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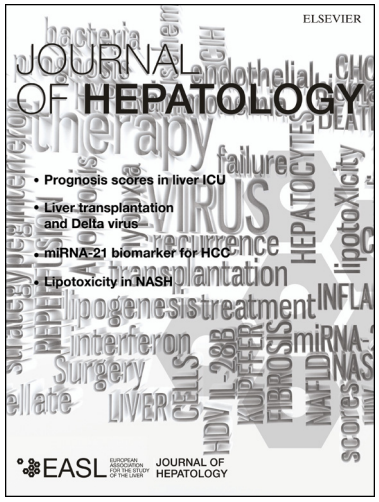

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THE INTRAHEPATIC SIGNALLING NICHE OF HEDGEHOG IS DEFINED BY PRIMARY CILIA POSITIVE CELLS DURING CHRONIC LIVER INJURY

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List of abbreviations:

LPC, liver progenitor cell

Hh, Hedgehog

SHH, Sonic Hedgehog

IHH, Indian Hedgehog

DHH, Desert Hedgehog

PTCH1, Patched 1

SMO, Smoothened

GLI, GLI-Kruppel family of transcription factors

GLI-A, GLI full-length activator

Pc, primary cilia

TAA, thioacetamide

Ptc1$^{+/-}$*, Ptc1-lacZ* reporter

NT2, non-targeting control

HSC, hepatic stellate cell

N-Hh, N-terminal Hedgehog signalling peptide

CK, cytokeratin

EpCAM, epithelial cell adhesion molecule

ALD, alcoholic liver disease

GLI-R, GLI cleaved repressor

α-SMA, α-smooth muscle actin

EGF, epidermal growth factor

HGF, hepatocyte growth factor

ALT, alanine aminotransferase

EMT, epithelial-to-mesenchymal transition

MCDE, methionine choline-deficient diet + ethionine

Conflicts of interest: The authors have none to disclose.

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Abstract:

Background: In vertebrates, canonical Hedgehog (Hh) pathway activation requires Smoothened (SMO) translocation to the primary cilium (Pc), followed by a GLI-mediated transcriptional response. In addition, a similar gene regulation occurs in response to growth factors/cytokines, although independently of SMO signalling. The Hh pathway plays a critical role in liver fibrosis/regeneration, however, the mechanism of activation in chronic liver injury is poorly understood. This study aimed to characterise Hh pathway activation upon thioacetamide (TAA)-induced chronic liver injury *in vivo* by defining Hh-responsive cells, namely cells harbouring Pc and Pc-localised SMO.

Methods: C57BL/6 mice (wild-type or *Ptc1*$^{+/-}$) were TAA-treated. Liver injury and Hh ligand/pathway mRNA and protein expression were assessed *in vivo*. SMO/GLI manipulation and SMO-dependent/independent activation of GLI-mediated transcriptional response in Pc-positive (Pc^{+}) cells were studied *in vitro*.

Results: *In vivo,* Hh activation was progressively induced following TAA. At the epithelial-mesenchymal interface, injured hepatocytes produced Hh ligands. Progenitors, myofibroblasts, leukocytes and hepatocytes were GLI2^{+}. Pc^{+} cells increased following TAA, but only EpCAM^{+}/GLI2^{+} progenitors were Pc^{+}/SMO^{+}. *In vitro*, SMO knockdown/h*GLI3-R* overexpression reduced proliferation/viability in Pc^{+} progenitors, whilst increased proliferation occurred

with h*GLI1* overexpression. HGF induced GLI transcriptional activity independently of Pc/SMO. *Ptc1*$^{+/-}$ mice exhibited increased progenitor, myofibroblast and fibrosis responses.

Conclusions: In chronic liver injury, Pc^{+} progenitors receive Hh ligand signals and process it through Pc/SMO-dependent activation of GLI-mediated transcriptional response. Pc/SMO-independent GLI activation likely occurs in Pc^{-}/GLI2^{+} cells. Increased fibrosis in Hh gain-of-function mice likely occurs by primary progenitor expansion/proliferation and secondary fibrotic myofibroblast expansion, in close contact with progenitors.

Chronic liver diseases are characterised by persistent injury directed at hepatic epithelial cells, specifically biliary cells and hepatocytes. The resulting epithelial damage and hepatic inflammation drives a progressive intrahepatic fibrotic response[1]. This is in concert with an epithelial regenerative response that combines repopulation from mature hepatocytes and activation, proliferation and differentiation from liver progenitor cells (LPCs). The LPC response, or ductular reaction, has been well characterised in human and experimental chronic liver disease[2]. These heterogeneous cells, sharing combinations of progenitor, biliary and hepatocyte markers, are located at the epithelial-mesenchymal interface, with isolated cells in the adjacent lobule.

