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Professor Lian-Sheng Ma, Science Editor, Company Editor-in-Chief

Dear Prof. Ma,

Thank you very much for your email communication on Feb. 28, 2021 regarding our manuscript “Hypoxia-Inducible Factor-1 α -Mediated Upregulation of CD99 Promotes the Proliferation of Mesenchymal Stem Cells by Regulating ERK 1/2” (Manuscript NO.: 63664, Basic Study). All changes to the content are indicated in highlight in the revised manuscript.

Round-1

Reviewers' Comments:

Reviewer #1:

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors:

1. In title “Hypoxia-Inducible Factor-1 α -Mediated Upregulation of CD99 Promotes the Proliferation of Mesenchymal Stem Cells by Regulating ERK 1/2” should refer to the stem cells used in the experiments (that is) and not use the generic term Mesenchymal Stem Cells.

Response: Thank you for your suggestion and we have specified the type of stem cells in the title which was highlighted in yellow in revised manuscript.

As follows:

Hypoxia-Inducible Factor-1 α -Mediated Upregulation of CD99 Promotes the Proliferation of Placental Mesenchymal Stem Cells by Regulating ERK 1/2

2. In the Abstract the authors state “Transcriptome profiling of MSCs under hypoxia was performed by RNA-sequencing”. Specify the type of MSC examined.

Response: We have specified the type of MSCs examined which was highlighted in yellow in revised manuscript.

As follows:

Transcriptome profiling of hP-MSCs and human umbilical cord (UC)-derived mesenchymal stem cells under hypoxia was performed by RNA-sequencing.

3. In the abstract the authors indicate only hP-MSCs. hUC-MSC are used only in the paragraph "Hypoxia induces CD99 expression via HIF-1 α " for analyzed their mRNA profile comparing with hP-MSCs profile. There are two possibilities: 1) delete hUC-MSC results from this paragraph and in the materials and methods; 2) do all the experiments performed for hP-MSCs also for hUC-MSCs. It is necessary to make a choice for the validity of the manuscript.

Response: We appreciate your kindly comments. In this study, hUC-MSCs are used as the control to screen and determine the key gene *CD99*. Then, we focused on the role of *CD99* in placenta-derived mesenchymal stem cells (hP-MSCs). In view of not all experiments performed for hP-MSCs also for hUC-MSCs, we have specified the type of MSCs (hP-MSCs) in the full text to avoid confusion.

4. In Material and Methods there is a paragraph named “Isolation and hypoxic culture of MSCs”. Please indicate the type of MSCs isolated.

Response: We agree with the reviewer’s point and have indicated the type of MSCs isolated in revised manuscript marked in yellow.

As follows:

Isolation and hypoxic culture of hP-MSCs and hUC-MSCs

5. Why did the authors use PD98059 as ERK inhibitor? There are other more recent and specific inhibitors. PD98059 is also a reversible inhibitor.

Response: We appreciate the reviewer's suggestions and comments. ERK pathways included Ras-Raf-MEK-Erk. We firstly need an ERK pathway inhibitor to investigate the role of ERK pathway in hypoxia promoting hP-MSCs proliferation. D98059 is a selective and reversible inhibitor of MAPK-activating enzyme, MAPK/ERK kinase (MEK) that inhibits the ERK1/2 phosphorylation. Of course, it is definitely better to use more recent and specific inhibitors. Since the duration of our experiment is not long and the inhibitor is still effective at the end of the experiment, PD98059, as a mitogen activated protein kinase (MAPK/ERK) specific inhibitor, has met the needs of our experiment.

6. Why did the authors evaluate CCNE1, CCNA2, CDK2 and p21 in the paragraph "Hypoxia promotes hP-MSC proliferation by modulating cell-cycle progression"? CCNE1 gene encodes cyclin E which controls G1/S. CCNA2 gene encodes cyclin A2, controls G1/S and G2/M transition. CDK2 gene encodes for Cdk2v acts as G1/S checkpoint control. p21 is a potent inhibitor of cyclin-dependent kinases (CKI) and regulates cell cycle progression at the checkpoint between the G1 and S phases. There is no evaluation of factors that regulate the G2/M phase. Also why did they label proteins with the names of genes? It doesn't seem appropriate.

