August 25, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 9313-revised-1st.doc).

Title: Tricistronic hepatitis C virus subgenomic replicon expressing double transgenes

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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. Some of suggestions did not require revision. This is the list of revisions.

(1) The authors fail to demonstrate the real advantage of this model compared to bicistronic models. (Reviewer 01489386)

(2) The aim does not imply the actual aim. (Reviewer 02447059)

(3) Introduction is too long and last paragraph include methodology details. (Reviewer 02447059)

(4) A lot of repetition of methodology details in result section and figure legends. (Reviewer 02447059)

(5) The authors reported a mean and SD for RNA copy. What does this mean represent? (Reviewer 02447059)

(6) alpha for (a). (Reviewer 02447059)

(7) DMEM, please indicate cat # and additives in details. (Reviewer 02447059)

(8) Add space before the unit or %. (Reviewer 02447059)

(9) What was the main advantages of the tricistronic over bicistronic replicon, please lead the reader to this information in the conclusion. (Reviewer 02447059)

3 References and typesetting were corrected

4 The detailed response to Reviewer 01489386
Reviewer's Comment:
I read with interest the manuscript focused on the tricistronic HCV subgenomic replicon with Rbm3 IRES expressing double transgenes. The authors tried to transfect different cell lines and study the replicative potentials and the effect of different antiviral agents. Although this replicon model showed a potential for future drug exploration trials, the authors fail to demonstrate the real advantage of this model compared to bicistronic models. The feasibility of using this replicon system should be accompanied by either efficiency of transfection, stability of the system or improved demonstrative capabilities in comparison to the traditional replicon models that unfortunately it does not seem to be noted in this manuscript. The study however is very well presented. The overall quality of language is good requiring minor revisions.

Response:
Thanks for your comment.

(1) Advantage over bicistronic models.

Sure, in this manuscript, we did not demonstrate by controlled experiments the advantage of the tricistronic model over bicistronic models, especially which use short proteolytic sequences linking a reporter gene and a selectable marker. This kind of bicistronic models forms fusion proteins of reporter and selectable marker. I discussed the structural and functional incompatibility of these fusion proteins in the introduction section. The impaired protein activity and solubility, and the insufficient cleavage efficiency of fusion protein had been well addressed by many articles. Thus we did not compare the tricistronic model and the bicistronic model in terms of protein activity, solubility and cleavage efficiency by controlled experiments.

As far as bicistronic models in which either reporter gene or selectable marker was incorporated are concerned, the advantage of tricistronic model over bicistronic model could be summarized as an unique feature of tricistronic model, by which the selection marker and reporter gene could be simultaneously expressed to construct stable cell lines with high replication of HCV replicon, as well as to ensure sufficient production of reporter proteins.

I have integrated the main advantages of the tricistronic over bicistronic replicon mentioned above into the revised section of introduction and discussion.

(2) The efficiency of transfection in comparison to the traditional replicon models.

The plasmids per se were not transfected into cells. Instead, the RNA, which was cell-free transcribed using these plasmids as templates, were transfected. If the transfection efficiency needed to be determined, the transcribed RNA had to be labeled with FITC or other fluorescence markers. It is not an easy mission, especially when considering the inevitable and uncontrollable degradation of RNA during the process of labeling. Thus, we did not compare the transfection efficiency of our tricistronic plasmids with those of pUC19-HCV, pUC19-HCV-hRLuc and Tri-JFH1.

However, we compared the transgene expression of four kinds of plasmids by hRluc activity assay (Figure 1 C,D,E), which directly determined the feasibility of using replicons to assess antiviral efficacy. As the transgene expression of the four plasmid was predominantly dependent on the replication efficiency of derived replicons and on the translation initiation efficiency of IRES, we considered that it might be unnecessary to present the result of transfection efficiency assay.

(3) Stability of our tricistronic replicon system in comparison to the traditional replicon models.

The replicon derived from our tricistronic plasmids was used to screen for replicon-harboring Huh-7 cells. After screening of 4-8 weeks, cell clones could be harvested and proliferated. hRLuc activity could be detected in these clones with different levels, suggesting this tricistronic HCV replicon could be stably maintained in Huh-7 cells. Thus, we assumed that the stability of the novel tricistronic replicon system was reliable.