Important pathogenic processes in the liver occur within anatomical *microenvironments* or *niches.* Hedgehog (Hh) signalling plays a critical role in mediating cell fate, growth and differentiation of epithelial stem/progenitor cell niches that reside within various adult tissues[3-6]. In the absence of Hh ligands, this family consisting of Sonic Hh (SHH), Indian Hh (IHH) and Desert Hh (DHH), the pathway is transcriptionally repressed. Binding of SHH/IHH/DHH to the receptor Patched1 (PTCH1) leads to the de-repression and subsequent activation of transmembrane protein Smoothened (SMO), which transduces the signal to downstream effectors. This results in stabilisation of the GLI-Kruppel (GLI) family of transcription factors into their full-length activator form (GLI-A), eliciting the Hh transcriptional response[7]. In recent years, two important observations have significantly changed our understanding of Hh signalling. Firstly, in vertebrates, transduction of Hh

ligand signal into a GLI transcriptional response is dependent on translocation of SMO into the primary cilia (Pc), an immotile membrane-bound sensory organelle found on most vertebrate cells[8-10]. Secondly, growing evidence suggests the expression of *GLI* genes *per se,* and consequently the downstream GLI-mediated transcriptional response, can be modulated by cytokines and growth factors; and decoupled from the Hh/PTCH1/Pc/SMO-cascade[11-15].

The Hh signalling pathway is classically recognised for its integral role in embryonic patterning during development, with aberrant activation of this pathway in adult tissues associated with malignancy. More recently, Hh pathway involvement has been studied in liver disease pathogenesis. A paradigm has emerged where Hh ligand production and SMO-dependent GLI signalling exists within multiple liver cell populations[16]. In particular, it has been proposed that Hh pathway activation is important in perpetuating an activated pro-fibrogenic phenotype[16, 17]. Studies have also reported the Hh pathway is involved in liver regeneration, and in the maintenance of intrahepatic stem/progenitor cells[16, 18]. A major limitation of these studies is that Hh signalling activation *in vivo* is only defined by GLI2 expression. As such, it has been impossible to define whether GLI regulation is SMO-dependent or SMO-independent, in the proposed paradigm.

In this paper, for the first time, we have used Pc detection to carefully identify Hh-responsive cells in chronic liver disease that co-express corresponding SMO and GLI proteins *in vivo*. We have defined the ligand-dependent, SMO-mediated induction of GLI expression in Pc^{+ve} LPCs as the 'Intrahepatic

Signalling Niche of Hedgehog'. High level GLI2 expression was also detected in several cell populations that lacked a Pc. Since Pc are crucially required for SMO-dependent Hh signalling[8], we hypothesise these cells elicit a SMO-independent GLI-mediated response, potentially driven by cytokines/growth factors associated with chronic liver disease[1]. Our observations demonstrate GLI activation in chronic liver disease occurs, at least, by two mechanisms. Such observations are critically important given the potential for Hh pathway-related therapeutics in liver disease treatment.

MATERIALS AND METHODS

Animal studies

Housing/experiments at the Centenary Institute performed in accordance with protocols imposed by the Animal Ethics Committee, University of Sydney (K75/2-2010/3/5209, K75/1-2010/3/5210). To induce progressive liver injury, eight-week old C57BL/6 male mice were thioacetamide (TAA)-treated (300mg/L; MP Biomedicals, USA) in drinking water *ad libitum* for 4, 8, or 20 weeks (wks) (*n*=3-10/group) prior to sacrifice. Non-treated control littermates were sacrificed at 20wks. Altered Hh pathway activation *in vivo* was studied using heterozygous *Ptc1-lacZ* reporter (*Ptc1*$^{+/-}$) mice[19]. Eight-week wild-type (wt) or *Ptc1*$^{+/-}$ male mice were TAA-treated for 8wks (*n*=4-9/group).

Quantitative mRNA analysis

Outlined Supplementary Material, Suppl. Table 1.

In situ hybridisation (ISH)

Murine specific RNA probe (sense/anti-sense) generation and ISH protocols were as previously described[20]. Primer sequences for probe generation outlined in Suppl. Table 2. Mouse 6µm paraffin liver sections were used. Specific hybridisation conditions/probe concentrations were 42^{o}C, 1.0µg/ml for *Gli1*; 45^{o}C, 2.5µg/ml for *Shh*. Sense RNA probes on an adjacent section served as a negative control, returning no specific signal.

Histology, immunohistochemistry and immunofluorescence

Outlined Supplementary Material. Isotype IgG controls were conducted in parallel (Supp. Fig. 1).

Cell culture/vector construction

Outlined Supplementary Material.

Viability assay

Cells (7.5x10^{5}) were transfected with 40nM non-targeting control (NT2) or Smoothened siRNA (α-*Smo*; Dharmacon) using Lipofectamine2000 (Invitrogen, USA) as per manufacturer's instructions. After 6h, cells were harvested and seeded into 96-well plates at 4000 cells/well. Viability was assessed by fluorescence (FLUOstar Omega, Ortenberg, Germany) using ALAMAR blue reagent (Invitrogen) per manufacturers instruction. The same method was applied to cells transfected with *hGLI1* and *hGLI3R*.pEF-DEST51

DNA (12µg) using Lipofectamine2000, after selection with blasticidin. Repeated minimum three times.

