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The hedgehog pathway in nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of obesity-associated liver diseases and it has become the major cause of cirrhosis in the Western world. The high prevalence of NAFLD-associated advanced liver disease reflects both the high prevalence of obesity-related fatty liver (hepatic steatosis) and the lack of specific treatments to prevent hepatic steatosis from progressing to more serious forms of liver damage, including nonalcoholic steatohepatitis (NASH), cirrhosis, and primary liver cancer. The pathogenesis of NAFLD is complex, and not fully understood. However, compelling evidence demonstrates that dysregulation of the hedgehog (Hh) pathway is involved in both the pathogenesis of hepatic steatosis and the progression from hepatic steatosis to more serious forms of liver damage. Inhibiting Hedgehog signaling enhances hepatic steatosis, a condition which seldom results in liver-related morbidity or mortality. In contrast, excessive Hedgehog pathway activation promotes development of NASH, cirrhosis, and primary liver cancer, the major causes of liver-related deaths. Thus, suppressing excessive Hh pathway activity is a potential approach to prevent progressive liver damage in NAFLD. Various pharmacologic agents that inhibit Hh signaling are available and approved for cancer therapeutics; more are being developed to optimize the benefits and minimize the risks of inhibiting this pathway. In this review we will describe the Hh pathway, summarize the evidence for its role in NAFLD evolution, and discuss the potential role for Hh pathway inhibitors as therapies to prevent NASH, cirrhosis and liver cancer.

Keywords

Nonalcoholic fatty liver disease; hedgehog; wound-healing response; treatment

INTRODUCTION

Liver cirrhosis is responsible for over one million deaths per year, 2% of all deaths worldwide (Mokdad et al., 2014). At the turn of the century, the main causes of liver cirrhosis were viral hepatitis and alcoholic liver disease (Mokdad et al., 2014). However, since then a vaccine to prevent hepatitis B infection and anti-viral treatments that can cure

hepatitis C infection have been developed, diminishing both the prevalence and incidence of cirrhosis caused by these infections. In contrast, the obesity pandemic has fueled new epidemics of diseases related to the metabolic syndrome, including nonalcoholic fatty liver disease (NAFLD). One fourth of the world's adult population now has NAFLD (Younossi et al., 2016), a disease that was once relatively restricted to affluent Western societies, such as the United States (Clark, Brancati, and Diehl, 2003). In countries where NAFLD has been prevalent for the past couple of decades, the health burden of NAFLD is now apparent. For example, NAFLD-related cirrhosis is surpassing cirrhosis caused by viral hepatitis as the leading general indication for liver transplantation in the U.S. and (Wong et al., 2015), and it has already become the most rapidly growing cause of liver transplantation for hepatocellular carcinoma (Wong, Cheung, and Ahmed, 2014).

Nonalcoholic fatty liver (NAFL) refers to hepatocyte accumulation of lipids (aka hepatic steatosis) that can not be attributed to alcohol consumption. NAFL strongly associates with obesity and related metabolic disorders, such as insulin-resistance, diabetes mellitus, dyslipidemia and hypertension (a.k.a, the metabolic syndrome). The metabolic syndrome is defined as a "disorder of energy homeostasis" (Wikipedia) and thus, it reflects metabolic stress. The association between NAFLD and metabolic stress is complex because metabolic stress not only induces NAFLD, but NAFLD can also amplify metabolic stress. This bidirectional relationship is thought to explain why mortality due to cardiovascular disease, and non-liver cancers are increased in NAFLD patients (Musso et al., 2011).

While most individuals with isolated ("simple") hepatic steatosis do not develop progressive liver damage, a minority (~20-30%) experience lipotoxicity, i.e., multi-factorial hepatocyte injury that results in hepatocyte death and liver inflammation, a condition dubbed nonalcoholic steatohepatitis (NASH). NASH is a dynamic condition that can regress, persist, or progress. Because chronic NASH invariably triggers wound healing responses that involve fibrogenesis and expansion of liver progenitor populations, it dramatically increases the risk for cirrhosis and primary liver cancer. (Machado and Cortez-Pinto, 2014). Epidemiological studies of NAFLD have revealed that liver-related morbidity and mortality in individuals with NASH are predicted by the severity of liver fibrosis, rather than by the degree of obesity, insulin-resistance, or hepatic steatosis (Angulo et al., 2015; Ekstedt et al., 2015; Dulai et al., 2017). These findings suggest that dysregulated repair of fatty liver damage (NASH) might distinguish the NAFLD patients who are destined to suffer bad liver outcomes from those whose liver condition will follow a relatively indolent course.

Much has been learned about the pathogenesis of NAFLD since it was first described by Ludwig *et al.* (Ludwig et al., 1980). However, lingering gaps in knowledge prevent optimal management of NAFLD patients because it remains impossible to accurately identify those who are at risk of progressive liver disease prospectively, and no therapy that reduces liver-specific and overall mortality has yet been discovered. In the last decade, researchers have uncovered a pivotal role for Hedgehog pathway dysregulation in NAFLD pathogenesis, particularly as a driver of disease progression to cirrhosis. Hence, Hedgehog has become a candidate target for the treatment of fibrosing NASH. This approach is immediately actionable since specific inhibitors of the pathway are already in clinical use for some cancers such as basal cell carcinoma and medulloblastoma (Guha, 2012); a phase 1 clinical

trial for the treatment of hepatocellular carcinoma with an oral hedgehog inhibitor, sonidebig, is ongoing (NCT02151864); and old inexpensive drugs (e.g., the antifungal itraconazole) are known to have strong anti-hedgehog activity (Kim et al., 2010). In this article we will review knowledge about the Hedgehog signaling pathway and summarize data regarding the role of the Hedgehog pathway in the pathogenesis and progression of NAFLD, as well as preclinical evidence that targeting the Hedgehog pathway may be an effective approach to inhibit the evolution of NAFLD-related cirrhosis.

