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Reply to: "The many faces of Hedgehog signalling in the liver: Recent progress reveals striking cellular diversity and the importance of microenvironments"

To the Editor:

We thank Matz-Soja and colleagues for their interest and critique of our article [1]. We agree with Matz-Soja *et al.* that generalisations regarding the contribution of Smoothened (SMO)-dependent and SMO-independent GLI-mediated signals during liver injury processes should be made with caution. We concur that more work is required to delineate how, within various liver cell niches, Hedgehog (Hh) pathway signalling components elicit canonical or 'non-canonical' Hh signalling responses during liver injury.

The focus of our article was to highlight the assumption that GLI2 expression alone as an accurate indicator of canonical Hh pathway activity in the liver, may be an oversimplification. Data from our *in vivo* model suggest that canonical SMO-dependent GLI-mediated signals can occur within primary cilia (Pc) positive/GLI2 positive liver progenitor cell (LPC) populations, while in Pc negative/GLI2 positive populations GLI-mediated signals may be driven in a SMO-independent manner.

Hh signalling pathway components coordinate diverse responses (both GLI-dependent and GLI-independent), categorised into five specific pathways (Table 1), recently reviewed [2,3]. Table 1 illustrates the complexity of Hh pathway signalling; a comprehensive understanding of Hh signalling processes within the liver would require that all five aspects be addressed. In our paper, we specifically studied canonical Hh (SMO-dependent GLI-mediated) signalling *in vivo*. Our results also point towards a role for SMO-independent GLI-mediated signalling involvement in chronic liver injury. We acknowledge that we have not incorporated type I (PTCH1) or type II (SMO-dependent GLI-independent) non-canonical Hh signals within our working model (see Table 1), which does not suggest the irrelevance of these in chronic liver injury. Indeed, Matz-Soja recently showed the importance of SMO-dependent GLI-independent signals within hepatocytes [4]. However, within our current understanding GLI is either activated via Pc/SMO, or through multiple SMO-independent mechanisms such as PI3K/AKT, S6K, RAS, TGFβ/SMAD, MEK/ERK, RTK, and EWS/FLI1 (reviewed in [3]). Currently, there is no evidence of Hh/PTCH1/SMO/GLI 'canonical' signalling occurring independently of the Pc.

We are perplexed by a few conclusions drawn by Matz-Soja *et al.* from the current literature. Matz-Soja *et al.* wrote, 'besides canonical (cilia- and SMO-dependent) signalling in endothelial cells, cholangiocytes, activated hepatic stellate cells (HSC) and progenitor cells, there is obviously cilia-independent but SMO-dependent signalling in mature healthy hepatocytes, Kupffer cells and, most probably, quiescent HSCs.' The conclusion that canonical Hh signalling occurs in liver endothelial cells (ECs) and activated HSCs has been drawn prematurely, as the presence of Pc (required for canonical Hh signalling) was not evaluated in the study referenced [5] or in previous studies.

The presence of Pc on HSCs is debatable. Confusingly, Matz-Soja *et al.* inferred that activated but not quiescent HSCs express Pc. Our data from TAA-induced chronic liver injury demonstrate that vimentin positive HSCs are Pc negative, suggesting that activated HSCs do not express a Pc, at least in mice. Further, a recent publication suggested myofibroblasts disassemble Pc during activation *in vitro* [6]. In human liver however, a minority of HSCs can assemble Pc under certain circumstances (http://www.bowserlab.org/primarycilia/cilialist.html) and we have observed Pc on a minority of vimentin positive HSCs in human alcoholic liver disease (unpublished data). Other electron microscopy (EM) studies have also reported <5% HSCs express a Pc in human cirrhotic injury [7].

The concern over the use of pan Hh antibodies is unwarranted, as the pan Hh antibody used detects both Sonic Hh (SHH) and Indian Hh (IHH). Ligand detection was corroborated separately with *in situ Shh* mRNA and IHH ligand protein detection. IHH positive ballooned hepatocytes were denoted as 'damaged' on pathologist recommendations, due to altered cell morphology and significantly increased AST/ALT serum levels in 20 week TAA mice, in keeping with previous studies that identify damaged hepatocytes as a source of ligand [8].

We note that Fig. 1 of the 'Letter to the Editor' inaccurately references our data with regard to LPC's 'known' involvement

Table 1. Summary of known canonical and non-canonical Hedgehog signalling cascades.

		Hh	Ptch	Smo	Gli	Cilia	Cascade	Effect
Canonical		Yes	Yes	Yes	Yes	Yes	Hh⟞PTCH1⟞SMO → GLI	Gene expression
Non-canonical	Type I	Yes	Yes	No	No	No	Hh⟞PTCH1/CDON → Caspase 9	Blocks extrinsic apoptosis
		Yes	Yes	No	No	No	Hh⟞PTCH1⟞ Cyclin B1	Promotes entry into G2/M
	Type II	Yes	Yes	Yes	No	No	Hh⟞PTCH1⟞ SMO → PI3K → Rho/Rac	Migration, chemotaxis
		Yes	Yes	Yes	No	No	Hh⟞PTCH1⟞ SMO → PI3K → PLCγ	Calcium signalling
	Metabolic	?	?	Yes	No	Yes	Hh⟞PTCH1⟞ SMO → AMPK	Warburg metabolism
	RTK	No	No	No	Yes	No	GF → RTK → GLI	Gene expression

in epithelial-to-mesenchymal transition (EMT). In actuality, we observed and described paracrine epithelial-epithelial (hepatocyte-LPC) canonical Hh signalling.

In summary, future research in this field should be focused towards dissecting out the contribution of all five potential Hh signalling responses. This includes identification of Hh ligand, Pc, SMO and GLI protein source(s) *in vivo*, coincident expression of Hh co-receptors (CDON, BOC, and GAS1), and the Hh pathway activation state (ratio of GLI-A:GLI-R, and *GLI1* Hh target gene expression) itself. These *in vivo* analyses combined with functional analysis of cellular proliferation, viability, migration, apoptosis, cell cycle progression, calcium signalling and metabolism will allow future studies to properly delineate what combination of Hh signal(s) occur within various liver cell niches. This will aid in addressing many of the current unknowns within the field. We thank the authors for their interest and comments and hope we have adequately addressed their concerns.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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