AUTHORS' RESPONSES TO THE REVIEWERS' COMMENTS

Manuscript ID: 47161

Ameliorating liver fibrosis in an animal model using the secretome released from miR-122-transfected adipose-derived stem cells

WORLD JOURNAL OF STEM CELLS

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2nd Sep 2019

We thank the reviewers and the associate editor very much for their insightful and valuable comments. We believe that *World journal of stem cells* is appropriate for our manuscript that we intend to be a practical paper that is helpful for future clinical applications. In this document, we quote the reviewers' comments in **bold type**; our replies follow in regular lettering. Moreover, we corrected a few minor improper expressions and grammatical errors that are not specifically mentioned here; we hope that this is acceptable.

Review Date:2019-06-06 14:53

Specific Comments To Authors:Interesting in vitro and small animal study reporting about the potential interest in using transfected ADSC secretome as a modality to inhibit liver fibrosis. Below are point by point comments

Abstract and running title. Do the authors consider a more specific terminology can be used instead of secretome? Conditioned media? Microvesicles? The manuscript seem to remain elusive in this respect failing to inform exactly what the authors have used for their study.

RESPONSE)

Thank you for your valuable comment. The term secretome was first mentioned by Tjalsma et al. and refers to a total sum of released materials from a cell [Microbiol Mol Biol Rev 2000; 64: 607-23]. The components of secretome are largely divided into secretory proteins and extracellular vesicles (EVs). Secretory proteins in humans comprise 13-20% of the total proteasome, and include growth factors, cytokines, chemokines, adhesion molecules, proteases, and shed receptors [Proteomics 2010; 10: 799-827]. EVs are typically 30–2000 nm in diameter and are divided into exosomes, microvesicles, and apoptotic bodies depending on their size [Front Physiol 2012; **3**: 359]. As we have removed the commercial media components as much as possible through a series of processes, including ultrafiltration and filtering, conditioned media is not an appropriate term for our experiment.

In addition, we have consistently used the term secretome in the title of our papers, as follows.

“Lipopolysaccharide Preconditioning of Adipose-Derived Stem Cells Improves Liver-Regenerating Activity of the Secretome.” Stem Cell Research & Therapy 6 (April 14, 2015): 75. https://doi.org/10.1186/s13287-015-0072-7.

“Secretome from Human Adipose-Derived Stem Cells Protects Mouse Liver from Hepatic Ischemia-Reperfusion Injury.” Surgery 157, no. 5 (May 2015): 934–43. https://doi.org/10.1016/j.surg.2014.12.016.

“Determination of Optimized Oxygen Partial Pressure to Maximize the Liver Regenerative Potential of the Secretome Obtained from Adipose-Derived Stem Cells.” Stem Cell Research & Therapy 8, no. 1 (03 2017): 181. https://doi.org/10.1186/s13287-017-0635-x.

“Contribution of Human Adipose Tissue-Derived Stem Cells and the Secretome to the Skin Allograft Survival in Mice.” The Journal of Surgical Research 188, no. 1 (May 1, 2014): 280–89. https://doi.org/10.1016/j.jss.2013.10.063.

Based on this, we would like to politely ask you to allow us to use the term “secretome” in the manuscript title.

Introduction. I would argue the veridicity of the first phrase. I am not convinced stem cell research is the most promising branch of biomedicine (what is biomedicine by the way, do authors mean biomedical research?) Stem cell research might be promising for researchers but from the clinician s and patient perspective it has not delivered so far too much compared with nano and advanced material science which is providing

increasingly performant implants, Aside of couple of approved therapies (one counts on the digits from one hand) the large majority (if we exclude hematopoetic stem cell transplantation) for otherwise untreatable diseases we do not have stem cells in the clinic as of 2019. One of the reason is indeed highlighted by the authors themselves when they try to argument the use of cell free therapies. I don t think they are miRNAs responsible for liver fibrosis rather involved in one way or another in the process.

RESPONSE)

We totally agree with your opinion. As you pointed out, we think that the superlative expression is not appropriate for the stem cell research. We fixed the first sentence as follows: stem cell research is one of the promising areas of biomedical research.

Material and methods. Please revise description of chondrogenetic assays. It is not clear how the cells were cultured (normally a mention about some form of high density culture should be there, if it was not the case please explain)

RESPONSE)

Thank you for your valuable comment. For chondrogenic induction, we performed micromass culture. We clarified the micromass culture in the method section as follows.

