ANSWERING REVIEWERS

August 17, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 12334-review.doc).

Title: FoxM1 overexpression correlates with hepatocellular carcinoma metastasis through Epithelial-Mesenchymal Transition

Author: Fan-di Meng, Ji-chao Wei, Kai Qu, Zhi-xin Wang, Qi-fei Wu, Ming-hui Tai, Hao-chen Liu, Rui-yao Zhang, Chang Liu

Name of Journal: *World Journal of Gastroenterology*

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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:

(1) The Western images of Figure 1C is not clear, please provide better pictures.

Answer: Thanks for your kindly suggestion. We have used a clear Western image to replace the previous.

(2) The author did not mention how much time they needed to detect CDH1, FoxM1 and vimentin expression. They also did not mention how much time did the HGF need to induce the typical morphology changes.

Answer: Thanks for your kindly suggestion. In the preliminary experiment, HCC cells were treated with HGF or siRNA for 12, 24 and 48 hours, then we detect the expression changes of CDH1, FoxM1 and Vimentin at different time points by western blotting and qRT-PCR. The results showed that the most significant expression changes were appeared in 48 hours. Therefore, the detection time of the next experiment was 48 hours. Detection of morphological changes is also the same as above. A detailed description of detection time has been added in the legend. Thanks for your kindly suggestion again.

(3) Avoid strong language: example is the title where "may contribute" or "likely contributes" rather then "affected" should be used.

Answer: Thanks for your kindly suggestion. We have used more appropriate term to replace the strong language in the text.

(4) Since EMT does not just induce cell motility and invasion, but also mediate drug-resistant in

cancer cells. Does FoxM1 factor have any drug-resistant effect on the HCC patients or cells?

Answer: Thanks for your kindly suggestion. According to previous studies, there are many chemotherapeutic drugs have drug resistance related to FoxM1, such as docetaxel, epirubicin, paclitaxel, lapatinib, cisplatin and so on. In our previous study, we found that FoxM1 mediates resistance to oxaliplatin in hepatocellular carcinoma via inhibits senescence (Qu K, Xu X, Liu C. Negative regulation of transcription factor FoxM1 by p53 enhances oxaliplatin-induced senescence in hepatocellular carcinoma. Cancer Lett 2013; 331(1): 105-114). Currently, our research group underway FoxM1 and sorafenib drug resistance-related research. Our nitial findings suggested FoxM1 expression is significantly associated with sorafenib-based chemotherapy resistance and poor prognosis in hepatocellular carcinoma patients.

(5) In the Figure 5A, I can not find the why the snai1 should be the most significant changed gene in response to FOXM1 overexpression. The real time PCR data also revealed the Snai2, ZEB1 and Twist1 may also be upregulated after enforced FOXM1 expression.

Answer: Thanks for your kindly suggestion. We observed that the expression of EMT-related molecules was altered under the effect of FoxM1, such as SNAI1, SNAI2, ZEB1 and TWIST1, suggesting that the HCC cells underwent EMT after transfection with FoxM1. In those EMT-related molecules, SNAI1 has a greater magnitude changes compared to other molecules (HepG2: SNAI1 5.610±0.515, SNAI2 3.270±0.599, ZEB1 2.510±0.393, TWIST1 3.930±0.674; HUH-7: SNAI1 5.110±0.556, SNAI2 2.740±0.587, ZEB1 2.331±0.369, TWIST1 4.130±0.973). Here we used some appropriate term to replace the strong language in the results as follows:

We observed that the expression of EMT-related molecules was altered under the effect of FoxM1, and the expression of SNAIL1 has a greater magnitude changes compared to other molecules (Fig. 5A).

Thanks for your kindly suggestion again.

Reviewer 2:

(1) Additional siRNA sequence against Foxm1 should be tested in order to avoid unexpected off-target effects (Figure 4).

Answer: Thanks for your kindly suggestion. According to your suggestions, we supplement an additional siRNA sequence against Foxm1 to avoid unexpected off-target effects in the results as follows:

To further confirm the effect of FoxM1 in HGF-induced EMT, we transfected two sequences of FoxM1-siRNA (1, and 2) or control-siRNA into HepG2 and HUH-7 cells. Western blotting and qRT-PCR showed that the expression of FoxM1 was suppressed by both FoxM1-siRNA #2 and FoxM1-siRNA #1 in the HepG2 and HUH-7 cells (Fig. 4E); thus, mixed siRNA was used in the subsequent experiments.

Thanks for your kindly suggestion again.

(2) Authours craimed that FOXM1-SNAl1 axis is necessary for EMT. However, I am wondering that other factors is associated with this process, since expression analysis displayed that forced over-expression of FOXM1 up-regulated Snal2, Zeb1 and so on. Thus, the dominance of FOXM1-SNAL1 is currently uncertain, even considering that Snal1 silencing down-regulated EMT. SNAL1 must be compared at least with SNAL2 (or perhaps others) in Figure 5, eg. promoter affinity, siRNA-based knockdown experiments.