Luciferase assays

Cells ($5x10^4$/well) were seeded into 12-well plates and transfected in quadruplicate with 8xGli.pGL4.10[*luc2*] (0.9µg) and Renilla control pRL-TK (0.1µg; Promega, USA) using Lipofectamine2000. Cells were pre-treated for 1h with inhibitors Erlotinib.HCl or SGX-523 (500nM; Selleck, USA) where appropriate, followed by 8h stimulation with murine recombinant SHH (1µg/ml; R&D Systems®, USA), EGF (20ng/mL; PeproTech Inc, USA) or HGF (20ng/mL; R&D Systems®) ± inhibitors. For *Smo* knockdown/luciferase assays, cells ($8x10^4$/well) were co-transfected with NT2 or α-*Smo* siRNA with both reporter plasmids. Luc2 and Renilla luciferase activities were determined using the Dual-Luciferase® Reporter System (Promega). Repeated minimum three times.

RESULTS

1. Hedgehog ligand expression

1.1 Intrahepatic ligand mRNA and protein expression

The TAA model of chronic liver injury shows features of cirrhosis with increased classical injury markers, gene expression, activated hepatic stellate cells (HSCs) and LPCs within portal tracts by TAA-20wks (Supp. Fig. 2-4). An incremental increase in pan-Hh (SHH, IHH, DHH) N-terminal Hh signalling peptide (N-Hh) was evident following TAA-treatment (Fig. 1A). To identify

which cells upregulated *Shh* mRNA, ISH was performed. *Shh*$^{+ve}$ cells increased around central veins/portal tracts with treatment. By TAA-20wk there was a marked increase in *Shh* localised to the epithelial-mesenchymal interface, compared with minimal expression in control tissue (Fig. 1B). Large *Shh*$^{+ve}$ cells exhibited characteristics of dysmorphic hepatocytes, often isolated and within the portal tracts (Supp. Fig. 5A). In addition, small portal tract cells were *Shh*$^{+ve}$, possibly implicating leukocytes, HSCs/myofibroblasts or LPCs as potential sources of Hh ligand.

1.2 Hedgehog ligand protein detection in vivo

To further characterise Hh-producing cells, immunohistochemistry was performed in liver tissue. Intense staining of IHH was localised to large damaged/dysmorphic hepatocytes in this chronic injury model (Fig. 1C). Confocal microscopy confirmed pan-Hh (SHH, IHH) protein expression within the fibrotic septa (Fig. 1D). The staining localised to large cells that co-expressed epithelial cell marker pan-cytokeratin (pan-CK; Fig. 1E), providing confirmation hepatocytes were the primary source of Hh ligands. In contrast to the ISH data, no pan-Hh ligand protein was detected in small portal tract cell populations (Fig. 1F, Supp. Fig. 5B). However, Hh-producing hepatocytes were in close proximity to epithelial cell adhesion molecule (EpCAM)$^{+ve}$ LPCs, CD45^{+ve} leukocytes and vimentin^{+ve} activated HSCs (Fig. 1F, Supp. Fig 5B). The same expression profile was observed in human alcoholic liver disease (ALD), with CK18^{+ve} hepatocytes at this interface the primary source of SHH (Suppl. Fig. 6).

2. Hedgehog pathway component expression

2.1 *Intrahepatic Hedgehog pathway expression*

The effectors of Hh pathway signalling are the GLI transcription factors, and since GLI proteins also regulate *Gli1* transcription, this gene has been conventionally used as a readout of GLI activity. An incremental increase in whole liver *Gli1* mRNA was observed from 4-20wks TAA (Fig. 2A). By TAA-20wks this was accompanied by a significant increase in *Ptc1* and *Gli3*, and a marked reduction in *Hhip*, a Hh pathway antagonist. Thus in TAA-induced cirrhosis, net upregulation of Hh signalling occurred with a combined 8.68-fold increase in *Gli1* and 9-fold decrease in *Hhip*. Furthermore, increased GLI1-A and reduced GLI3-R (R, truncated/cleaved GLI3 repressor) proteins occurred by TAA-20wks (Fig. 2B). Protein levels of GLI target genes (OSTEOPONTIN, CYCLIN D1, BCL-2) were also increased.

2.2 Gli1 mRNA localisation

The cellular source(s) of GLI1 in chronic liver injury were yet to be determined. Since a reliable commercial anti-GLI1 antibody was not available, ISH was performed on TAA-20wk liver tissue to detect *Gli1* mRNA. *Gli1* transcript was readily detectable in various cell populations at the epithelial-mesenchymal interface (Fig. 2C). Notably, *Gli1* was detected in large dysmorphic hepatocytes, as well as small portal tract cell populations, in TAA-20wk liver.