THE HEDGEHOG SIGNALING PATHWAY

Nobel Prize laureates Wieschaus and Nussland-Volhard first described the Hedgehog pathway in 1980. While using a genetic screen in *Drosophila melanogaster* to identify genes that regulate body plan and segmentation, they discovered that fly larvae acquired spiny denticles resembling those of the mammalian hedgehog when one gene was mutated, which they dubbed hedgehog (Nusslein-Volhard and Wieschaus, 1980). A decade later, three mammalian hedgehog counterparts were identified, and named Sonic hedgehog (Shh, after the videogame character), and Indian (Ihh) and Desert (Dhh) (after two hedgehog species) (Echelard et al., 1993). Shh and Ihh are widely expressed, whereas Dhh expression is restricted to the nervous system and testis (Merchant and Saqui-Salces, 2014).

Canonical hedgehog pathway

The four major components of the Hedgehog pathway in the fly are: the ligand (hedgehog), the receptor (Patched), the signal transducer (smoothened) and the effector transcription factor (Gli). Signaling via this pathway regulates the expression of several target genes (Figure 1). In mammals, the canonical hedgehog pathway requires the presence of the primary cilium (PC) (Arendsdorf, Marada, and Ogden, 2016). Primary cilia (PC) are small immotile cilia comprised of polymerized tubulin. PC are assembled during interphase in most differentiated cells. Cells possess only one PC, which is composed by a basal body and a filamentous axoneme that projects into the extracellular space. The basal body derives from the mother centriole at the end of mitosis (Roy, 2012). Two checkpoints separate the PC from the plasma membrane: the ciliary pockets and the transition zone (Goetz, Ocbina, and Anderson, 2009). The PC is crucial for Hh pathway activation, establishing cellular compartments that regulate the pathway by controlling the entry and exit of pathway components (Ramsbottom and Pownall, 2016). Within the PC, a complex trafficking system allows movement of the different Hh pathway components along the cilia. Movement towards the tip of the PC is mediated by a kinesin motor protein-based transport system which is facilitated by the ciliary Bardet-Biedl syndrome proteins (BBS) and intraflagellar transport proteins (ITF). Movement towards the base of the PC is mediated by a dynein motor protein-based transport system which is facilitated by the BBS, ITF and Kif7 (Merchant and Saqui-Salces, 2014; Liu, Wang, and Niswander, 2005).

Hedgehog ligand (Hh) is a morphogen, i.e., it is secreted by several cells, diffuses into the extracellular space, and determines the fate of Hh-target cells as a function of its concentration (Briscoe and Therond, 2013). Hh is synthesized as a 45 kDa precursor protein, with two domains, N-terminal and C-terminal. In the endoplasmic reticulum (ER), the

precursor protein undergoes autocatalytic cleavage through the action of the C-terminal domain to generate an N-terminal 19 kDa fragment (Hh-N) (Chen et al., 2011). The Hh-N fragment then undergoes sequential lipidation that is essential for its function (Ramsbottom and Pownall, 2016). Hh-N has intrinsic cholesterol transferase activity which allows covalent attachment of a cholesterol group to its carboxyl end (Porter, Young, and Beachy, 1996). Subsequently, it undergoes palmitoylation through the action of the membrane-bound O-acyltransferase (MBOAT) skinny hedgehog (SKI), resulting in a highly hydrophobic molecule (Pepinsky et al., 1998). As detailed below, Hh uses ingenious mechanisms to diffuse into the extracellular matrix despite its hydrophobicity.

Release of Hh from ligand-producing cells requires the protein dispatched (Dis) (Burke et al., 1999) which binds to the cholesterol group of Hh-N (Tukachinsky et al., 2012). A soluble glycoprotein, SCUBE-2 binds to different regions of the cholesterol group of the Hh-N and forms complexes with sheddases which cooperate with heparan sulfate proteoglycans to prune away the lipid groups in the plasma membrane that sequester Hh, freeing Hh for release into the extracellular space (Jakobs et al., 2014) as either monomers or multimeric complexes. Multimeric complexes of Hh ligand are assembled using heparan sulfate proteoglycans as scaffolds (Goetz et al., 2006); they are more stable and more active than monomeric Hh (Goetz et al., 2006; Chen et al., 2004). In the absence of sheddases, however, multimeric complexes cannot be released to the extracellular space (Jakobs et al., 2016) and are internalized with the help of endosomal sorting complexes required for transport (ESCRT) proteins (Matusek et al., 2014), packaged into intraluminal vesicles, and released as exosomes (Parchure et al., 2015) via a mechanism that also requires Dispatched (D'Angelo et al., 2015). Lastly, Hh can be released with very low-density lipoproteins. This process is not completely understood but seems to depend on glycosylphosphatidylinositol-linked glypicans (Panakova et al., 2005). Glypicans can also act as co-receptors for Hh and thus, are important modulators of the Hh signaling pathway, capable of either inhibiting signal transduction by sequestering ligand or perpetuating signaling by stabilizing the binding of Hh to its receptor (Ramsbottom and Pownall, 2016).

The 12-pass transmembrane protein patched (Ptch) is the receptor for Hh ligand. Unlike most membrane spanning receptors, Ptch can block pathway activity. In the absence of Hh ligand, it constitutively inhibits the pathway through a negative effect on the downstream effector smoothened (Smo). The mechanism by which Ptch inhibits Smo is not fully elucidated, but it does not seem to require physical interaction between the two molecules (Taipale et al., 2002). When Hh binds to Ptch, it represses Ptch, blocking its inhibitory effect on Smo, and hence allowing signaling through the pathway (Ramsbottom and Pownall, 2016). After Hh binds to Ptch, the Hh-Ptch complex is internalized and degraded (Denef et al., 2000). Ptch internalization involves a complex mechanism: Hh induces accumulation of Ptch in lipid rafts, enabling Ptch to be ubiquitinated by the E3 ubiquitin ligases Smurf1 and Smurf2, and targeted to lysosomes for degradation (Yue et al., 2014).