Although we did not compare the duration of our tricistronic replicon system in Huh-7 cells to those of the traditional replicon models, we compared the replication of our tricistronic HCV replicon in Huh-7 cells in a transient transfection setting to those derived from pUC19-HCV (parental), pUC19-HCV-hRLuc (bicistronic) and Tri-JFH1 (tricistronic, EMCV IRES) plasmids. We found that the replication of the novel tricistronic HCV replicon in Huh-7 cells was similar to the replication of replicon derived from the parental plasmid and bicistronic plasmid, and was higher than the replication of tricistronic plasmid using EMCV IRES (Figure 1B). Therefore, we were confident about the stability of the newly-formed tricistronic replicon.

(4) The improved demonstrative capabilities in comparison to the traditional replicon models.

We used several types of anti-HCV antivirals to quantitatively test the feasibility of our tricistronic replicon and presented the results in the manuscript (Figure 2, Figure 4). We think the demonstrative capabilities of this novel tricistronic replicon to evaluate the efficacy of antivirals could be confirmed by these observes.

In comparison to the bicistronic replicons with only transgene of antibiotic marker, our tricistronic replicon system could provide a convenient and quantitative solution to evaluate antiviral efficacy, which might be assumed as an improvement of demonstrative capability.

In comparison to the bicistronic replicons with only transgene of fluorescence marker, our tricistronic replicon system could provide a solution to enrich the cells with high replication efficiency of replicon, which might be a better target of antiviral to fulfill their potency of replication inhibition.

As for the tricistronic replicon of Tri-JHF1, we found that the replication of our tricistronic replicon in Huh-7 cells was higher than the replication of Tri-JFH1 replicon (Figure 1B). So did the expression of the inserted hRLuc (Figure 1B,C,D). Thus we think that the demonstrative capability of our replicon might be not less than that of Tri-JFH1 replicon, though we did not compare the demonstrative capabilities of these two replicons in a controlled assay.

5. The detailed response to Reviewer 02447059

Reviewer's Comment:

The authors reporting the tricistronic HCV subgenomic replicon with Rbm3 IRES expressing double transgenes in different cell lines and studying the replicative potentials, suppression of replication by different types of antivirals (DAA and the SOF IFN/RBV. They concluded that the tricistronic replicon had best replicative potentials in four of the tested strains, namely sH7. The inhibitory activity was demonstrated for DAA but not for IFN which, as concluded by the authors, might be attributed to suppressed IFN response pathway. The study is very interesting, well designed. The results are well presented and discussed. Yet, some points of concern were raised during revision. Comments to the authors Abstract: 1- The aim does not imply the actual aim, it is rather a background. Please be specific. Introduction 1- Too long and last paragraph include methodology details. Methods 1- A lot of repition of methodology details in result section and figure legends leading to incoherence Results 1- Page 6, the authors reported a mean and SD for RNA copy. What does this mean represents multiple independent experiments or multiple culture wells in the same experiment. 2- Page 12, line 8, alpha for (a) 3- Page 14, DMEM, please indicate cat # and additives in details (glutamine or not, antibiotics type/conc… etc.) Figures 1- Please indicate significance on the figures and not in the legends 2- In the Y axis title of figures 1B, 2A, 2C, 3B, add space before the unit or % 3- Page 27, figure 3D, NS5B was probed in 4 strains only. What is the explanation for deficient probing from other strains? 4- Page 29, Fig 4, what about cell viability in relation to timing? What was the negative control of the experiment? 5- Page 30, figure 5, was the inability of IFN to inhibit replication in sH7 only? Did you try other cell lines such as Huh-7 for comparison of IFN response? Discussion and conclusion 1- What was the main advantages of the tricistronic over bicistronic replicon, please lead the reader to this information in the conclusion.

Response:

Thanks for your comment.

(1) The aim does not imply the actual aim.

I have rewritten the section of aim in the abstract according to the advice of the reviewer. It is more specific now.

(2) Introduction is too long and last paragraph include methodology details.

I have made the introduction section more concise and deleted the redundant details of methodology in the revised manuscript.

(3) A lot of repetition of methodology details in result section and figure legends.