2.3 GLI2 expressing cell populations in TAA-treated liver in vivo

To determine which cell sub-population(s) could potentially respond to Hh signal, co-staining for cellular markers and GLI2 was performed. GLI2 was expressed by EpCAM^{+ve} LPCs, as well as in a significant number of hepatocytes at the injury interface and lobule (Fig. 2D, Supp. Fig 7). Vimentin^{+ve} HSCs/myofibroblasts and CD45^{+ve} leukocytes were also GLI2^{+ve} (Fig. 2E). Interestingly, co-expression of GLI2 and pan-Hh (SHH, IHH) ligand was observed in a subset of large cells with hepatocyte morphology (Suppl. Fig. 7B). This was consistent with our ISH data and suggests the large ligand-producing cells exhibiting an active GLI response are injured hepatocytes, located at the epithelial-mesenchymal interface (Fig. 1,2C, Suppl. Fig 5A).

2.4 Identification of ciliated cells in vivo

In vertebrates, Hh ligands act on cells via SMO translocation to the Pc[8]. In TAA chronic liver injury, Pc were identified on numerous cells located within the portal tracts adjacent to the hepatic lobule (Fig. 3A). These cells expressed EpCAM (Fig. 3A, Suppl. Fig 8A) and SMO was localised within the Pc axoneme, a commonly used readout of SMO signalling (Fig. 3A). As identified earlier, EpCAM^{+ve} cells also express GLI2 (Fig. 2D). Thus, these findings indicate the EpCAM^{+ve}/Pc^{+ve}/SMO^{+ve}/GLI2^{+ve} cell to be the compartment receiving Hh ligand signal *in vivo*. Further, ~32% of EpCAM^{+} cells were found to express Pc *in vivo* (Suppl. Fig 9A). However, this number most likely underrepresents the true number of Pc^{+}/EpCAM^{+} cells due to loss of Pc through sectioning (with only one Pc per cell), and reabsorption of the Pc during cell-cycle re-entry. Moreover, although vimentin^{+ve}, CD45^{+ve} and hepatocyte populations expressed GLI2, these cells did not harbour Pc

(Suppl. Fig 8B, 9B and data not shown), suggesting these cell populations do not signal through Pc/SMO *in vivo*. These findings were corroborated in three independent laboratories on several occasions (McCaughan, Watkins, Tirnitz-Parker).

2.5 Characterisation of EpCAM^{+ve} liver progenitor cells

The Hh-responsive EpCAM^{+ve} population, in close proximity to Hh-producing hepatocytes (Fig. 1F), was additionally always noted in close spatial association with fibrosis-driving α-smooth muscle actin (α-SMA)$^{+ve}$ HSCs (Suppl. Fig. 10A); a phenomenon commonly seen in chronic liver injuries with a LPC response[21]. Further, most EpCAM^{+ve} cells co-expressed biliary LPC marker CK19, and LPC/cancer stem cell marker CD133 (Suppl. Fig. 10B,C).

3. A Pc/SMO-dependent GLI-mediated response is important for liver progenitor cell survival *in vitro*

To study the function of Hh signalling in Pc^{+ve} liver cells *in vitro,* confocal microscopy was performed to determine which liver cell lines harbour Pc. BMOL1.2 LPCs were shown to have Pc (Suppl. Fig. 11A). Consistent with our *in vivo* observations, hepatocyte line H2.35 and HSC line LX-2 did not express Pc (Suppl. Fig 11B,C). SMO knockdown in Pc^{+ve} LPC line BMOL1.2 achieved between 70-90% reduction in nascent SMO (75 kDa), resulting in significantly (p<0.005) reduced cell proliferation by 48h treatment, and viability (Fig. 3B). Abrogation of SMO also reduced GLI-A, PTCH1, BCL-2 and CYCLIN D1 proteins, consistent with overall reduced cell viability. Further, BMOL1.2 cells expressing V5-tagged human GLI1-A (*hGLI1*) or GLI3-R

(*hGLI3R*) exhibited significantly enhanced ($p<0.0001$) or reduced ($p<0.0001$) proliferation/viability (respectively) by day 6, as compared to controls (Fig. 3C). This confirmed the importance of Hh pathway signalling in cell growth/expansion of Pc^{+ve} LPCs.