There are two isoforms of Ptch in mammalian cells, Ptch-1 and Ptch-2, the former being more widely expressed, and more critical for pathway activity (Carpenter et al., 1998). Three Hh co-receptors positively regulate the pathway, enhancing Hh-Ptch binding: CAM-related down-regulated by oncogenes (Cdo), brother of Cdo (Boc) and growth arrest-specific

(GAS)-1. To activate the signaling cascade, Hh must bind to each of these co-receptors (Izzi et al., 2011). Conversely, Hh interaction with Ptch is blocked by the soluble co-receptor hedgehog interacting protein (Hip) which binds to Hh and prevents it from coupling to Ptch. Hence, Hh-Hip interaction negatively regulates the signaling pathway (Chuang and McMahon, 1999).

Smo, a 7-pass transmembrane G protein-coupled receptor, is the signal transducer of the Hh pathway, and has been the most frequent therapeutic target for modulating Hh pathway activity. In the absence of Hh, Ptch is on the PC and blocks Smo from entering the PC (Corbit et al., 2005) and hence, Smo localizes in intracytoplasmic vesicles. When Hh binds to and inactivates Ptc (Rohatgi, Milenkovic, and Scott, 2007), Smo can move onto the PC (Arensdorf, Marada, and Ogden, 2016). The movement of Smo onto the PC is mediated by the kinesin motor protein Kif3A via an interaction that is dependent on beta-arrestins (Kovacs et al., 2008). Accumulation of Smo in the PC requires Smo binding to the anchoring Ellis van Creveld Syndrome (EVC-2) ciliary complex (Dorn, Hughes, and Rohatgi, 2012). Smo must then be phosphorylated by casein kinase-1 (CK1) and G-protein-coupled receptor kinase-2 (GRK-2) to become fully activated (Rohatgi et al., 2009; Chen et al., 2011). The localization of Smo in the PC membrane depends on its activation state: it localizes at the base of the PC when inactive, and at the ciliary tip when active (Milenkovic et al., 2015). Smo activation can be modulated by endogenous lipids such as oxysterols (which activate Smo) and vitamin D3 (which inhibits Smo) (Bijlsma et al., 2006). Interestingly, Ptch-1 induces vitamin D3 secretion, providing another layer of Hh signaling regulation in distant cells (Bijlsma et al., 2006).

Gli proteins are transcription factors that belong to the Kruppel-like family and contain a zinc-finger DNA-binding domain (Hu et al., 2015). Mammals have 3 Gli proteins: Gli-1, Gli-2 and Gli-3. In the absence of Hh ligand, Smo is inactivated, and the transcription factor Gli is prevented from entering the nucleus, bound to a suppressor protein complex, composed of fused kinase (Fu), suppressor of fused (Sufu) and Costal-2 (Cos) (Teperino et al., 2014). Gli can then be sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase-3 (GSK3) and CK1. Phosphorylated Gli binds to β -transducin repeat containing protein (β TrCp), and the Gli- β TrCp complex is ubiquitinated by Cul-1-base E3 ligase. Ubiquitinated Gli is targeted to the proteasome, where it can be either processed in a truncated transcription repressor (Gli-R) (Wang and Li, 2006), or it can be fully degraded (Wang, Pan, and Wang, 2010). When Smo is inactive, Gli-2 predominantly undergoes proteasomal degradation and this limits the transcription of Gli-1, a Gli-2 target gene (Ikram et al., 2004). Under these conditions, Gli-3 is efficiently processed into Gli-3-R, re-enforcing suppression of genes that would otherwise be activated by Gli-1 and/or Gli-2 (Pan and Wang, 2007). When Smo is active, Gli-2 predominately acts as transcription enhancer. This induces expression of Gli-1, which generally functions as a transcriptional activator, and Gli-1 accumulates because it does not undergo PKA-dependent proteasomal degradation. Gli-1 is a target of atypical protein kinase Cc/ λ (aPKC/ λ) which phosphorylates Gli-1 to enhance Gli-1 DNA binding and transcriptional activity (Atwood et al., 2013). Conversely, phosphorylation of Gli-1 by AMP-activated protein kinase (AMPK) promotes Gli-1 degradation and links cellular metabolism with the hedgehog pathway (Di Magno et al., 2016; Li et al., 2015). (Ikram et al., 2004).

Activated Smo can dissociate Gli from Sufu at the tip of the PC (Pak and Segal, 2016). Kif7, which also localizes to the tip of the PC when Smo is active, enhances Gli-Sufu localization in this compartment (He et al., 2014)(He et al., 2014)(He et al., 2014)(He et al., 2014)(He et al., 2014)(He et al., 2014)(He et al., 2014). and full-length Gli dissociates from Sufu, moves back to the cytoplasm, and enters the nucleus, where it promotes the transcription of several genes, including vascular endothelial growth factor (VEGF), angiopoietin-1 and -2 (in endothelial cells); snail, twist-2, FoxF1, α -smooth muscle actin (α -SMA), vimentin, cyclin D, interleukin (IL)-6 (in fibroblasts/myofibroblasts); and nanog, sox-2 and -9 (in stem/progenitor cells) (Merchant and Saqui-Salces, 2014; Hanna and Shevde, 2016).

