^{flox/flox}$ HSCs treated with control adenoviral vectors (adenovirus encoding green fluorescent protein). Jagged-1 significantly increased expression of Notch 2 and Notch target genes in control HSCs, but had no effect in Smo-depleted HSCs (Supporting Fig. 3B). Thus, the aggregate *in vivo* and *in vitro* data suggest that the Hh pathway modulates Notch signaling downstream of Jagged-1 in liver cells, at least in part, by promoting expression of Notch-2. Abrogating

canonical Hh signaling prevents Jagged-1 from inducing Notch-2 and is sufficient to cause liver cells to become relatively resistant to Jagged-1, thereby inhibiting Jagged-Notch signaling and blocking induction of Notch target genes. This blocked the outgrowth of both myofibroblastic and ductular cells and reduced fibrosis during cholestatic liver injury (present data and previous work[9]). Given that blocking Notch inhibited Hh in cultured MFs (Fig. 5B), and inhibiting Notch signaling also decreased liver fibrosis in rats treated with CCl_4,[13] it seems likely that the Hh and Notch pathways interact to control HSC fate *in vivo*, as they do *in vitro*. Future experiments that conditionally disrupt Notch signaling in MFs are needed to resolve that issue.

Discussion

This study demonstrates, for the first time, that primary HSCs use the Notch-signaling pathway to regulate their transdifferentiation. We found that as HSCs become MFs in culture, they up-regulate their expression of the Notch ligand, Jagged-1, as well as the Notch-2 receptor, while down-regulating their expression of Notch-1 receptor and Numb, a Notch-signaling inhibitor. Our findings in primary mouse HSCs differ somewhat from those that were reported on recently in a T-antigen-transformed rat HSC line, which was shown to express mainly Notch-3.[12] However, as was noted in that immortalized rat HSC line, we also found that primary MFs/HSCs reverted to a less myofibroblastic phenotype when treated with DAPT, a specific Notch-signaling inhibitor. Moreover, we showed that inhibiting Notch permitted the primary MFs/HSCs to reacquire markers of Q-HSC (e.g., GFAP and PPAR-γ), reaccumulate lipid, become less proliferative, and express several genes that typify epithelial cells (e.g., E-cadherin and Desmoplakin). Evidence that blocking Notch signaling permits a mesenchymal-to-epithelial–like transition in primary MFs/HSCs is novel, but consistent with the known ability of Notch to promote epithelial-to-mesenchymal transitions.[39] Indeed, we observed that DAPT also decreased Notch signaling and mesenchymal gene expression in an immature ductular cell line (603B) with multipotent liver epithelial progenitor features. During this process, we observed that 603B exhibited not only the expected down-regulation of ductular progenitor markers (e.g., HNF-1β, HNF-6, and Krt19) and reciprocal up-regulation of hepatocytic progenitor markers (e.g., HNF-4α and AFP), but also showed increased expression of the Q-HSC gene, GFAP.

Evidence that a Notch-regulated progenitor for hepatocytes and cholangiocytes can also differentiate into Notch-sensitive cells that express markers of HSCs is consistent with an earlier lineage tracing study in adult mice, which suggested a common lineage for such bipotent liver epithelial progenitors and HSCs,[32] as well as a more recent lineage tracing study, which proved that α-SMA- and GFAP-expressing cells give rise to hepatocytes and ductular cells during adult liver injury.[9] MFs derived from HSCs express several markers of multipotent progenitors, including Oct4.[40] Other adult epithelial tissues are known to harbor subpopulations of differentiated (nonstem) cells that are capable of dedifferentiating into stem-like cells[41]; passage of such nonstem cells through epithelial-to-mesenchymal transitions has been closely connected to their entrance into the stem cell state.[42] These findings have prompted speculation that stem cell compartments in adult tissues might be replenished by contextual signals within the microenvironment that reactivate pluripotency factors, such as Oct4, in subpopulations of mature cells with intrinsic phenotypic plasticity.[41]

