

Format for ANSWERING REVIEWERS



Jan 12, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (ESPS Manuscript NO: 15264).

Title: Hepatocyte Nuclear Factor 4 α Induces a Tendency of Differentiation and Activation of Rat Hepatic Stellate Cells

Author: Kai Liu, Minggao Guo, Xiaoli Lou, Xiaoya Li, Yang Xu, Weidan Ji, Xuandong Huang, Jiahe Yang, Jicheng Duan

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 15264

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

Reviewer #1

The authors present interesting results suggesting that a mesenchymal to epithelial transition occurs in stellate cells following forced expression of HNF4 after infection with an adenovirus vector. The authors state that the infection forced expression of albumin and alfa fetoprotein, but no data are giben in any of the figures in that regard. While the overall findings are interesting, it would take more information to validate the assertion made by the authors in their abstract, which states "HNF4 α can induce the differentiation of HSCs into mature hepatocytes".

The following additional information would be required to validate that assertion:

- (1) Provide data to demonstrate that expression of HNF4 in stellate cells is also associated with expression of albumin and alpha-fetoprotein, as claimed but not demonstrated in the manuscript.

Answer: In Figure 3, when the hepatic stellate cells were overexpressed HNF4 α after infected with AdHNF4 α , albumin and alpha-fetoprotein were positively expressed, demonstrating the relations between HNF4 α and albumin/alpha-fetoprotein. According to the reviewer's good suggestion, we also provided the data that the expression of albumin/alpha-fetoprotein was downregulated after the HNF4 α -induced HSC-T6 cells were re-inhibited HNF4 α expression, as showed in Figure 5. The statement "HNF4 α can induce the differentiation of HSCs into mature hepatocytes" has revised to "HNF4 α can induce a tendency of differentiation of HSCs into hepatocyte-like cells".

- (2) Provide gene expression data by gene array or RNAseq to demonstrate that expression of HNF4 is associated with a broad spectrum of gene expression changes. This approach, even if it does not provide evidence for a complete hepatocyte transdifferentiation, it will at least document the extent of the mesenchymal to epithelial transition of the stellate cells after HNF4 expression.

Answer: The gene array or RNAseq may need a long time and more money. We couldn't complete this experiment during the 2-weeks revision time. And, this work was supported by a project of National Natural Science Foundation of China, this project ended two years before and the money had been run out.

- (3) Provide electron-microscopy evidence about morphologic changes associated with formation of hepatocytes after expression of HNF4. A cell expressing albumin is not necessarily a hepatocyte. Hepatocytes have characteristic structural features.

Answer: We have provided the observation under electron microscope, the results found that the HNF4 α -induced cells had more mitochondria and ribosomes compared with the parental cells, as showed in Figure 1E.

Reviewer #2

The authors indicate that HNF-4alpha induces transdifferentiation from rat stem like HSCs into hepatocyte like cells but not the activation. Furthermore, the readers cannot believe the phenomenon in the present study. Major comments:

- (1) Title: Please should change it. In the activated HSCs, upregulation of collagen I, alpha-SMA and TIMP-1 are observed. However, HNF-4alpha inhibited them in the present study.

Answer: We thank reviewer for this good suggestion. We really demonstrated that the expression of collagen I, alpha-SMA and TIMP-1 was decreased by HNF4 α . But the HSCs had no obvious changes in morphology. As suggested by Reviewer #1, we provided the observation under electron microscopy, and found that the HNF4 α -induced cells only showed to have more mitochondria and ribosomes compared with the parental cells. We considered that the expression of HNF4 α may result in a tendency of differentiation of HSCs to hepatocytes and there is a far way to differentiate to mature hepatocytes, the cells only showed changes of molecular markers but not in morphology. We have revised the title and the description about differentiation, and emphasized it is a tendency of differentiation and the cells are hepatocyte-like cells.

- (2) Abstract: Please should rewrite it like as title and delete the last sentence.

Answer: Thanks, we rewrote the abstract and deleted the last sentence.

- (3) Introduction: Please add the more information of stem like HSCs.

Answer: Thanks, we rewrote and add more information of stem like HSCs in the Introduction.

- (4) Figure 1: Please add the clear phase contrast images of HSC-T6 and HSC-T6/HNF-4alpha and write the state of cell shape.