The canonical Hh pathway is highly regulated. Once the Hh pathway is activated, it initiates mechanisms of self-restraint. For example, 3 Hh inhibitors, Ptch, Hip and Foxa2 are direct transcriptional targets of Gli-2. In addition to the inhibitory actions of Ptch and Hip on pathway activity (see above), Foxa2 directly inhibits Gli-2 transcript (Mavromatakis et al., 2011). Like P-AMPK (which increases during energy depletion), protein kinase A (PKA) and cAMP (sensors of glucose deprivation) also negatively regulate Hh signaling. PKA and GSK3 sequentially phosphorylate Sufu to stabilize Sufu in a complex with Gli-2 and Gli-3. This blocks nuclear localization of the Gli factors and suppresses expression of Gli-target genes (Chen et al., 2011). When Smo is inactive, the orphan G-protein-coupled receptor Gpr161 also localizes in the PC and activates adenylyl cyclase, increasing local cAMP levels and PKA activity to assure the Hh pathway remains inactive (Mukhopadhyay et al., 2013). When Hh binds to Ptch and Smo enters the PC, Gpr161 leaves the PC, cAMP levels decrease, and reduced PKA activity amplifies Hh pathway activation. The Smo-induced removal of Gpr161 from the PC is dependent of β -arrestin (Pal et al., 2016). Furthermore, ciliary accumulation of Gpr161 is modulated by the lipid composition of the PC membrane, which is kept relatively enriched in Pi(4)P by the action of the cilium-located phosphatase Inpp5e. Inpp5e dephosphorylates Pi(4,5)P₂ to generate Pi(4)P and thus Pi(4,5)P₂ accumulates when Inpp5e activity is inhibited., Pi(4,5)P₂ recruits Tubby-like protein 3 (Tulp3) and IFP to the PC, and these proteins promote trafficking of Gpr161 into the PC, resulting in inhibition of the Hh pathway (Chavez et al., 2015; Garcia-Gonzalo et al., 2015). The Hh pathway can also be regulated by factors that can modulate Gli activity independent of Smo. For example, transforming growth factor (TGF)- β can directly activate Gli-2 (Dennler et al., 2007; Johnson et al., 2011). Osteopontin, a Gli-target gene, also inhibits GSK3 β , thus promoting Gli activation (Das, Samant, and Shevde, 2013). K-ras seems to induce, whereas p53 and Notch-target Hes-1 repress, Gli-1 expression in a Smo-independent manner (Nolan-Stevaux et al., 2009; Stecca and Ruiz i Altaba, 2009; Schreck et al., 2010). Finally, Pi3K-AKT signaling enhances, while protein kinase C δ inhibits, Gli-1 transcriptional activity (Stecca et al., 2007; Cai et al., 2009).

Noncanonical hedgehog pathway

Two types of noncanonical Hh signaling have been described: type 1, which is Ptch-dependent but Smo independent; and type 2, which is Smo-dependent, but Hh-ligand and PC-independent. In type 1 signaling, Ptch-1 can trigger apoptosis through activation of caspase-3 when Hh ligand is removed, and restoring binding of Hh ligand to Ptch inhibits this process (Chinchilla et al., 2010). In the absence of Hh ligands, Patch-1 can also inhibit

proliferation by preventing nuclear localization of cyclin D. This anti-proliferative effect of Patch-1 is also inhibited by Hh ligand (Barnes et al., 2001).

In type 2 signaling, Smo's G α i activity regulates cellular processes such as metabolism, proliferation, calcium flux and migration (Teperino et al., 2014; Polizio et al., 2011; Belgacem and Borodinsky, 2011; Teperino et al., 2012). For example, Smo can induce a Warburg-like effect promoting a glycolytic metabolism in muscle and adipose tissue through a calcium-AMPK kinase axis (Teperino et al., 2012). Independently of Hh ligands or Ptc, Smo can also stimulate GTPases, which promote cytoskeletal rearrangement and induce migration of fibroblasts and endothelial cells (Polizio et al., 2011; Bijlsma et al., 2007; Razumilava et al., 2014).

PHYSIOLOGY OF THE HEDGEHOG PATHWAY IN THE LIVER

During embryogenesis, the Hh pathway regulates growth, differentiation, patterning, and vascularization in multiple tissues, including liver (Hanna and Shevde, 2016; Deutsch et al., 2001). The actual role of Hh pathway in liver embryogenesis is not completely understood, but Shh is highly expressed in the ventral foregut endoderm which will differentiate into the hepatic bud, and Shh expression fades as the liver bud forms. Later in development there is transient expression of Hh in the hepatoblasts that disappears once hepatoblasts differentiate into hepatocytes (Omenetti et al., 2011).

Thereafter, the pathway gradually shuts down in the liver by adolescence (Omenetti et al., 2011). Hence, Hh ligand is barely expressed and the Hh pathway is relatively dormant in healthy adult liver (Sicklick et al., 2006). However, during hepatic regeneration after partial hepatectomy (Ochoa et al., 2010), acute liver injury (Pratap et al., 2010) or in response to chronic liver injury (Omenetti et al., 2008), the Hh pathway strongly reactivates and acquires a major role in the wound healing response (Michelotti et al., 2013).

NASH is a form of obesity-related chronic liver disease that results when fat accumulated in hepatocytes induces lipotoxicity (Machado and Diehl, 2016), causing tissue injury and related proinflammatory, profibrogenic and procarcinogenic responses. The injured and dying liver cells release signals that elicit a multi-faceted wound-healing response that: *i*) mobilizes an inflammatory reaction to clear dead and irreparably-injured cells; *ii*) stimulates vasculogenesis to optimize blood flow to the damaged parenchyma; *iii*) activates hepatic stellate cells to remodel the matrix; and *iv*) induces outgrowth of the progenitors to replace the lipotoxic hepatocytes that died. These various aspects of the response must be coordinated perfectly for wound healing to be effective, and they must ultimately terminate when repair has successfully regenerated healthy hepatic parenchyma. Hence, recovery from NASH requires a dynamic dialogue among various types of cells that fulfill different missions in the wound healing process. The Hh pathway plays an essential role in orchestrating the proper reconstruction of damaged adult liver, just as it assures the appropriate formation of various tissues during embryogenesis. However, when the pathway becomes dysregulated or cannot be shut-down because noxious stimuli are persistent, sustained Hh signaling perpetuates wound healing activities, resulting in chronic

inflammation, vasculogenesis, fibrogenesis, and progenitor cell accumulation. Over time, this increases the risk for cirrhosis and primary liver cancer (Machado and Diehl, 2016).

During the past decade, research has revealed how the Hh pathway becomes activated in the liver during NAFLD and shown that this controls the progression of NASH (see below). These insights are particularly provocative given growing evidence that obesity itself may reflect a state of generally sub-normal Hh activity. In several species, for example, globally inhibiting Hh pathway activity promotes obesity, and stimulating Hh signaling inhibits adipogenesis (Fleury et al., 2016). Although more research is needed to clarify tissue-specific mechanisms that control Hh signaling, local availability of Hh ligands appears to be an important variable in the liver.