Response: We appreciate the reviewer's point. We have changed the names of the corresponding genes to those of the protein in the text and pictures which was highlighted in yellow in revised manuscript. We evaluate CCNE1, CCNA2, CDK2 and p21 based on following reasons. Firstly, we observed a drastical increase in S-phase cell population under hypoxia. This phenomenon pushed us to focus on the process of G1-S transition. Secondly, CDK2 is a catalytic subunit of the cyclin-dependent kinase complex, whose activity is maximal during S phase and G2; activated by interaction with cyclin E during the early stages of DNA synthesis to permit G1-S transition, and subsequently activated by cyclin A2 during the late stages of DNA replication to drive the transition from S phase to mitosis, the G2 phase.

Indeed, there is no evaluation of factors that regulate the G2/M phase, which is a shortcoming of this study. Thank you for your valuable comments; we will add this content in subsequent experiments.

7. The authors evaluate the protein level of CD99 by immunostaining. Using this method, only a qualitative and not a quantitative analysis is carried out. To evaluate the protein level it is necessary to carry out Western Blot experiments with subsequent quantization.

Response: We concur with the points raised. The immunostaining result is a qualitative analysis, so we performed Western Blot experiments with subsequent quantization, as shown in revised figure 3E.

8. In figures 4A, 5A, 5C, 6A and 6C a generic p-ERK and ERK cannot be written. It is necessary to specify in the western blots which band corresponds to ERK1 and which to ERK2.

Response: We have specified ERK1 and ERK2 band in the western blots results in revised Figure 4-6.

9. The concentration of PD98059 used in the different experiments is not specified. Please specify.

Response: We have indicated the concentration of PD98059 (Page 10, line 215).

10. In figure 6C, in the presence of the inhibitor, an important residual ERK2 phosphorylation is observed. In my opinion as the inhibition is not total the subsequent results are not reliable.

Response: We are sorry for misleading you because of our unclear statement. In figure 6C, we used CD99-specific siRNA (siCD99#1) instead of inhibitor PD98059 and we found that hypoxia-induced phosphorylation of ERK1 and ERK2 was decreased when CD99-specific siRNA (siCD99#1) was used. In Figure 5D, we can see that 50 μ M PD98059 can effectively inhibit the phosphorylation of ERK1/2 and in

this experiment, the incomplete inhibition of the inhibitor can also meet our experimental needs and support our conclusions.

11. In the paragraph “Hypoxia-induced CD99 regulates hP-MSC proliferation via the MAPK/ERK pathway”, the authors state “Western blotting and immunofluorescence analysis revealed that hypoxia-induced phosphorylation of ERK1 and ERK2 decreased as the expression of CD99 decreased (Fig. 6A, B)”. But this sentence is unclear as in the western Blot 6A siCD99 # 1 is indicated and do not correspond to the text. Rewrite the sentence.

Response: Thank you for your suggestion. This sentence has been rewritten as follows: “Western blotting and immunofluorescence analysis revealed that hypoxia-induced phosphorylation of ERK1 and ERK2 was decreased when CD99-specific siRNA (siCD99 #1) was used”.

12. The authors used a kit to separate Nuclear and Cytoplasmic Proteins. There is the possibility of contamination between the two fractions. It is necessary to demonstrate that the nuclear fraction is only nuclear otherwise the results obtained are not valid.

Response: We concur with the points and made the appropriate changes. The bands of Histone H3 and GAPDH were added to the cytoplasmic and nuclear components, respectively. The results showed that there is almost no contamination between the two fractions. Even if we can vaguely observe bands, we think it will not significantly affect our results.

