ANSWERING REVIEWERS

December 20, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 15090-review.doc).

Title: Loss of Dicer1 impairs hepatocyte survival and leads to chronic inflammation and progenitor cells activation

Author: Xu-Feng Lu, Yong-Jie Zhou, Lei Zhang, Hong-Jie Ji, Li Li, Yu-Jun Shi, Hong Bu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 15090

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) Answer to the Reviewer 02936191.

Comments To Authors: Authors generated a hepatocyte-selective Dicer1 knockout mouse and observed the gradual hepatic histopathological changes in the mutant liver, including hepatocyte proliferative and apoptosis, liver necrosis and inflammation, HCC development and so on. Although related work has been previously published, authors did great jobs on the further detail continuous hepatic histopathological processes in response to the loss of Dicer1. It's a good complement to the previous research. However, a minor language polishing is required.

Answer: We appreciate the positive comments on our manuscript. To make the manuscript more readable and understandable, the language has been thoroughly revised by the American Journal Expert.

(2) Answers to the Reviewer 02943371.

Comments To Authors: The article "Loss of Dicer1 impairs hepatocyte survival and leads to chronic inflammation and progenitor cells activation" by Lu et al., describes the generation of a hepatocyte-selective Dicer1 knockout mouse model and the investigation of the continuous hepatic histopathological processes in response to the loss of Dicer1 in this model. The results obtained in this study are interesting. The experiments have been done properly, and the interpretations of the data are mostly appropriate.

Specific points:

● Fig 4B : There is no increase of serum IL-1β in the 2 months-old Dicer1-deficient mice. The authors should discuss about that.

Answer: It is hard to discuss the mechanism of the decrease of serum IL-1β in the 2 months-old Dicer1-deficient mice. However, necrosis hepatocytes release a high level of IL-1α, which may repress serum IL-1β level, remaining total level of IL-1 unchanged.

● Fig 5B : There is no increase of Nanog expression the 2 months-old Dicer1-deficient mice. The authors should discuss about that.

Answer: The expression of pluripotency transcription factor genes especially Nanog decreased with age, however it is too complex to explain the reasons, just as like previous study (Sekine S et al Gastroenterology 2009), we show a pluripotency state of young mutant liver.

● The authors should mention the Figure 6D in the text.

Answer: We have mentioned the Figure 6D in the text to confirm Dicer1 was kept negative in the HCC and the non-tumor tissue.

(3) Answers to the Reviewer 02770708.

Comments To Authors: MicroRNAs have important roles in liver biologies. Therefore it is important to examine their roles in liver development and diseases using animal models. It was previously reported that liver-specific Dicer1 knockout mice showed impaired lipid metabolism and eventually develop hepatocellular carcinoma. In this paper, the authors further characterized the gradual histological processes of spontaneous development of HCC. HCC development and defects in lipid metabolism have been already reported. Therefore, the novelty of this work is that they analyzed types of cells death as well as inflammation and fibrosis. However, the authors should provide clearer data to show necrosis, inflammation, and fibrosis and be more careful to discuss how fibrosis and HCC are developed in Dicer1-/- mice.

Answers: we have performed additional experiments to manifest the tissue necrosis, inflammation and progressive fibrosis. All these results have been added in the revised manuscript.

Specific points:

● Fig. 1B is not clear. I could see Dicer1 band in the lane for Dicer1-/- at 4month. Clearer Western blot data should be provided to conclude that Dicer 1 could not be detected at all the time points.

Answer: We performed the immunoblotting using liver tissue homogenate including non-parenchymal cells in the liver. These non-parenchymal cells, which account for approximately 25% total liver cells, are kept Dicer 1 positive, therefore, a slight Dicer 1 band could be seen in the western blotting result.

● The authors described that in addition to apoptosis, hepatocyte necrosis could be observed in Dicer1-/- mice. How did the authors detected necrotic hepatocytes? Is it possible to provide a quantitative data for necrosis as the authors provided for TUNEL+ and Ki67+ cells in Fig. 3C?

Answer: Necrotic hepatocytes were observed on the HE staining results and the examination of hepatic enzymes. To clearly show the necrosis, we provided a quantitative data of the necrotic area and the IL-1α level which reflects the death of hepatocytes.

● In Fig. 4, the authors described that inflammatory cells including neutrophils and eosinophils infiltrated into KO liver tissues. How did the authors know those infiltrated cells were neutrophils and eosinophils?

Answer: We examined neutrophils and eosinophils on HE staining sections by our clinical pathologist. To be rigorous, we deleted this sentence. In addition, we performed flow cytometric analysis for the pan-leucocyte marker CD45 to determine the total inflammatory infiltration of the leukocytes in the liver.