Answer: We are sorry for the mistake labeling in Figure 1D. The first-row images are HSC-T6 and HSC-T6/HNF4 α under bright field, the second row images are HSC-T6 and HSC-T6/HNF4 α that were stained by DAPI and the third row images are showed HNF4 α expression. We have corrected the image labeling and also readjusted the phase contrast in Figure 1D.

- (5) Figure 4 and 5: Please should indicate the images of E-cadherin and vimentin.

Answer: We added the indicators for E-cadherin and vimentin in Figure 4 and 5.

- (6) Discussion: The authors should discuss about the mechanisms of transdifferentiation from rat stem like HSCs into hepatocyte like cells via HNF-4alpha.

Answer: Thanks, we revised the discussion.

Reviewer #3

This manuscript describes the function of HHNF-4a in the regulation of the differentiation and activation of hepatic stellate cells. The authors provided a reasonable hypothesis and clear results to support their notion. However, there are several points to be revised:

- (1) In Figure 1, the resolution of Fig. 1D is not so good to support your notion. Please replace the figure with

another clear one.

Answer: We thank reviewer to give a good suggestion. We have readjusted Figure 1D.

- (2) In Figure 2, please indicate the method to analyze the image intensity (ie. name of used software).

Answer: The image analysis was performed with the Image-Pro Plus V6.0 (Media Cybernetics, Inc., Rockville, MD, USA). We have added the information in legend of Figure 2.

- (3) In Figure 3, the description of method is not clear. In the case of AFP, ALB, G-6-P, and PEPCK, the authors performed RT-PCR and Western blot analyses (3A and 3B). However, In Fig. 3C, they performed only RT-PCR. Therefore, it will be clearer to describe the two sets of experiment separately. Did you perform Western blot analysis for the genes in Fig. 3C? It will be nice if you include the data set, too.

Answer: We thank reviewer to give a good suggestion. We have added the data of Western blot analysis for the genes in Fig. 3C.

- (4) In Figure 4, I can't find Western blotting data. Please check the legend and figure to make them be consistent.

Answer: We thank reviewer's keen eyesight. We have corrected the error in figure legend.

- (5) In the abstract, please check the first sentence. "~ liver repair and regeneration of after liver injury."

Answer: The abstract was thoroughly revised, and this sentence is deleted.

Reviewer #4

Liu, Lou, and Li, et al. reported that HNF4 α can induce the differentiation of HSCs into mature hepatocytes by transient expression of HNF4 α and the phenomenon was inhibited by HNF4 α shRNA. Although the form of the manuscript does not follow the instruction at all, the finding is interesting. Their conclusion of the finding is to provide novel donor cell for cell transplantation. However, it is jumping too high in conclusion.

- (1) First of all, you should add several immunofluorescence studies such as ALB-HNF4 α , CK8-HNF4 α , CK18-HNF α and CEBP α -HNF4 α .

Answer: Although this is a very good suggestion, our experiment used the rat HSC cell line, and we have some primary anti-rat antibodies for PEPCK, G-6-P, Collagen I or TIMP-1 that are limited to use in Western blot, only Nanog antibody can be used in immunofluorescence study. During the experiments, we couldn't get the primary anti-rat antibodies for CK8, CK18 and CEBP α that can be used in immunofluorescence studies. So, we didn't examine the expression of CK8, CK18 and CEBP α . We had examined the expression of CK8 and CK18 by RT-PCR, the results showed that both of them were positive in HSC-T6 cells, and no statistical changes were found after transfection of HNF4 α , therefore, we didn't include the data of CK8 and CK18 expression.

- (2) Second, you should re-running PCR data in Fig.2. Because actin product is small and others are large. So, you could present all data with actin product like multiplex PCR.

- (3) **Answer:** Thanks for this good suggestion. We performed the amplification of these markers by PCR, including many factors such as CD133(370 bp), CD105(369bp), Nestin(450bp), AFP(402bp), α -SMA(292bp), G-6-P(392bp), PEPCK(396bp), etc., the fragments ranges from from 370 to 450 bp, the inner control β -actin is 390bp and closely near the size of some markers, we have to run them simultaneously in different tubes and different bands.

- (4) Third, the conclusion was overstated, so it should be changed. Otherwise, overall writing is interesting if it is true.

Answer: We thank reviewer to give a good suggestion. The conclusion was re-wrote and the whole text was revised thoroughly.

3 References and typesetting were corrected

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

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Peter L. Lakatos', with a stylized, cursive script.

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