Healthy adult hepatocytes generally do not produce Hh ligands (Sicklick et al., 2006) but hepatic synthesis of Hh ligands increases dramatically in many types of liver injury, including NASH. In NASH, the primary sources of Hh ligands are the injured hepatic epithelial cells, particularly lipotoxic hepatocytes (Rangwala et al., 2011; Guy et al., 2012) and reactive cholangiocytes (Natarajan et al., 2014; Natarajan et al., 2017). In hepatocytes, fatty acid toxicity provokes ER and apoptotic-stress, resulting in disruption of the cytoskeleton and swelling (a.k.a., ballooning). Ballooned hepatocytes are the hallmark of hepatocyte injury in NASH and immuno-stained human NASH livers readily demonstrate Hh ligand accumulation in such cells. Further, the Hh-positive hepatocytes in human NASH are typically surrounded by nonparenchymal cells with Gli2-positive nuclei, suggesting that the injured liver epithelial cells are activating Hh signaling in the adjacent stroma (Guy et al., 2012). *In vitro* models proved that expression of Sonic and Indian Hh mRNAs and proteins are induced in ballooned hepatocytes and showed that these cells release biologically active Hh ligands that are able to trigger Hh signaling in Hh-target cells (Rangwala et al., 2011; Kakisaka et al., 2012; Machado et al., 2014). Further, treatments that improve liver injury and hepatocyte ballooning in humans with NASH also reduce hepatic accumulation of both Hh-positive hepatocytes and Gli2-positive stromal cells (Guy et al., 2014). Other types of cells that accumulate in injured livers during hepatic wound healing are also capable of producing Hh ligands, such as immune cells (e.g., NKT cells (Syn et al., 2012) and macrophages (Pereira et al., 2013)), activated progenitor cells (Jung et al., 2007), activated hepatic stellate cells/myofibroblasts (Sicklick et al., 2005; Yang et al., 2008; Choi et al., 2009) and activated liver sinusoidal endothelial cells (Xie et al., 2013). Hence, local production of Hh ligands by injured liver epithelial cells, as well as by cells that are mobilized to repair liver injury, is able to initiate Hh signaling in neighboring cells, even in obese individuals who may maintain low Hh pathway activity in other tissues. Conversely, eliminating lipotoxic stress in hepatocytes of such subjects removes Hh-generating hepatocytes. This abolishes a key stimulus for Hh pathway activation in Hh-responsive cells, and rapidly permits hepatic Hh signaling to subside to its typically low level of basal activity. Decreasing/inactivating Hh-producing inflammatory cells, such as NKT cells and macrophages, would further help inhibit disease progression (Kwon et al., 2016).

The cellular targets of Hh ligands in the liver are somewhat uncertain because it appears that the Hh-responsiveness of liver-resident cells is influenced by various factors, including circadian periodicity, age, and health (Borjigin et al., 1999; Duesterdieck-Zellmer et al.,

2015). Mature hepatocytes in healthy adult livers are believed to be Hh unresponsive (Sicklick et al., 2006) because they do not have a PC (Wheatley, Wang, and Strugnell, 1996).. However, some peri-portal hepatocytes exhibit nuclear Gli-2 in fatty livers, consistent with induction of Hh signaling (Fleig et al., 2007; Syn et al., 2009; Swiderska-Syn et al., 2013). It is possible that Gli-2 expression in those hepatocytes might have resulted from non-canonical (i.e., Smo- independent) activation of Gli-2 (Grzelak et al., 2014; Grzelak et al., 2017) by factors other than Hh ligands, such as TGF- β (Dennler et al., 2007; Johnson et al., 2011). Alternatively, the Gli-2-positive hepatocytes might be the immature progeny of Hh-responsive progenitor cells that still retain PC that permit ligand-dependent canonical activation of the Hh pathway (Ochoa et al., 2010). Finally, perhaps hepatocytes can acquire a PC when injured, similar to endothelial cells which do not express PC in the resting state but acquire a PC when exposed to increased hydrostatic pressure (Ke and Yang, 2014).

In any case, Smoothened is definitely activated in some hepatocytes following liver injury because cyclopamine, a direct Smoothened antagonist, inhibits proliferative activity in hepatocytes harvested from regenerating livers after partial hepatectomy (Ochoa et al., 2010). Further, Gli-expressing hepatocytes have been shown to release other Hh-inducible factors, such as osteopontin, that promote inflammation and fibrogenesis (Kwon et al., 2016). Hepatic progenitor cells strongly react to canonical Hh pathway activation with increased proliferation and viability but decreased hepatocytic differentiation (Sicklick et al., 2006; Fleig et al., 2007; Syn et al., 2009; Grzelak et al., 2017; Jung et al., 2010). Hh-responsive progenitor cells also release many factors that can promote the wound-healing response, such as platelet-derived growth factor (PDGF) and osteopontin which activate hepatic stellate cells (Omenetti et al., 2008; Syn et al., 2011), and proinflammatory chemokines (for example, CXCL16 and osteopontin) (Omenetti et al., 2009) which recruit immune cells to the liver. Also, hepatic stellate cells are strongly activated by Hh ligands, becoming more proliferative, myofibroblastic, and fibrogenic (Yang et al., 2008; Choi et al., 2009). Sinusoidal endothelial cells respond to Hh with loss of fenestration and a vasoconstriction-inducing phenotype, which promotes capillarization and vascular remodeling (Xie et al., 2013). Lastly, immune cells (e.g., NKT cells and macrophages) respond to Hh by becoming more pro-fibrogenic and promoting immune tolerance (Pereira et al., 2013; Syn et al., 2009).