4. GLI-mediated transcriptional activity can be induced by growth factors in a SMO-independent manner *in vitro*

Following our *in vivo* observations, we hypothesised that liver injury-related growth factors might induce SMO-independent GLI-mediated transcriptional responses in liver cells. To address this, we examined Hh pathway activation within the Pc^{+ve} BMOL1.2 LPC line, using a *Gli-luciferase* reporter assay system. As predicted, BMOL1.2 cells treated with recombinant SHH (rSHH) significantly upregulated luciferase activity compared to controls (Fig. 3D). Injury factors epidermal growth factor (EGF) and HGF also significantly increased GLI-dependent transcription, and to the same degree as rSHH (Fig. 3D). Upregulation of a GLI-mediated response by EGF/HGF was completely abolished using specific inhibitors (Erlotinib, SGX523) whilst having no effect on the basal GLI-mediated response (Fig. 3E). To assess the contribution of SMO-dependent and SMO-independent pathways in basal and induced GLI-mediated transcriptional responses (luciferase activity), BMOL1.2 cells were treated with non-targeting control or α-*Smo* siRNAs (Fig. 3F). Intriguingly, α-*Smo* inhibited EGF-dependent induction of GLI, but did not prevent HGF-induced GLI signal. Also, although a significant decrease in basal luciferase activity was detected on SMO knockdown, it did not completely abolish luciferase activity.

5. Hh pathway manipulation *in vivo*: the functional role of SMO-dependent Hh pathway activation in chronic liver injury

Ptc1 heterozygous mice (*Ptc1*$^{+/-}$) were used to test the contribution of SMO-dependent Hh signalling in the TAA-induced liver injury model. Previous studies indicate *Ptc1*$^{+/-}$ mice have an impaired ability to regulate SMO-dependent Hh pathway signal, thus have increased baseline Hh pathway activation[19, 22]. Following TAA-8wks, *Ptc1*$^{+/-}$ mice exhibited exacerbated liver injury compared to wt counterparts as determined by liver function testing and Picrosirius red staining for collagen I (Fig. 4A,D). Upregulated *Gli1* expression correlated with increased serum alanine aminotransferase (ALT) levels (Fig. 4B,C). Expansion of EpCAM^{+ve} and vimentin^{+ve} populations was evident when compared with wt controls, indicating amplified regenerative and fibrotic responses (Fig. 4D). Further, whole liver EpCAM and α-SMA protein levels were elevated in *Ptc1*$^{+/-}$ mice (Fig. 4E). Importantly, N-Hh protein increased with TAA-treatment, but levels were equivalent in wt and *Ptc1*$^{+/-}$ mice, although *Ptc*$^{+/-}$ mice exhibited increased Hh pathway activity (Fig. 4B,E).

As Hh signalling has been implicated in epithelial-to-mesenchymal transition (EMT)[23], a selection of EMT genes were quantitated in wt vs. *Ptc*$^{+/-}$ TAA-treated mice (Suppl. Fig 12A,B). The results show upregulation of EMT-related genes (*Vim, Bcl2, Snai1, Zeb2*) in *Ptc*$^{+/-}$ mouse liver. However, the significant upregulation (p<0.01, *Ptc*$^{+/-}$ vs. wt TAA-treated) rather than downregulation of E-cadherin (*Cdh1*) does not support a specific EMT process. Additionally, it was of interest to note the significant correlation of

Gli1 expression with EMT-related Hh target genes (*Bcl2*, *Snai1*, *Zeb2*) (Suppl. Fig 12C).

DISCUSSION

The key observations in this paper define the intrahepatic niche of the Hh pathway in the TAA model of experimental cirrhosis. The major novel aspects of this paper are: (1) For the first time, Hh-responsive cells in the liver have been identified using Pc as a novel marker. (2) Characterisation of Hh signalling within the TAA injury model. (3) Use of ISH to detect cellular intrahepatic *Shh*/*Gli1* mRNA in experimental chronic liver injury. The first finding is of particular importance given Pc are required to transduce the Hh ligand signal in vertebrates[8-10], and the emerging relevance of SMO-independent GLI-mediated responses in injury and cancer[11-15].

Hh ligand production has previously been reported by injured biliary[22] and hepatocyte epithelium[24] in experimental models/human injury. Looking specifically at hepatocellular damage, we reported similar results with hepatocytes at the epithelial–mesenchymal border producing copious Hh ligand in human ALD (SHH) and following TAA (SHH, IHH). ISH indicated small cell populations also produced *Shh*. The mRNA/protein discrepancy is unexplained at present as no technical issues were identified. We can only conclude that in the TAA model Hh protein is produced by injured hepatocytes. Other studies have shown Hh ligands to be released by hepatocytes following ER stress[25] and apoptosis[24]. Further, in non-TAA injury models Hh ligands were detected in HSCs/myofibroblasts[17],

endothelial cells[26], leukocytes[27] and LPCs[18] following primary culture or *in vitro*.

Potential Hh-responsive cells have been identified in several models of liver injury and human liver diseases[16, 22, 23, 25, 28]. However, in these papers Hh-responsiveness *in vivo* has been defined by GLI2 staining only, without considering signalling via the Pc. Intrahepatic cell populations reported to be Hh-responsive include HSCs/myofibroblasts[17], endothelial cells[26], leukocytes[27] and LPCs[18]. Such a model proposes promiscuous Hh signalling to almost all cell populations within the liver during injury. We cannot refute the findings, as we noted *Gli1* and GLI2 expression in similar populations. However Pc, the organelle required to transduce SMO-dependent GLI transcriptional signals to downstream genes in vertebrate cells[8-10], were not detected in hepatocytes, leukocytes or myofibroblasts *in vivo* despite an intensive search. Previous studies have established that hepatocytes and leukocytes do not express a Pc[29]. A study of Pc expression by HSCs in human cirrhosis established 2.4% of HSCs (5 of 210 cells) had an evident Pc[30]. The presence of SMO within the Pc, expressed by $EpCAM^{+ve}$ cells *in vivo* confirmed that these cells are competent at receiving and responding to Hh ligand signals[8].