“For chondrogenic induction, micromass cultures were plated by seeding 5㎕ droplets of 8×

10^4 cells into the center of 48-well plate (Swioklo et al., 2016 and Voga et al., 2019). After incubating micromass cultures for 2 hours at 37℃, chondrogenic medium (StemPro, GIBCO) was added to 400㎕ per culture wells and cultured for 3 weeks."

Results. In figure A what is the significance of "Mock"? Are they ADSCs transfected with vector only? In this case, a control with non transfected ADSCs should have been added for comparison.

RESPONSE)

We are really sorry to confuse you. In Figure A, mock refers to nontransfected ASCs. To avoid confusion, we modified the description and legend of figure 1.

he subchapter "Determination of the antifibrotic effects of the secretome released from miR-122-transfected ASCs in an in vitro model of liver fibrosis: remains esoteric as there is no description of how this has been performed.

RESPONSE)

Thank you for bringing this discrepancy to our attention. In this subchapter, TAA-treated LX2 cells correspond to the *in vitro* model of liver fibrosis. We think that it appeared to be

difficult because we did not explain it in detail. According to the reviewer's comment, we changed the title of the subchapter as follows; *In vitro* experiments validating the effects of miR-122transfection into ASCs. In addition, we incorporated the definition of in vitro model of liver fibrosis in the paragraph as follows; The *in vitro* model of liver fibrosis was generated by treating human HSCs cells (LX2 cells) with a hepatotoxin (TAA).

On what kind of samples and using what methods. Please resolve this as it is important to understand what kind of secretome the author are referring to. Is it the conditioned media? Have the microvesicles have been extracted or not. This important aspect in the context of this paper should be very clearly described.

RESPONSE)

Thank you for pointing it to us. As answered above, we did not use microvesicles or conditioned media but secretome in this experiment. However, as you pointed out, we found that there was lack of a detailed description of how we attained secretome from the conditioned media wherein ASCs had been cultured. Again, we really appreciate your valuable comment that led us to find the deficit. We incorporated the following paragraph to the method section.

"ASCs with or without miR-122 transfection were grown in a 100 mm cell dishes (Corning Glass Works, Corning, NY). After reaching 70–80% confluence, 1.0×10^6 ASCs were cultured in 5 mL serum-free low-glucose DMEM for 48 h. Therefore, to obtain 0.2mL amount of secretome from 1.0×10^6 ASCs, the conditioned media were concentrated 25-fold

using ultra filtration units with a 3-kDa molecular weight cutoff (Amicon Ultra-PL 3; Millipore, Bedford, MA). We then injected 0.1 mL amount of secretome per mouse. This means that one mouse is injected with the secretome obtained from $1x10^5$ ASCs. In this study, NCM refers to the secretome shed from ASCs after 48 h of incubation, and MCM refers to the secretome shed from miR-122-transfected ASCs after 48 h of incubation."

Same remark about the affirmation "we treated LX2 cells" how were the cells treated (methods, doses, timing) and most of all exactly what were they treated with (conditioned media? MV?)

RESPONSE)

The *in vitro* model of liver fibrosis was generated by treating human HSCs cells (LX2 cells) with 5.0 mM TAA. We then treated the TAA-treated LX2 cells with NCM or MCM for 24 h, and investigated the expression of fibrosis-related markers using western blot analysis. As previously stated, NCM refers to the secretome shed from ASCs after 48 h of incubation, and MCM refers to the secretome shed from miR-122-transfected ASCs after 48 h of incubation. We clarified these findings in the revised manuscript.

For the histological evaluation of rat liver fibrosis how was the collagen content quantitatively determined? Figure 3 and 4 legend inform the graphs below the pictures show the relative density of the markers. How was this assessed quantitatively?

RESPONSE)

Thank you for your good comment. In Sirius red A and Masson's trichrome stains, percentages of fibrotic areas were measured using NIH image J and expressed as relative values to those in normal livers. In the immunohistochemical stains, percentages of immunoreactive areas were measured using NIH image J and expressed as relative values to those in normal livers. The relative densities of individual markers in the western blot analysis had been quantified using Image Lab 3.0 (Bio-Rad) software and then were normalized to that of β-actin in each group. We clarified such things in the revised manuscript.

Discussion chapter is well written. Resulting arguments collected from the study seem to supporting the use of transfected ADSCs and antifibrotic agents with improved potential compared to native ADSCs.

Once again, we thank you for your response and hope we have been thorough in answering your comments. Your comments have aided us immensely in improving our manuscript. We hope our revision is satisfactory to your high standards and we readily await your next feed-back.

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