In conclusion, NASH (like other forms of chronic liver disease) evokes a wound healing response that is coordinated by crosstalk among different types of cells that are involved in tissue repair. The Hh pathway orchestrates the orderly activation all of these cells and regulates their functions so that healthy hepatic parenchyma is regenerated. Hedgehog producing cells, such as dying hepatocytes and inflammatory macrophages, activate and increase the pool of hedgehog responsive cells, including hepatic progenitor cells and hepatic stellate cells. This suggests that defective repair (i.e., cirrhosis and liver cancer) result from dysregulated Hh signaling and positions the Hh pathway as a major therapeutic target in NAFLD.

HEDGEHOG PATHWAY IN NONALCOHOLIC FATTY LIVER DISEASE

Although sustained and excessive Hh pathway activity plays a pivotal role in driving the progression of NAFLD to fibrosing NASH, liver cirrhosis and hepatocellular carcinoma (see below), transient Hh pathway activation is necessary to recover from NASH, and some basal threshold of Hh activity may be necessary to prevent hepatic steatosis. Viewed from this perspective, Hh signaling might be necessary to prevent the onset of NAFLD and thus, Hh pathway activation could be a compensatory response to ectopic fat accumulation in the liver.

Matz-Soja *et al.* showed that conditional liver-specific deletion of Smo in adult mice downregulated the Hh pathway and induced liver steatosis. The steatogenic effect of Gli suppression resulted from a skewing of lipid metabolism towards lipogenesis through the induction of lipogenic factors such as sterol regulatory element-binding protein (SREBP)-1c and adiponutrin/patatin-like phospholipase domain-containing protein-3 (PNPLA-3). Furthermore, activation of the Hh pathway (through SUFU knockdown, small molecule Shh agonists or Gli overexpression) reversed the steatogenic phenotype in hepatocytes derived from genetic models of obesity-related NAFLD, i.e., ob/ob mice and melanocortin-4 knockout mice (Matz-Soja *et al.*, 2016).

Once hepatocyte lipotoxicity develops, Hh signaling is increased and evidence that failure to constrain Hh pathway activity appropriately drives progression of fibrosing NASH is striking. Briefly, the Hh pathway has been studied in different animal models of NASH, including genetically obese leptin-deficient ob/ob mice and wild type mice with NASH induced by feeding either diets deficient in methionine and choline or diets enriched with various fats (Fleig *et al.*, 2007; Syn *et al.*, 2009; Hirsova *et al.*, 2013; Machado *et al.*, 2015; Wang *et al.*, 2016). In all models, hepatic Hh pathway activation has been observed consistently. Similarly, in human NASH the Hh pathway is activated and the level of pathway activity correlates with the severity of liver damage (as assessed by numbers of ballooned hepatocytes, levels of portal inflammation, markers of liver repair (aka accumulation of progenitor cells and activated myofibroblasts), and fibrosis severity) (Guy *et al.*, 2012; Grzelak *et al.*, 2017; Syn *et al.*, 2009; Machado *et al.*, 2015).

As mentioned earlier, the main source of Shh is the injured ballooned hepatocyte in human NASH (Guy *et al.*, 2012). The number of Shh-expressing hepatocytes correlates with fibrosis severity, and the severity of the ductular reaction (Guy *et al.*, 2012; Machado *et al.*, 2015) which is known to associate with fibrogenesis and carcinogenesis (Richardson *et al.*, 2007; Ye *et al.*, 2014). Once the Hh pathway is activated, it can induce a strong self-perpetuating response. Indeed, Chung *et al.*, evaluated a transgenic mouse model that initially achieved Shh expression in only 2–5% of hepatocytes, and observed advanced liver fibrosis after 6 months and hepatocarcinogenesis after one year (Chung *et al.*, 2015). At these late time points, Gli-2-positive (Hedgehog responsive) cells included not only ductular progenitor cells, hepatic stellate cells, and immune cells but also 30–50% of the hepatocytes (Chung *et al.*, 2015). Genetically modified mice with Ptch haploinsufficiency also have enhanced Hh pathway activation. Such mice develop worse liver disease and fibrosis in different models of NASH (Syn *et al.*, 2009; Syn *et al.*, 2011; Syn *et al.*, 2010). Conversely,

mice with conditional deletion of Smo exhibit inhibited Hh signaling and are protected from liver disease in different models of NASH (Michelotti et al., 2013; Kwon et al., 2016).

Excessive Hh pathway activity has also been implicated in liver carcinogenesis. In different animal models of hepatocellular carcinoma, the Hh pathway is activated in pre-cancerous tissues and cancerous tumors (Cai et al., 2016). In precursor cancerous cells, Shh promotes proliferation through induction of cyclin B1 and CDK1 mitotic proteins (Cai et al., 2016), while preventing apoptosis (Zhang et al., 2014; Wang et al., 2013). Hh also induces an epithelial to mesenchymal transition in malignant cells, favoring migration and invasiveness (Chen et al., 2014; Byrne et al., 2015). Finally, cancer-associated stromal cells release Hh ligands that promote malignant cell proliferation, angiogenesis and metastasis (Chan et al., 2014; Liu et al., 2016; Li et al., 2016). In human hepatocellular carcinoma, excessive Hh signaling occurs relatively frequently (Sicklick et al., 2006; Cheng et al., 2009; Al-Bahrani et al., 2015; Dugum et al., 2016), and the level of Hh pathway activity positively correlates with tumor size, invasion, chemoresistance, metastasis and mortality (Sicklick et al., 2006; Cheng et al., 2009; Chen et al., 2011; Lu et al., 2012; Che et al., 2012). Finally, adenomas that associate with obesity and hepatic steatosis also tend to have an overly active Hh pathway (Nault et al., 2017), and adenoma transformation is one possible mechanism for carcinogenesis in non-cirrhotic NAFLD (Michelotti, Machado, and Diehl, 2013).

In summary, while the role of the Hh pathway in the initial stages of NAFLD (i.e. isolated steatosis) is not fully understood, it is consistently activated in NASH and the best-studied pathway for regulating NASH-related fibrosis progression and hepatocarcinogenesis. (Machado and Diehl, 2016). As such, it is an attractive therapeutic target for the management of patients with progressive NAFLD.