During liver injury, the hepatic microenvironment changes dramatically, and factors that are not expressed in healthy adult livers, such as Jagged and Hh ligands, accumulate. Many of the cell types required for liver repair are Hh responsive, including HSCs and bipotent liver progenitors. Activating Hh signaling in such cells globally affects their fate, provoking epithelial-to-mesenchymal–like transitions, stimulating proliferation, and enhancing survival.[43] Here, we demonstrate, for the first time, that Hh interacts with Notch to orchestrate these cell-fate changes in primary HSCs. We showed that blocking Notch signaling with DAPT inhibited expression of Hh target genes, such as Ptc, whereas GDC-0449, a direct antagonist of Smoothened, reduced expression of Notch-2, Hes1, Hey2, and HeyL. MFs/HSCs require cross-talk between the Notch and Hh pathways to retain their myofibroblastic phenotype, because blocking either pathway suppressed expression of typical MF markers (e.g., α-SMA and collagen) while inducing reexpression of quiescent markers (e.g., PPAR-γ and GFAP). Parallel studies in 603B cells confirm that similar Hh-Notch interactions regulate cell-fate decisions in multipotent liver progenitors. In addition, cross-talk with other key repair-related signaling pathways is likely to be involved because we found that DAPT suppressed expression of TGF-β mRNA in both MFs/HSCs and the progenitor cell line, and GDC-0449 has been reported to inhibit TGF-β expression in MFs/HSCs.[44] TGF-β interacts with its receptors to initiate signals that activate

Gli-family factors independently of Smoothened,[45] suggesting that Notch-Hh cross-talk might promote activation of other signaling pathways that reenforce their actions on downstream targets.

Therefore, to clarify the ultimate biological relevance of Hh-Notch interactions in adult liver repair, we used a Cre-recombinase-driven approach to target α-SMA-expressing cells and deleted Smoothened to abrogate canonical (i.e., TGFβ-independent) Hh signaling in mice with ongoing cholestatic liver injury induced by BDL. We found that knocking down Hh signaling in MFs significantly inhibited Notch signaling, decreasing whole-liver expression of various Notch target genes by 40%-60%. This inhibited accumulation of cells that express ductular markers, such as Krt19 and HNF-6 ($P < 0.05$ and 0.005 versus respective vehicle-treated controls). As expected by data generated here and in our earlier work,[9,31] blocking Hh signaling in MFs significantly decreased accumulation of collagen-producing cells and decreased liver fibrosis post-BDL. However, contrary to our prediction, depletion of MF did not appreciably reduce hepatic expression of Jagged-1. IHC localized Jagged-1 to Desmin(+) stromal cells that persisted after Smo depletion, suggesting that MFs/HSCs that revert to quiescence when Hh signaling is abrogated *in vivo* retain Jagged-1. However, Hh-deficient cells are relatively resistant to Jagged-Notch signaling, because treating Smo-depleted cells with recombinant Jagged-1 failed to evoke induction of Notch-2 or increase expression of Notch-regulated genes. Given present and published evidence for the inherent plasticity of HSCs and HSC-derived MFs,[40] additional research will be necessary to determine whether the outcomes observed after Smo knockdown in MFs of BDL mice reflect disruption of Hh-Notch interactions that control epithelial-to-mesenchymal–like/mesenchymal-to-epithelial–like transitions in these wound-healing cells. In any case, the new evidence that Hh signaling influences Notch-pathway activity in the injured adult mouse livers complements data that demonstrate mutually reenforcing cross-talk between these two signaling pathways in cultured adult liver cells. Stated another way, both *in vitro* and *in vivo*, activating the Hh pathway stimulates Notch signaling, and the latter further enhances profibrogenic Hh signaling. The newly identified positive feedback loop provides a previously unsuspected mechanism that helps to explain why a recent study found that treating rats with a Notch inhibitor reduced CCl_4-induced liver fibrosis.[13]

In summary, our latest discoveries complement work by other groups and, together, extend growing evidence that adult liver repair is controlled by reactivated morphogenic signaling pathways that orchestrate organogenesis during development, such as Notch and Hedgehog. These pathways clearly act in concert during adult organ repair and likely coordinate during development as well. In the adult liver, these mechanisms appear to involve modulation of fundamental fate decisions in subpopulations of adult liver cells that retain high levels of inherent plasticity. Although additional research is needed to clarify the nuances of this insight, it has already identified a myriad of novel diagnostic and therapeutic targets that might be exploited to improve outcomes of adult liver injury.

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