ANSWERING REVIEWERS

Name of Journal: World Journal of Gastroenterology

ESPS Manuscript NO: 26274

Title:
MicroRNAs in liver fibrosis: focusing on the interaction with hedgehog signaling

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We have now revised our manuscript according to the comments and suggestions from reviewers. We marked the changed parts in the revised manuscript with blue-colored characters for the convenience. We hope that our revised manuscript would meet your criteria and eventually get published in your journal.

Answer to Reviewer 1 (code: 03387541)

In this review, Hyun and collaborators discuss the regulation of Hedgehog (HH) pathway by MicroRNA in liver fibrosis. The authors discuss the dysregulation of miRNAs closely associated with fibrotic diseases such as liver fibrosis. Aberrant reactivation of HH pathway has been previously implicated in the reactivation of Hepatic stellate cells (HSCs) during liver fibrogenesis. They review growing evidence showing the association of miRNAs with HH signaling. For instance, recent studies suggest that HH-regulating miRNAs induce inactivation of HSCs, leading to decreased hepatic fibrosis. The topic of this review is interesting but several points should be clarified:

1. In the paragraph “Signal transduction of the HH signaling pathway”, a schematic of the HH pathway could help the reader to better understand this complex pathway (miRNA targeting the different components of the pathway could be also included). In addition, major aspects of the pathway are missing such as 1) the involvement of the primary cilium

in mammalian cells and 2) that GLI2 and GLI1 are rather strong transcriptional activators while GLI3 may act as strong transcriptional repressor.

As you recommended, we added a schematic diagram of the Hh signaling pathway and Hh signaling-associated miRNAs in Figure 1.

1) We added the explanation of Hh signaling in the primary cilium, "The canonical Hh signaling is well-known in the primary cilium in vertebrates. Hh signaling is activated by the translocation of Smo into the primary cilium, a single, tiny, microtubule-based organelle that projects from the surface of most vertebrate cells [*J Hepatol* 2011; 54(2): 366–373, *Nature* 2005; 437: 1018–1021, *Science* 2007; 317: 372–376]. Inherited ciliary defects, such as Bardet-Biedl syndrome and Meckel syndrome, was reported to have the disrupted Hh signaling [*J Hepatol* 2011; 54(2): 366-373, *Cur Top Dev Biol* 2008; 84: 249-310]. In addition, ciliary dysfunction blocks the proteolytic process of full-length Gli3 to the truncated repressor form because of the localized SUFU-Gli3 in the tip of cilia where proteolytic process occurs. Therefore, it induces the aberrant activation of various Hh-target genes, causing developmental failure [*J Hepatol* 2011; 54(2): 366-373, PLoS Genet 2005; 1(4): e53]".

2) The C-terminal-cleaved form of Gli3 is known to dominantly act as a repressor and reduce the expression of Gli1/2 and Gli-target genes including Pax2, Sall1l, Cyclin D1 and N-myc in embryonic development, whereas Gli1 and Gli2 function as the transcriptional activators [*Development* 2006; 133(3): 569-578, *Cell* 2000; 100: 423–434]. In canonical Hh signaling pathway, active Smo inhibits the proteolytic processing of Glis and allows Glis to act as a transcriptional activator, triggering the activation of Hh signaling in the presence of Hh. Hence, the full-length Gli3 as well as Gli2 activates Hh signaling [*Dev Biol*. 2005 Jan 15;277(2):537-56]. In addition, the active form of Gli3 was report to be upregulated in colorectal cancer [*Cancer Sci* 2013; 104: 328–336] and liver fibrosis [*Nat Commun* 2016; 7: 10993]. To clarify this point, we added this explanation in the revised manuscript.

2. On one hand, the authors describe the differentiation of HSCs into myofibroblast-HSCs (MF-HSCs). On the other hand, they mention the role in EMT in HSCs activation. The link between these two processes is missing and should be clarified.

As your comments, we presented more explanation of explained of EMT and activation of HSCs. "When Q-HSCs are activated into MF-HSCs, the expression of quiescent markers (e.g., PPARγ and GFAP) and epithelial genes (e.g., BMP7, desmoplakin, and E-cadherin) is

downregulated but the expression of myofibroblastic markers (e.g., α-SMA, vimentin, fibronectin, and Col1α1) and mesenchymal genes (e.g., Snail and Lhx2) is upregulated in MF-HSCs [*Am J Physiol Gastrointest Liver Physiol* 2009; 297(6): G1093-G1106, *J Biol Chem* 2010; 285(47) 36551-36560]. Leptin, an anti-adipogenic and pro-EMT factor, promotes the activation of HSCs by inducing the expression of Hh signaling components [*J Biol Chem* 2010; 285(47) 36551-36560]. These findings indicate that EMT process characterizes the transdifferentiation of the Q-HSC into MF-HSCs.

3. In the paragraph "MiRNAs interacting with HH signaling in liver fibrosis", the authors describe the anti-fibrotic effects of MiR-378a-3p, which targets GLI2 and GLI3. In the light of GLI3 repressor activity and GLI2 activating properties, the authors should discuss this apparent discrepancy.