HEDGEHOG PATHWAY AS A THERAPEUTIC TARGET IN NONALCOHOLIC FATTY LIVER DISEASE

In humans, improvement in NASH histological activity correlates with a decrease in the activation of the Hh pathway, suggesting a therapeutic role of targeting Hh in NASH. A major clinical trial in NASH, the PIVENS trial (Pioglitazone, Vitamin E for Non-alcoholic Steatohepatitis), showed histological improvement of NASH in patients who responded to treatment. A *post-hoc* analysis of PIVENS evaluated pre and post-treatment biopsies from 59 patients treated with either vitamin E or placebo, and showed that biochemical and histological response, including a decrease in liver fibrosis, strongly correlated with loss of Shh expressing hepatocytes (Guy et al., 2014).

The Hh pathway can be pharmacologically targeted through inhibition of Hh, inhibition of Smo, or inhibition of Gli transcriptional activity (Figure 2). When considering therapeutic targeting of the Hh pathway, it is important to recall that some tissues require Hh signaling to avoid toxicity, such as gonads, gastrointestinal tract, and bone marrow (Pak and Segal, 2016).

In preclinical studies, a monoclonal antibody against Hh (5E1) was able to inhibit activation and proliferation of hepatic stellate cells (Sicklick et al., 2005), as well as the Shh-dependent

paracrine effects of hepatic stellate cells on the proliferation of progenitor cells (Michelotti et al., 2013) and on the metabolic reprogramming and proliferation of malignant hepatoma cells (Chan et al., 2012). Shh can also be inhibited by robotnikinin, a small molecule that binds Shh, preventing it from engaging Ptch-1 and hence, inhibiting activation of the Hh pathway (Stanton et al., 2009). Although robotnikinin was discovered in a small-molecule microarray-based screen in 2009, there is lack of preclinical or clinical experience with robotnikinin in liver cells.

Inhibition of Smo is the best-studied approach to target the Hh pathway and this strategy has moved to clinical applications. Cyclopamine was the first available Smo antagonist. It was first identified in the late 1960s, from the observation of several birth defects (for example craniofacial deformities including cyclopia) in the offspring of lambs that ingested corn lilies (*Veratrum californicum*) in the Western US (Lee et al., 2014). From those lilies, investigators isolated the steroidal alkaloid cyclopamine. Dr. Philip Beachy *et al.*, showed that cyclopamine inhibited the Hh pathway by directly binding to, and inhibiting, Smo (Cooper et al., 1998; Incardona et al., 1998). Chemically, cyclopamine has some characteristics that limit its clinical utility; for example it has low water solubility, and it is not stable in acid conditions (such as the stomach pH) (Pak and Segal, 2016). A semisynthetic derivative, 3-keto-N-(aminoethyl-amicaproyl-dehydrocinnamoyl) cyclopamine-2 (KAAD-cyclopamine) has increased solubility and activity, being 10–20 fold more potent than cyclopamine (Taipale et al., 2000). Syn *et al.* studied the effect of cyclopamine in the methionine-choline deficient diet supplemented with ethionine mouse NASH model, and showed decreased expression of mesenchymal and fibrogenic genes, suggesting a potential role in the prevention of NASH-related fibrogenesis (Syn et al., 2009). Furthermore, in different mouse models of hepatocellular carcinoma, treatment with cyclopamine decreased tumor size (Jeng et al., 2012) and increased radiosensitivity (Tsai et al., 2015). A synthetic cyclopamine analogue, saridegib or IPI-926, achieved improved stability, kinetic profile and potency as compared to cyclopamine (Peluso et al., 2014). Phase I and II clinical trials of saridegib in basal cell carcinoma showed potent anti-tumoral effect, but saridegib showed no effect or deleterious effects in patients with myelofibrosis, chondrosarcoma or pancreatic cancer (Jimeno et al., 2013; Ko et al., 2016; Sasaki et al., 2015).

Vismodegib (also called GDC-0449) is a potent Smo antagonist that was identified by high-throughput *in vitro* screening (Sandhiya et al., 2013) by Curis Genentech. The US Food and Drug Administration (FDA) approved it for the treatment of advanced basal-cell carcinoma in January 2012 (Guha, 2012). Vismodegib is well-absorbed orally, but has several drug interactions: P-glycoprotein inhibitors such as macrolides increase vismodegib adverse effects and inhibitors of gastric acid secretion decrease vismodegib bioavailability.

Vismodegib has shown anti-fibrogenic and anti-inflammatory effects in different mouse models of NASH (Kwon et al., 2016; Hirsova et al., 2013). Furthermore, vismodegib was also strongly anti-fibrotic in the Mdr2 deficient mouse model (Philips et al., 2011). This is relevant for NASH because Mdr2 deficient mice have altered bile acid homeostasis and bile acids are known to have an important role in NASH pathogenesis (Arab et al., 2017).

Vismodegib also showed anti-carcinogenic effects in different animal models of hepatocellular carcinoma, decreasing tumor size, invasion, angiogenesis and metastatic disease (Philips et al., 2011; Jeng et al., 2015; Pinter et al., 2013). Vismodegib has been

studied in clinical trials for basal cell carcinoma, medulloblastoma, gastrointestinal tract cancers, chondrosarcoma, pancreatic, lung and hematological cancer. The best effects were noted in basal cell carcinoma, with phase IV studies showing good response rates, although with frequent acquired resistance due to *de novo* mutations (Rimkus et al., 2016). There are several other small molecules that inhibit Smo, under study, including sonidegib (also called erismodegib or LDE225), glasdegib (PF-04449913) and taladegib (LY2940680). Sonidegib also has FDA-approval for basal cell carcinoma and is currently being tested on patients with advanced hepatocellular carcinoma (NCT02151864). Furthermore, sonidegib has demonstrated anti-fibrotic and anti-inflammatory actions in a mouse model of NASH (Kwon et al., 2016). In addition, itraconazole, an old, commonly used and inexpensive anti-fungal drug, has anti-Smo activity mediated via direct binding to Smo (Kim et al., 2010). It is currently being studied in clinical trials for different solid tumors and has shown anti-fibrotic effects in animal models of lung fibrosis (Naranjo et al., 2010; Naranjo et al., 2011). No studies have been done yet in other fibrotic diseases.