● They mentioned that CK19+ progenitor cells increased in KO mice (Fig. 5). However, in contrast to previous reports, proliferating CK19+ ductular cells were negative for CD133- and OV6-. Since CD133 is expressed in normal bile ducts (Kamiya et al Gastroenterology 2009), it is weird that those CK19+ cells are negative for CD133. The authors should provide data of co-staining of CD133 and CK19 to show CK19+ proliferating ductular cells in Dicer1-/- mice are negative for CD133-.

Answer: We performed CD133/CK19 and OV6/CK19 co-staining to confirm OV6 and CD133 were indeed negative in CK19+ ductular cells. Besides, OV6 and CD133 antibodies worked very well in our

other Alb-Cre conditional knockout mouse models with ductular reaction.

● Do hepatocytes express OCT4 and SOX2 (Fig. 5B)? The authors should perform the staining of OCT4 or SOX2 with a hepatocyte marker such as HNF4? and C/EBP?

Answer: We are sorry for that we did not perform this experiment because all these mentioned antibodies against OCT4, SOX2, HNF4α and C/EBP are rabbit polyclonal antibodies. But we can determine those cells are hepatocytes based on their shape and spatial distribution. Previous study also showed that mutant liver expressed the same level of HNF4α compared with control (Sekine S et al Gastroenterology 2009).

● CK19 staining was performed at 2 and 4 months, whereas SOX2 and OCT4 staining was at 1 month. Why the authors performed those experiments at different stages?

Answer: The proportion of CK19+ ductular cells increased with the age, while the expression of SOX2 and OCT4 decreased with the age. That's why we performed those experiments at different stages to show the pathological changes.

● In Fig. 6A. why did the authors show Masson staining? It is hard to know whether there were fibrotic areas or not.

Answer: Yes, it is hard to know whether there were fibrotic areas or not in HCC. We deleted the part of Masson staining.

● Staining in panels in Fig. 6B are not clear. Did the authors try to show HCC are CK19+ or HNF4?+?

Answer: The IHC of CK19 and HNF4α was performed to show the tumor is HCC but not cholangiocellular carcinoma.

● Parts of HCC tissue were Dicer 1+ (Fig. 6C)? I could see brown area in the image at 12 month. cat 4 and 12 months slightly expressed Dicer1? (Fig. 6D)

Answer: Inflammatory cells and hepatic non-parencymal cells in Dicer1-/- tissues and HCC tissue are Dicer1 positive (Fig. 6C and 6D).

● Page 9. The authors proved a data that 4 out of 20 mutant mice developed HCC.

Answer: We proved a summary of incidence and characteristics of the tumors that developed in the 4- to 12-month-old mutant mice.

● How about fibrosis? All the mutant mice developed liver fibrosis?

Answer: Yes, all the mutant mice developed liver fibrosis.

● In Discussion, the authors described that progenitor cells expand and become hepatocytes to compensate hepatocyte death. However, as the authors mentioned in Page 9 that hepatocytes expressed SOX2 and OCT4, suggesting hepatocytes, which escaped from apoptosis and necrosis, may alter their characteristics. Recent papers showed that progenitors do not majorly contribute to tissue repair by providing new hepatocytes (Yanger et al Genes and Dev. 2012, Tarlow et al Hepatology 2014). In addition, since the authors described that CK19+ cells were negative for CD133 and OV6, it is not clear whether those cells are liver progenitors or not. Therefore, the authors should carefully discuss how damaged liver tissue is compensated and how HCC is developed in Dicer1 KO mice.

Answer: Indeed, growing studies showed that progenitors do not majorly contribute to liver repair by providing new hepatocytes. Nevertheless, it is still controversial and remains further study.

The continuous proliferation of mutant hepatocyte took majorly responsibility in compensatory proliferation in our mutant mouse model, however, activated progenitors might contribute to tissue repair, the clear roles of progenitors were undetermined. Loss of Dicer1 induced progenitors expressing SOX2 and OCT4, which may repress the expression of CD133 and OV6. The relationship initiated by hepatocytes death leading to inflammation, fibrosis, compensatory proliferation and HCC promotion is

summarized in Figure 8C.

● Other points. Page 8, the section "Dicer1-deficient mice develop chronic inflammation and fibrosis" What are "histocytes"?

Answer: We feel sorry for our incorrect description of inflammation cells, and we have corrected it.

● Page 9 KlF4 is probably KLF4.

Answer: Thank you for pointing out our spelling mistake, we have already corrected it.

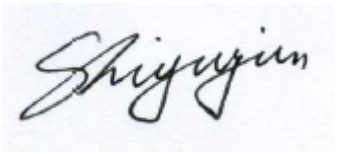
● Fig. 6D Dicer1-/- means "non-tumor tissue"?

Answer: No, all Dicer1-/- means mutant livers without HCC, because nearly half mutant livers spontaneously developed HCC.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



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