I have eradicated the repetitive details from the result section and figure legends in the revised manuscript.

(4) The authors reported a mean and SD for RNA copy. What does this mean represent?

The mean and standard deviation for RNA copy represent the average RNA copy numbers of quadruplicated experiments. I have clarified the meanings of all average data in the revised section of results.

(5) alpha for (a).

I have changed all "a" or "α" into "alpha" in the revised edition if needed.

(6) DMEM, please indicate cat # and additives in details.

I have added the information of DMEM in revised section of materials and methods.

(7) Please indicate significance on the figures and not in the legends.

I have added the mark of significance "*" into the revised figures.

(8) Add space before the unit or %.

I have modified the labels of the figures following the reviewer's advice.

(9) Page 27, figure 3D, NS5B was probed in 4 strains only. What is the explanation for deficient probing from

other strains?

Fifty stable cell clones were screened with G418 from Huh-7 cells transfected with our tricistronic HCV replicon RNA. Then, four of the 50 clones with the highest hRLuc expression were selected and probed for NS5B expression evaluation. In these four clones, the hRLuc expression could be confirmed as related to the levels of replicon replication and the levels of NS5B expression (Figure 3 A, B, C, D). In those 46 cell clones other than these four clones, the approximate consistency of the NS5B, RNA level and hRLuc activity could be observed. i.e., in the cell clones with higher expression of hRLuc, the replicon copy numbers and NS5B levels were roughly higher than those in cell clones with lower hRLuc activity. Although the accurate consistency was not determined in our assay in all of the 50 cell clones, the trend could be observed. Thus we only presented the results of hRLuc activity, replicon copy number and NS5B levels in the four selected cell clones with highest hRLuc expression.

(10) Page 29, Fig 4, what about cell viability in relation to timing? What was the negative control of the experiment?

When we cultured the sH7 cells (stably transfected with our tricistronic HCV replicon) with ribavirin, simvastatin, atorvastatin, telaprevir and boceprevir for different durations (Figure 4 B, D, F), we measured the cell viability in parallel plates by CCK-8 procedure. We found that the cell viability of sH7 cells treated with antivirals at each time point was almost the same with the cell viability of untreated sH7 cells as control at each counterpart time point.

Furthermore, the numbers of relative light unit (RLU) of hRLuc at each time point in treated sH7 cells were normalized to the RLU in untreated sH7 cells. So the influence of cell viability was eradicated after the normalization. Thus we did not present the data of cell viability.

As for the negative control of the experiment, we think that the untreated sH7 cells might be considered as the negative control.

(11) Page 30, figure 5, was the inability of IFN to inhibit replication in sH7 only? Did you try other cell lines such as Huh-7 for comparison of IFN response?

We found that the replicon replication could be inhibited by IFN in Huh-7 cells which were transiently transfected with our tricistronic replicon, as well as by other anti-HCV antivirals (Figure 2 A, B).

In Huh-7 derived cell clones which were stably transfected with our tricistronic replicon, such as sH1, sH20, the inhibition of replicon replication in response to IFN treatment was not as significant as the inhibition in response to ribavirin treatment and simvastatin, telaprevir treatment. These data were not shown in the manuscript. In sH7 cells, the absence of inhibition of replicon replication in response to IFN treatment was the most prominent in comparison to other stably transfected cell clones. So we focused on the resistance to IFN in sH7 cells (Figure 5A).

(12) What was the main advantages of the tricistronic over bicistronic replicon, please lead the reader to this information in the conclusion.

Sure, in this manuscript, we did not demonstrate by controlled experiments the advantage of the tricistronic model over bicistronic models, especially which use short proteolytic sequences linking a reporter gene and a selectable marker. This kind of bicistronic models forms fusion proteins of reporter and selectable marker. I discussed the structural and functional incompatibility of these fusion proteins in the introduction section. The impaired protein activity and solubility, and the insufficient cleavage efficiency of fusion protein had been well addressed by many articles. Thus we did not compare the tricistronic model and the bicistronic model in terms of protein activity, solubility and cleavage efficiency by controlled experiments.

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I have integrated the main advantages of the tricistronic over bicistronic replicon mentioned above into the revised section of introduction and discussion.

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

Sincerely yours,

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