Our findings confining Hh ligand cognate signalling to the $EpCAM^{+ve}/Pc^{+ve}/SMO^{+ve}/GLI^{+ve}$ cell is compelling and is in keeping with Hh signal regulation that occurs in adult epithelial progenitor cell compartments in other diseases and niches[3-6]. $EpCAM^{+ve}$ cells in the TAA model share LPC

markers and further support the concept that Hh signalling is involved in maintaining the intrahepatic progenitor cell niche[18].

In light of our observations, previous studies that report widespread GLI-mediated responses in multiple cell types without a Pc are likely to represent SMO-independent activation GLI via growth factors. Indeed, we demonstrated that EGF and HGF specifically induce a GLI-mediated transcriptional response. Interestingly, EGF/HGF induced a comparable response to rSHH in the LPC line BMOL1.2. To assess the contribution of SMO-dependent and SMO-independent GLI-mediated responses in BMOL1.2 cells *in vitro*, EGF/HGF treatment was coupled with α-*Smo*. HGF still induced GLI luciferase signal in SMO-depleted cells, suggesting that the GLI-mediated transcriptional response via HGF is SMO-independent. Emerging *in vivo* data supporting the concept of SMO-independent GLI-mediated responses was recently documented in kidney fibrosis[12] and pancreatic carcinogenesis[15].

Studies using BMOL1.2 cells suggested that growth factors can elicit a GLI-mediated transcriptional response in Pc^{+ve} cells in addition to the SMO-dependent GLI-mediated response. As such, growth factors present during chronic liver injury may contribute to facilitation of a GLI-mediated transcriptional response in Pc^{+ve} (e.g. LPCs) and Pc^{-ve} (e.g. hepatocytes) cell compartments. Further, GLI activity was not abolished in unstimulated SMO-depleted BMOL1.2 cells, indicating that SMO-independent processes of GLI activation may maintain basal GLI pathway signal. It is possible that multiple mechanisms of transduction e.g. SMO-dependent and SMO-independent co-

exist to exert a GLI-mediated signal in the LPC compartment, as maintenance of this signal is integral to cell survival within this population.

Finally, we addressed whether genetic manipulation of the Hh/PTCH1/Pc/SMO cascade *in vivo* increased liver fibrosis in the TAA model. Prior studies have shown increased liver fibrosis in *Ptc1*$^{+/-}$ mice in other injury models[22, 23], and *Ptc1*$^{+/-}$ mice also exhibited increased fibrosis following hepatocellular TAA damage. Further, increased Hh activation, liver injury and amplified LPC and HSC/myofibroblast responses were observed. Importantly, expression of Hh ligands was not increased, confirming manipulation of the pathway itself rather than by a feedback loop.

Thus, we confirmed with the current literature that *in vivo* manipulation of the Hh pathway alters the outcome of fibrosis[22, 23]. However, our interpretation using the TAA model and *in vitro* experimentation has led us to a paradigm distinct from the current literature (Fig. 4F). The literature proposes a chronic liver repair model whereby Hh ligands produced by multiple cell types drive SMO-dependent pathway activation in many cell types to promote inflammatory, regenerative and fibrogenic responses[16]. In particular, the pro-fibrogenic model suggests SMO-dependent signaling in the HSC/myofibroblast, which perpetuates the fibrotic response[17]. This model has recently been expanded to include SMO as a master regulator of many cell types including the transformation of HSC/myofibroblasts into liver progenitor cells and hepatocytes[31]. However, this paper did not use Pc as a marker for Hh-responsive cells, and studied alternate liver injury models (bile duct ligation, methionine choline-deficient diet + ethionine (MCDE))[31]. We

propose a more refined model of SMO-dependent pathway activation in TAA-induced injury, where Hh ligands are produced locally by injured hepatocytes at the epithelial-mesenchymal interface. These cells are in close proximity to the LPC population, which receives paracrine Hh ligand signal via Pc. This drives subsequent expansion/growth of the LPC population. The close proximity of LPCs with HSCs/myofibroblasts implies cross talk between these populations. Other studies suggest LPCs may perpetuate HSC/myofibroblast activation/proliferation via cytokine/growth factor secretion[21]. We speculate $GLI2^{+ve}/Pc^{-ve}$ populations (hepatocytes, leukocytes, myofibroblasts) also located within the Hh niche, are not responding to Hh ligand through SMO given they do not have a Pc and cannot transduce the Hh signal *in vivo*[8]. Instead, we suggest SMO-independent activation of GLI-mediated transcriptional response is occurring in these cell populations, driven by growth factors/cytokines and independent of Hh/PTCH1/Pc/SMO.

