Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

# The intrahepatic signalling niche of hedgehog is defined by primary cilia positive cells during chronic liver injury

Candice Alexandra Grzelak[1], Luciano Gastón Martelotto[4,6], Nicholas David Sigglekow[1], Bramilla Patkunanathan[1], Katerina Ajami[1], Sarah Ruth Calabro[1], Benjamin James Dwyer[5], Janina Elke Eleonore Tirnitz-Parker[5], D. Neil Watkins[4], Fiona Jane Warner[1], Nicholas Adam Shackel[1,2,3], Geoffrey William McCaughan[1,2,3,*]

[1]*Liver Injury and Cancer, Centenary Institute, Camperdown, NSW, Australia;* [2]*A. W. Morrow Gastroenterology and Liver Centre, R.P.A.H., Camperdown, NSW, Australia;* [3]*Faculty of Medicine, University of Sydney, Sydney, NSW, Australia;* [4]*Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, VIC, Australia;* [5]*School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley, WA, Australia;* [6]*Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

**Background & Aims**: 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.



## Introduction

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

Keywords: Hedgehog signalling pathway; Primary cilia; Liver progenitor cells; Non-canonical cell signalling; GLI; Smoothened; Thioacetamide.
*Received 9 April 2013; received in revised form 2 July 2013; accepted 5 August 2013; available online 23 August 2013*

* Corresponding author. Address: Liver Injury and Cancer, Centenary Institute, Locked Bag No. 6, Newtown, NSW 2042, Australia. Tel.: +61 2 9565 6125/2 9515 8578; fax: +61 2 9565 6101.
*E-mail address:* g.mccaughan@centenary.org.au (G.W. McCaughan).

*Abreviations:* 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.

(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 that 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 (300 mg/L; MP Biomedicals, USA) in drinking water *ad libitum* for 4, 8, or 20 weeks (wk) (n = 3–10/group) prior to sacrifice. Non-treated control littermates were sacrificed at 20 wk. 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 8 wk (n = 4–9/group).

### *Quantitative mRNA analysis*

Outlined in Supplementary Data, Supplementary Table 1.

### *In situ hybridisation (ISH)*

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

### *Histology, immunohistochemistry and immunofluorescence*

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

### *Cell culture/vector construction*

Outlined in Supplementary Data.

### *Viability assay*

Cells ($7.5 \times 10^5$) were transfected with 40 nM non-targeting control (NT2) or Smoothened siRNA (α-*Smo*; Dharmacon) using Lipofectamine2000 (Invitrogen, USA) as per manufacturer's instructions. After 6 h, 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) as per manufacturer's instruction. The same method was applied to cells transfected with *hGli1* and *hGli3R*.pEF-DEST51 DNA (12 μg) using Lipofectamine2000, after selection with blasticidin. Repeated minimum three times.

### *Luciferase assays*

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

### *Hedgehog ligand expression*

#### *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-20 wk (Supplementary Figs. 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-20 wk 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 (Supplementary Fig. 5A). In addition, small portal tract cells were $Shh^{+ve}$, possibly implicating