Answer: Thanks for your kindly suggestion. We observed that the expression of EMT-related molecules was altered under the effect of FoxM1, such as SNAI1, SNAI2, ZEB1 and TWIST1, suggesting that FoxM1-induced EMT may occur through a variety of EMT-related factors. In previous studies, FoxM1 induced EMT may be generated by a variety of pathways, such as ZEB1, ZEB2, Cav-1, uPAR and so on. These pathways have played an important role in the FoxM1-mediated EMT. Our study confirmed SNAI1 also play a crucial role in the EMT-mediated FoxM1, and this effect directly due to

the direct interaction between FoxM1 and SNAI1. The article does not deny the role of other relevant factors in proving SNAI1 effect simultaneously. To further confirm the effect of SNAI1 in FoxM1-mediated EMT, we knockdown of SNAI1 by mixed siRNA when transfected pcDNA3.1-FoxM1 into HCC cells. We found that the FoxM1-mediated EMT was prevented by siRNA knockdown of SNAI1, and this indicates that SNAI played an important role in FoxM1-mediated EMT, even we could not proved this role is decisive. We believe that this does not affect the core idea of this article. Thanks for your kindly suggestion again.

(3) Concentrations of plasmid or siRNA is missing. The authors must mention how to ensure negligible off-target effects in siRNA experiment. In addition, control siRNA should be defined.

Answer: Thanks for your kindly suggestion. In this study, we used 1ug/ml of plasmid and 100 nmol/L siRNA to transfect HCC cells. We have added this content into the legend as follows:

HepG2 and HUH-7 cells were transfected with 1ug/ml of pcDNA3.1 or pcDNA3.1-FoxM1 for 48 h.

The HepG2 and Huh7 cells were transfected with 100 nmol/L of each siRNA for 48 h.

To avoid unexpected off-target effects, we used mixed siRNA to ensure negligible off-target effects in siRNA experiment. We have added this content into the results as follows:

Western blotting and qRT-PCR showed that the expression of FoxM1 was more suppressed by FoxM1-siRNA #2 in the HepG2 and HUH-7 cells (Fig. 4E);thus, mixed siRNA was used in subsequent experiments to avoid unexpected off-target effects.

Western blotting and qRT-PCR showed that the FoxM1-mediated upregulation of SNAI1 was suppressed by both SNAI1-siRNA #1 and SNAI1-siRNA #2 in the HepG2 and HUH-7 cells (Fig. 5D); thus, mixed siRNA was used in subsequent experiments to avoid unexpected off-target effects.

In addition, we have defined control siRNA in the results as follows:

To further confirm the effect of FoxM1 in HGF-induced EMT, we transfected two sequences of FoxM1-siRNA (1, and 2) or control-siRNA (consists of a scrambled sequence that will not lead to the specific degradation of any cellular message) into HepG2 and HUH-7 cells.

Thanks for your kindly suggestion again.

(4) Morphological changes is needed to be defined in results sections (Figure 3C, 4C and 5E). What is 'typical morphology'?

Answer: Thanks for your kindly suggeston. We have defined morphological changes in the results as follows:

HGF clearly mediated both cell scattering and the elongation of the cell shape, and resulted in morphologic changes from tightly packed colonies to scattered growth structure in HepG2 and HUH-7 cell lines, which consistent with mesenchymal morphology (Fig. 3C).

(5) In the present immunobrot experiments, FOXM1 usually detected as double bands (Figure 1C, 4E and 5F). However, in Figure 4A, FOXM1 was detected as single bands even in the same cell lines. Is this correct?

Answer: Thanks for your kindly suggestion. In our laboratory, we use the same FoxM1 antibody sometimes get different bands, this phenomenon only appeared when using FoxM1 antibodies. By changing the experimental conditions, we excluded electrophoresis and protein modification or degradation of these possible causes. Finally, we believe that this phenomenon is due to the FoxM1 polyclonal antibodies. The same results also appear in our previous study (Qu K, Xu X, Liu C, et al. Negative regulation of transcription factor FoxM1 by p53 enhances oxaliplatin-induced senescence in hepatocellular carcinoma. Cancer Lett 2013; 331(1): 105-114. Jie Tao, Xin-Sen Xu, Chang Liu, et al. Down-regulation of FoxM1 inhibits viability and invasion of gallbladder carcinoma cells, partially dependent on inducement of cellular senescence. World J Gastroenterol 2014; 20(28): 9497-9505.)

(6) This manuscript contains many typographical errors; eg. lever instead of level, further more insteed of furthermore.

Answer: Thanks for your kindly suggestion. Our manuscript has been modified in the language

professional English language editing company, which was recommend by WJG and could provide language certificate letter. Those typographical errors have been modified.

(7) Multiple comparisons should be conducted in Figure 1A-1D, 2C-2D, 5B-5D.

Answer: Thanks for your kindly suggestion. We have used multiple comparisons to replace the previous in the results.

(8) Statistical analysis in Figure 1E, Figure 2B should be mentioned.

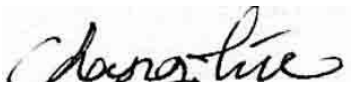
Answer: Thanks for your kindly suggestion. We have added this content into the Materials as follows:

The cumulative recurrence and overall survival rates were performed by the Kaplan–Meier method and the log-rank test.

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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