There is no question the Hh signalling pathway plays an important role in chronic liver disease pathogenesis. Given the great potential for use of small molecule inhibitors of SMO in the treatment of liver disease, it is critical to dissect the contributions of both SMO-dependent and SMO-independent GLI-mediated transcriptional responses in chronic liver disease. This has not been previously undertaken. The use of Pc as a marker to define Hh/PTCH1/SMO-GLI responses will undoubtedly bring more clarity to this evolving field.

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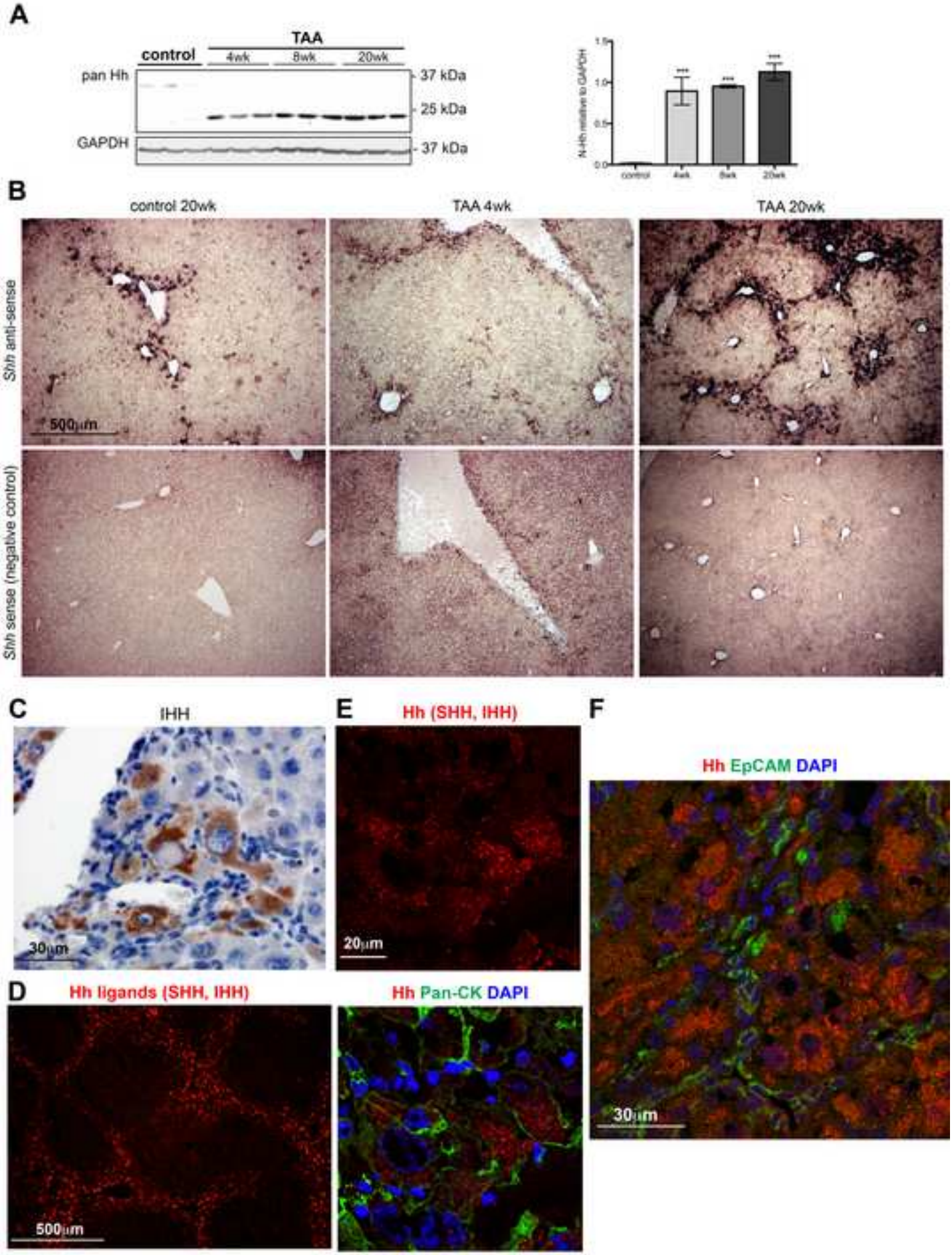
Fig. 1. TAA-treatment induces Hh ligand mRNA/protein in hepatocytes located at the injury interface. (A) Western blot, pan-Hh N-terminal signalling peptide (N-Hh, <25 kDa) in whole liver lysates and densitometry. Mean±S.E.M., One-way ANOVA, Dunnett's multiple comparisons test; ***p<0.001 vs. control 20wk. **(B)** *In situ* hybridization detected *Shh* mRNA in TAA model. Brown/purple precipitate indicates *Shh*$^{+ve}$ cells. 10x objective. **(C)** Immunohistochemistry detected IHH protein (brown) in hepatocytes. 40x objective. **(D)** Immunofluorescence detected Hh ligand proteins (red) at the injury interface. 10x objective. **(E)** Hh ligands (red) within pan-cytokeratin (pan-CK)$^{+ve}$ hepatocytes. Confocal, 63x objective. **(F)** Hh-producing hepatocytes (red) adjacent to EpCAM^{+ve} LPCs (green). 40x objective. **(C-F)** TAA-20wk liver.