Reviewer #2:

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: The manuscript entitled as “Hypoxia-Inducible Factor-1 α -Mediated Upregulation of CD99 Promotes the Proliferation of

Mesenchymal Stem Cells by Regulating ERK 1/2" cover an important aspect of stem cells proliferation. The experiments were finely designed and executed with conclusion. However, there are few minor issues which need to be addressed.

1. Language need to be improved.

Response: Thank you for your pertinent comments on our work. We have made appropriate changes following your suggestions and we revised the grammar, layout, etc. of the manuscript. All changes to the content are indicated in yellow in the revised manuscript.

2. For characterization of MSCs, chondrogenic differentiation is missing.

Response: We fully agree that the potential of chondrogenic differentiation is one of the important characteristics of MSCs. The experiment takes nearly 28 days to complete, so please forgive us for not being able to get the pictures of osteogenic differentiation in time. However, we believe that even without chondrogenic differentiation, the quality of our MSCs can also be guaranteed. On one hand, the isolated MSCs showed plastic adherent properties under normal culture conditions and had a fibroblast-like morphology. The cultured MSCs also expressed CD73, CD90 and CD105, while lacking the expression of CD11b, CD34, CD45 and HLA-DR surface markers. On the other hand, these types of MSCs have been reported in our previously studies [1, 2, 3, 4]. Although we are unable to meet your requirements now, we are very willing to adopt your opinion and add chondrogenic differentiation experiments in follow-up studies to make the characteristics of MSCs more complete.

[1] **Cao H**, Yang J, Yu J, Pan Q, Li J, Zhou P, Li Y, Pan X, Li J, Wang Y, Li L*. Therapeutic potential of transplanted placental mesenchymal stem cells in treating Chinese miniature pigs with acute liver failure. BMC Med. 2012 Jun 6;10:56.

[2] Jiong Yu, Guangshu Hao, Dan Wang, Jingqi Liu, Xiaotian Dong, Yanni Sun, Qiaoling Pan, Yang Li, Xiaowei Shi, Lanjuan Li, and **Hongcui Cao***.Therapeutic Effect and Location of GFP-Labeled Placental Mesenchymal Stem Cells on Hepatic

Fibrosis in Rats. Stem Cells International. 2017:1798260,doi:10.1155/2017/1798260

[3] Pan Q, Wang D, Chen D, Sun Y, Feng X, Shi X, Xu Y, Luo X, Yu J, Li Y, **Cao H***, Li L. Characterizing the effects of hypoxia on the metabolic profiles of mesenchymal stromal cells derived from three tissue sources using chemical isotope labeling liquid chromatography-mass spectrometry. Cell Tissue Res. 2020 Apr;380(1):79-91.

[4] Feng X, Liu J, Xu Y, Zhu J, Chen W, Feng B, Pan Q, Yu J, Shi X, Yang J, Li Y, Li L, **Cao H***. Molecular mechanism underlying the difference in proliferation between placenta-derived and umbilical cord-derived mesenchymal stem cells. J Cell Physiol. 2020 Oct;235(10):6779-6793.

3. In figure 1A the western blot results need to be quantified.

Response: We have supplemented the quantitative results of western blot in the form of fold change in revised figure 1A.

4. In figure 1B need to be shown that if the data is significant at day 4 only, on other day if it is non-significant, it need to be mentioned.

Response: Following your suggestion, we have added statistical analysis of the data at day 1, 2, and 3, as shown in revised figure 1B. We found that the data is significant on each day.

5. Scale bar should be added at figure 1C.

Response: We have added scale bars in revised figure 1C.

6. Figure 4 and Figure 5 are exactly same; I think this may be a technical error. Authors need to correct it.

Response: Thank you for carefully examining our data and we have already corrected the Figure 4 and 5 in the revised manuscript.

7. Housekeeping protein need to be added in figure 4 for western blot.

Response: We have supplemented the housekeeping protein in figure 5A (original

figure 4 was changed to figure 5 in revised manuscript) and figure 6A.