Gli2 and Gli3 have both of the activator and the repressor domains in the C- and N-terminal region, respectively, whereas Gli1 has only an activator domain. Although the C-terminal-cleaved form of Gli3 is known as a repressor, active Smo inhibits the proteolytic process of full-length of Gli3, which also activates Hh signaling with Gli2. In addition, it has been reported that the expression level of Gli2 and Gli3 is upregulated in activated HSCs and CCl_4-induced liver fibrosis [*Hepatology* 2012; 56(3): 1108-1116, *Sci Rep* 2015; 5: 14135, *Nat Commun* 2016; 7: 10993]. As we described above (answer for 2), this explanation was inserted in the revised manuscript.

4. In the paragraph "miRNAs interacting with HH signalling in others tissues, besides liver", the authors mentioned the interaction between miR-21 downstream of TGF-b1 signaling in HVC infections… Basically, they discuss the effects of miRNA targeting both TGF-b1 and HH pathways. To broaden their discussion, the authors could also mention and discus the fact that the TGF-b pathway can regulate major components of the HH pathway in a smo-independent manner in skin and lung fibroblast, pancreatic cancer cells for examples.

As you requested, we discussed TGF-β-regulated Hh signaling in the revised manuscript, "it is possible that miR-21 enhances the Hh signaling by up-regulating TGF-β expression in the chronic liver of patients with HCV infection, because the TGF-β signaling is known to promote the expression of Gli1/2 in a Smo-independent manner in various cell types, such as skin and lung fibroblasts and pancreatic cancer cells [*Cancer Res* 2011; 71(17): 5606–5610, *Cancer Res* 2007; 67(14): 6981–6986]. These findings indicate that miR-21 is involved in the

crosstalk between Hh and TGF-β signaling.".

5. In the paragraph "Improvement of the therapeutic application of miRNAs in liver fibrosis", the authors mentioned very superficially the other HH pathway inhibitors already validated. 1) They should broaden the discussion to the others inhibitors (not only to cyclopamine). They 2) never also clearly discuss the major caveat of miRNA: their potential to generate false positive by targeting multiples targets in addition of HH pathway components (with regards to the topic of this review).

1) As you requested, we added the explanation for another Hh inhibitor, vismodegib, in the revised manuscript. "Vismodegib targeting Smo-dependent Hh signaling has been approved by the FDA for the treatment against advanced basal cell carcinoma [*Nat Rev Drug Discov* 2012; 11(6): 437–438] and it has shown the therapeutic effects on both liver fibrosis and hepatocellular carcinoma in mice [*PLoS One* 2011; 6(9): e23943, *PLoS One* 2013; 8(7): e70599]. However, vismodegib also has side effects, such as muscle spasms, alopecia, dysgeusia, weight loss, fatigue, nausea, diarrhea, decreased appetite, constipation, arthralgia, vomiting, ageusia, hyponatremia, pyelonephritis and presyncope [*Infect Agent Cancer* 2012; 7(1): 29, *J Pharmacol Pharmacother* 2013; 4(1): 4–7]. Especially, vismodegib is not allowed to be prescribed to pregnant women due to its teratogenicity, embryotoxicity and fetotoxicity. In addition, it does not work for patients having mutations in Smo receptor [*Infect Agent Cancer* 2012; 7(1): 29, *J Pharmacol Pharmacother* 2013; 4(1): 4–7]; thus the novel therapeutic strategies should be developed. A recent study reports that the co-treatment of vismodegib with miR-29b-1 targeting several pro-fibrotic genes, such as Col1α1, FN-1 and PDGF-β, regresses the hepatic injuries and fibrosis in bile duct ligated livers of mice [*Biomaterials* 2016; 76: 144-156]. Compared with the single treatment with miR-29b-1 or vismodegib, this combination therapy was more effective in reducing the levels of injury-related enzymes and the expression of fibrotic proteins in liver tissue, implicating the synergistic action of miRNA and small molecular inhibitor in treating liver fibrosis [*Biomaterials* 2016; 76: 144-156]."

2) We also discussed about the potential of miRNAs to generate false positive effects by targeting multiple targets that you pointed out in the revised manuscript as followed; "In addition, therapy utilizing miRNAs is complex because miRNAs are possible to generate false positive effects by targeting multiple target genes. For example, miR-125b that directly targeted Smo in medulloblastoma [*EMBO J* 2008; 27(19): 2616-2627] was shown to have an anti-fibrotic effect by regulating Hh signaling in CCl_4-injured liver of rats [*Sci Rep* 2015; 5:

14135]. Zhou et al. also reported that miR-125b directly targeted SMAD4, which inhibited EMT process in HCC cells [*Hepatology* 2015; 62(3): 801-815]. Because EMT is closely associated with HSC activation, it is possible that miR-125b exerts its anti-fibrotic role through targeting SMAD4 and other EMT-related genes, including Hh signaling, in CCl_4-induced liver fibrosis. Therefore, baseline expression of various target genes in each patient should be carefully considered for miRNA therapy."

Answer to Reviewer 2 (code: 00002232)

Hyun et al. wrote a well-organized and comprehensive review on the regulation of the Hedgehog signaling pathway by miRNAs in liver fibrosis. I only have one comment:

-I would recommend adding a figure explaining graphically how miRNA regulates HSC activation by interacting with Hh signaling pathway. This figure would make clear the significance and impact of this topic for readers.

As you recommended, we added a schematic diagram of the Hh signaling pathway and miRNAs interacting with the different components of the Hh signaling as in Figure 1 for helping the readers to better understand the complex regulatory pathway.