Fig. 2. Progressive activation of the Hh pathway occurs with TAA-treatment, and multiple liver cell populations express *Gli1* and GLI2 *in vivo*. (A) qRT-PCR for *Gli1, Ptc1, Gli3, Hhip* transcripts in whole liver. Mean±S.E.M. Significant (*) difference between means. **(B)** Western blot; GLI1, GLI3-R, downstream Hh target (OSTEOPONTIN, CYCLIN D1, BCL-2) proteins in whole liver lysates. **(C-E)** TAA-20wk liver. **(C)** *In situ* hybridisation for *Gli1* mRNA. Purple precipitate indicates *Gli1*$^{+ve}$ small cells (solid arrows) and hepatocytes (dashed arrows) at injury interface. 10x (top), 40x (bottom) objective. Negative control, *Gli1* sense (inset). **(D)** Immunofluorescence detected GLI2^{+ve} nuclei (red) within EpCAM^{+ve} LPCs (solid arrows). GLI2^{+ve} hepatocyte nuclei (dashed arrow). 100x objective. **(E)** GLI2^{+ve} nuclei (green) within vimentin^{+ve} HSCs/myofibroblasts or CD45^{+ve} leukocytes (red). Confocal, 63x objective.

Fig. 3. Identification of primary cilia on Hh-responsive LPCs *in vivo* and the contribution of SMO-dependent/-independent GLI responses *in vitro*. (A) Immunofluorescence identified primary cilia (Pc) structures in TAA-20wk liver, at the

injury interface. Inset, fully assembled Pc: basal body (γ-tubulin, red) and axoneme (α-acetylated tubulin, green). Pc^{+ve} cells expressed LPC marker EpCAM (white). SMO (green) localisation within ciliary axoneme (red). Confocal, 60x/100x objective. **(B)** SMO knockdown in Pc^{+ve} BMOL1.2 LPC line. α-*Smo* reduced cell proliferation (fluorescence, Em 590nm) and viability vs. control (NT2, non-targeting). Concurrent decrease in Hh pathway/target proteins. **(C)** Proliferation (fluorescence)/viability following expression of V5-tagged GLI-A (*hGLI1*) and GLI-R (*hGLI3R*) vs. control (*lacZ*) in BMOL1.2s. Mean+S.E.M (*n*=6); Two-way ANOVA, multiple comparisons testing, α=0.001, ***p<0.005, #p<0.0001. Vector expression confirmed using anti-V5 antibody by Western blot. **(D-F)** Co-transfection (8xGli-luciferase, Renilla luciferase constructs) assessed GLI luciferase activity in BMOL1.2 cells. **(D)** rSHH, EGF and HGF significantly upregulated GLI activity vs. untreated cells. **(E)** Pre-treatment with inhibitors (Erlotinib/SGX523) prior to EGF/HGF treatment abolished EGFR/c-MET induced GLI activity, respectively. **(F)** EGF/HGF treatment coupled with co-transfection of *α-Smo* or NT2. **(B, D-F)** Mean±S.E.M. (*n*=4-6); Two-sided student *t*-test; *p<0.05, **p<0.01, ***p<0.005, #p<0.0001.

Fig. 4. *$Ptc^{+/-}$* mice exhibit increased liver injury, Hh pathway activation, regenerative and fibrotic responses. (A) Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were increased in *$Ptc^{+/-}$* vs. wt mice, following TAA. **(B)** qRT-PCR for *Gli1* mRNA in whole liver. **(C)** Significant correlation of *Gli1* (Hh activation) with serum ALT levels (U/L; hepatocyte damage) (ALT vs. *Gli1* mRNA; Spearman r=0.6907; p=0.0011). **(D)** Representative Picrosirius red (PSR) stain, immunofluorescence for EpCAM (LPCs), vimentin (HSCs/myofibroblasts). 10x objective. **(E)** Western blot; EpCAM (39 kDa), α-SMA, N-Hh (<25 kDa) in whole liver lysates. **(F)** Proposed *in vivo* model. Mean±S.E.M., Mann-Whitney U test; *p<0.05, **p<0.01.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TAA 20wk
Shh anti-sense
Shh sense (negative control)
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Hh ligands (SHH, IHH)
500μm
Hh Pan-CK DAPI
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