Reviewer #3:

Scientific Quality: Grade B (Very good)

Language Quality: Grade A (Priority publishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: Authors have reported on HIF1 α -mediated upregulation of CD99 promotes the proliferation of MSCs by regulating ERK 1/2 pathways. It has a scientific merit and contribute to the understanding on MSC biology.

Response: Thank you very much for the positive comment on our work.

Science editor's Comments:

–Self-cited references: There are 5 self-cited references. The self-referencing rates should be less than 10%. Please keep the reasonable self-citations that are closely related to the topic of the manuscript, and remove other improper self-citations. If the authors fail to address the critical issue of self-citation, the editing process of this manuscript will be terminated.

Response: We removed 2 improper self-cited references in revised manuscript.

–The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s).

Response: We uploaded the approved grant application form(s) to the website.

–The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response: We have uploaded the original figure documents to the website.

–The “Article Highlights” section is missing. Please add the “Article Highlights” section at the end of the main text.

Response: We have added the “Article Highlights” section at the end of the main text in revised manuscript.

Taken together, we feel that your comments and suggestions are very helpful in improving our manuscript, and therefore have made corresponding additions and modifications (marked in yellow in the re-submitted version). Please let us know if you need further clarification. Thank you once again for your professional and timely assistance.

Sincerely,

Hongcui Cao

Round-2

March 17, 2021

Professor Lian-Sheng Ma, Founder and CEO, Baishideng Publishing Group Inc

Dear Prof. Ma,

Thank you very much for your email communication on March 15, 2021 regarding our manuscript “Hypoxia-Inducible Factor-1 α -Mediated Upregulation of CD99 Promotes the Proliferation of Placental Mesenchymal Stem Cells by Regulating ERK 1/2” (Manuscript NO.: 63664, Basic Study). All changes to the content are indicated in highlight in the revised manuscript.

Reviewers' Comments:

Reviewer's code: 03814168

SPECIFIC COMMENTS TO AUTHORS

The revised version of manuscript is improved. All the queries and concerns are successfully addressed in revised version. Therefore, the manuscript is accepted in present form.

Response: Thank you very much for the positive comment on our work.

Reviewer's code: 02567328

SPECIFIC COMMENTS TO AUTHORS

I thank the authors for their exhaustive answers to my questions and for making the required changes. I still have some observation:

1. Answer 3: The authors did not answer my question. But is it necessary to have hUC-MSCs as a control for CD99? If it is not necessary please delete it from the paragraph and from the materials and methods. If, on the other hand, it is necessary to explain why

Response: We appreciate your kindly comments. We have deleted the relevant content of hUC-MSCs in revised manuscript to ensure the validity and logic of manuscript.

2. Answer 8: In this answer the authors state “We have specified ERK1 and ERK2 band in the western blots results in revised Figure 4-6”. But in Figure 4 there is no ERK1/ERK2 western blot. As reported by Reviewer #2, Figure 4 and Figure 5 are exactly same. The authors recognized the mistake and inserted the right figure. In the answer to my question, it would have been appropriate to underline that the corrections concerned fig 5 and 6 as there was an error for figure 4

Response: We are very sorry for our negligence for not underlining the revision we made to Figure 4 when answering your question. The response should be as follows:
We have specified ERK1 and ERK2 band in the western blots results in revised Figure 5-6.

3. Answer 10: Thanks for the answer, but I believe that the observed residue phosphorylation for ERK1 and ERK2, may have its own relevance in the conclusions drawn from the experiment. However this is my opinion and I accept your answer

Response: We concur with the points and we believe that this phenomenon may have a deep significance, which is worthy of further study in follow-up experiments.

Taken together, we feel that your comments and suggestions are very helpful in improving our manuscript, and therefore have made corresponding additions and modifications (marked in yellow in the re-submitted version). Please let us know if you need further clarification. Thank you once again for your professional and timely assistance.

Sincerely,

Hongcui Cao