Finally, GANTs are Gli antagonists that act in the final step of the Hh pathway (Lauth et al., 2007). GANT-58 and GANT-61 both inhibit Gli-mediated gene activation, however the latter is more specific for Gli and more potent in the inhibition of Gli-mediated transcriptional activity (Rimkus et al., 2016). GANT-61 can induce autophagy, apoptosis and cytotoxicity in hepatocellular cells *in vitro* and can inhibit hepatocellular tumor formation and growth in SCID mice (Wang et al., 2013). GANTs have not been evaluated in clinical trials (Rimkus et al., 2016).

CONCLUSIONS

NAFLD has become pandemic, with an increasing prevalence paralleling the increase in obesity and related metabolic disturbances. Currently, we do not have effective treatment for NAFLD, although we now know that patients with NAFLD-related liver fibrosis are at risk of progression to end-stage liver disease and pre-mature death. Both NAFLD and the Hh pathway were first described in 1980. This coincidence is intriguing since the Hh pathway is both heavily lipid-regulated and the best-studied driver pathway for fibrogenesis in NAFLD. Preclinical data consistently support a causal role for excessive Hh pathway activation in NAFLD progression to cirrhosis. Overly active Hh signaling is also involved in the pathogenesis of primary liver cancer. Those preclinical data are supported by association studies in human NAFLD. As such, the Hh pathway is an obvious therapeutic target to prevent bad liver outcomes in NAFLD. Hh signaling can be blocked pharmacologically at different steps of the pathway. Many drugs are under clinical investigation, and some of them are already approved to treat different malignant diseases. Preclinical studies also suggest that inhibiting the Hh pathway with these agents improves fibrosing NASH and its complications, namely hepatocellular carcinoma. To date, however, there have been no clinical trials of Hh inhibitors in NAFLD. This likely reflects the suspicion that the window for therapeutic efficacy may be narrow since the Hh pathway is highly complex, tightly regulated and critical for controlling growth responses that are necessary to maintain tissue homeostasis, as well as to repair tissue damage. Thus, it remains to be determined if long-term inhibition of the pathway is possible and safe. Further research is necessary to address

this issue because there is a desperate need for therapies that can safely and effectively prevent/treat NASH-related cirrhosis and liver cancer.

Abbreviations

AMPK	AMP-activated protein kinase
aPKC/λ	atypical protein kinase-Cc/ λ
BBS	Bardet-Biedl syndrome proteins
Boc	brother of Cdo
Cdo	CAM-related down-regulated by oncogened
CK-1	casein kinase-1
Dhh	Desert hedgehog
Dis	dispatched
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
GAS	growth arrest-specific
GRK-2	G-protein-coupled receptor kinase-2
GSK3	glycogen synthase kinase-3
Hh	hedgehog
Hip	hedgehog interacting protein
Ihh	Indian hedgehog
IL	interleukin
ITF	intraflagellar transport proteins
MBOAT	membrane-bound O-acyltransferase
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PC	primary cilium
PDGF	platelet derived growth factor
PKA	protein kinase A
Ptch	patched
Shh	Sonic hedgehog

SKI	skinny hedgehog
SMA	smooth muscle actin
Smo	smoothened
Sufu	suppressor of fused
TGF	transforming growth factor
TrCp	transducin repeat containing protein
VEGF	vascular endothelial growth factor

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Figure 1. The hedgehog pathway

A. In the absence of the hedgehog (Hh) ligand, patch (Ptc) constitutively inhibits smoothened (Smo). This prevents the dissociation of the transcription factor Gli with its inhibitor Sufu, and allows Gli sequential phosphorylation by protein kinase A (PKA), casein kinase-1 (CK1) and glycogen synthase kinase-3 (GSK). Phosphorylated Gli is ubiquitinated through the action of transducin repeat containing protein (β -TrCP), which targets Gli to proteasomal degradation. The net result is the repressor form of Gli (Gli-R), which inhibits transcription of Gli-target genes.

B. In the presence of Hh ligand, the Hh-Ptc complex is internalized and degraded. This removes the inhibitory effect of Ptc on Smo, and allows Smo entry into the primary cilium (PC). To become activated Smo is then phosphorylated through the action of CK1 and G-protein-coupled receptor kinase-2 (GRK2). This allows the entry of the complex Sufu-Gli into the PC. At the tip of the PC, Sufu dissociates from Gli. Gli then returns to the cytoplasm where it undergoes partial degradation in the proteasome in an activated form. Activated Gli enters the nucleus, where it mediates transcription of Gli-target genes.

	Hepatocytes	Hepatic stellate cells	Progenitor cells	Kupffer cells NKT cells	Endothelial cells
Source of ligands	No expression in healthy cells. Injured and ballooned cells express ↑↑ Shh, ↑ Ihh	Quiescent cells ↓ expression of ligands and ↑ of inhibitor. Activated cells ↑↑ Shh and Ihh.	Activated cells ↑↑ Shh and Ihh.	Activated Kupffer cells ↑↑ Ihh and NKT cells ↑↑ Shh.	Activated cells express ligands (↑↑ Shh).
Ligand responsive cells	During liver regeneration, Smo activity ↑ Peri-portal Gli-2+ cells in chronic liver disease (non-canonical activation?)	Hh activation promotes transdifferentiation in myofibroblasts, ↑ survival and proliferation. Canonical vs. non-canonical Hh pathway activation.	Hh activation ↑ survival and proliferation, and ↓ differentiation in mature cells. Canonical Hh pathway activation.	Hh activation promotes M2 polarization in Kupffer cells and fibrogenic IL-13 secretor phenotype in NKT cells.	Hh activation promotes capillarisation

Figure 2.
Pharmacological targeting of the hedgehog pathway.