Dear Editor and Reviewers,

Thank you very much for giving us this opportunity to revise our manuscript entitled "ACE2 improves liver fibrosis in mice by regulating autophagy of hepatic stellate cells" (Manuscript NO:85885, Basic Study). We have revised our manuscript carefully according to the reviewer's comments. Those comments are all valuable and very helpful for improving the quality of our manuscript, as well as the important guiding significance to our researches. We have studied comments carefully and have made extensive corrections to our previous draft. The responses and corrections are listed below.

Responses to review #1:

Response to comment: The present manuscript describes data which strongly suggest that ACE2 plays a crucial role in HSC activation, thus modulating ECM formation, sinusoidal capillarization, and sinusoidal fibrosis. The authors present several lines of evidence: ACE2 overexpression was achieved in an animal model by injection of a viral vector containg the ACE2 gene. Fibrosis was induced by CCl_4. ACE2 overexpression alleviated CCl_4-induced fibrosis, as shown by immunohistochemistry, e.g. Fibronectin and alpha-SMA, reduced serum markers of fibrosis, e.g. PDGF-BB and VEGF. TEM and TUNEL staining demonstrated reduced apoptotic bodies in HSC. The role of the AMPK/mTOR pathway was investigated using the mTOR inhibitor rapamycin. Western blot analysis showed characteristic overexpression or dowregulation of constitutents of the pathway and the effect of rapamycin. Generally, the manuscript presents evidence to prove a hypothesis established earlier about the role of HSCs in development of hepatic fibrosis. In addition, it opens a novel possibility to mitigate or even reverse liver firbrosis or even cirrhosis in the clinical context. ACE2 may a novel target for pharmacological interventions.

Response: Thank you very much for the professional review work on our manuscript. We are very grateful for your recognition of our research work, which gives us great encouragement.

Responses to review #2:

Response to comment: The research paper presents a study investigating the effects of ACE2 overexpression on liver fibrosis and hepatic sinusoidal remodeling using a mouse model induced by CCl_4. The authors explore various aspects, including autophagy, the AMPK/mTOR signaling pathway, HSC activation and apoptosis, intrahepatic angiogenesis, and LSEC capillarization. Overall, the study provides valuable insights into the potential mechanisms underlying the beneficial effects of ACE2 in liver fibrosis.

Response: Thank you very much for the professional review work on our manuscript. Your comments are very helpful to our research. We greatly appreciate your acknowledgment of our research work and have carefully revised the manuscript in response to your comments.

1.Response to comment: The introduction provides a general overview of liver fibrosis and its significance but lacks a clear research objective. Please revise the introduction to state the aim and objectives of the study clearly.

Response: Thank you very much for your helpful comments. Following your comments, we added the study objectives on page 7 of the manuscript.

2.Response to comment: The methods section requires more detailed information. Specify the number of animals used, the specific protocols and techniques employed, and the statistical analyses performed. This will enhance the re-productivity of the study.

Response: Thank you very much for your suggestion. Following your suggestion, we have supplemented the methods section. As in most articles, to keep the methods section concise, we did not specify each step. Detailed instructions are available to the reader via email from the corresponding author.

3.Response to comment: The results section presents findings in a concise manner but lacks interpretation and discussion of the results. Provide a more in-depth analysis and relate the findings back to the research objectives.

Response: Thank you for your valuable comments, and we have added interpretation and discussion in the results section, as detailed on pages 11-14.

4.Response to comment: The discussion section briefly touches on the results but lacks a comprehensive analysis and comparison with existing literature. Include a more extensive discussion of the implications of the findings and their relevance to the field and cite this article https://www.mdpi.com/1999-4915/15/6/1231 Scientific and technical improvements.

Response: Considering your suggestion, we have added the content of discussion of the implications of the findings and their relevance to the field, as detailed on pages 15-18. In addition, we cite this article https://www.mdpi.com/1999-4915/15/6/1231,as seen in Reference [29].

5.Response to comment: Long-term follow-up studies to assess the sustained effects of ACE2 overexpression on liver fibrosis regression and potential side effects.

Response: Thank you for your suggestion, because of time constraints, we will conduct a long-term follow-up study in future studies.

6.Response to comment: Investigation of the interplay between ACE2 and other signaling pathways or molecules involved in liver fibrosis to provide a more comprehensive understanding of the underlying mechanisms.

Response: AMPK/mTOR pathway is an important upstream pathway that regulates autophagy, and our previous studies have shown that this pathway can affect the proliferation of HSCs. In addition, ACE2 has been reported to affect AMPK/mTOR pathway in animal models. Therefore, we hypothesized that ACE2 may regulate autophagy through the AMPK/mTOR pathway to inhibit HSC proliferation and improve liver fibrosis. There are numerous pathways involved in liver fibrosis, and there is no evidence to show whether they regulate autophagy and whether they are affected by ACE2. We selected this pathway based on our previous research results and review of relevant literature.

7.Response to comment: Inclusion of human samples or clinical data to validate the findings in a translational context and increase the relevance to human liver fibrosis.

Response: Thank you very much for your suggestion. Due to time and sample size limitations, human samples and clinical data were not included in this study. Based on this study, we plan to include human samples and clinical trials in the next step of our

research.

8.Response to comment: Exploration of the impact of ACE2 overexpression on liver function, systemic effects, and potential interactions with existing therapies or interventions for liver fibrosis.

Response: Thank you very much for your suggestion. The liver-specific recombinant adeno-associated virus vector rAAV2/8-ACE2 was used in this study. ACE2 could be overexpressed specifically in the liver, with little effect on systemic and liver function. In addition, because this experiment used a mouse model of liver fibrosis, the amount of blood taken was limited, and the serum was used for luminex and Elisa assays. We are so sorry that the remaining blood volume was not enough to test the liver function, and we will test the liver function in the next experiment.

Etiologic therapy is the most important treatment for liver fibrosis and cirrhosis. Up to now, there is no specific and effective anti-hepatic fibrosis drug in clinical practice.

9.Response to comment: Consideration of additional techniques, such as gene expression profiling or proteomics, to provide more detailed insights into the molecular changes associated with ACE2 overexpression in liver fibrosis.

Response: Thank you very much for your suggestion. Due to the high cost of the experiment, gene expression profiling or proteomics techniques were not used in this study. We will consider the application of gene expression profiling or proteomics techniques in our next study.

10.Response to comment: Discuss the potential adverse effects or safety concerns associated with ACE2 overexpression. This is particularly important if considering the clinical translation of ACE2-based therapies.

Response: Adeno-associated virus (AAV) is a naturally defective virus, which has not been found to be associated with any human disease, and has a high level of biosafety. The liver-specific recombinant adeno-associated viral vector rAAV2/8-ACE2 was used in this study. ACE2 is specifically overexpressed in the liver, with minimal systemic effects. Moreover, enhanced expression and activity of liver tissue specific ACE2 can reduce local Ang II levels, increase local Ang- (1-7) levels, and minimize

off-target effect. ACE2 gene therapy provides a therapeutic strategy that transfers the balance between the two arms of the RAS towards the protective pathway. We have added the discussion section, as detailed on page 15-16.

11.Response to comment: Statistical Analysis: Specify the statistical tests used and provide appropriate p-values for all comparisons made in the results section. This information is crucial for assessing the significance of the findings.

Response: Thank you for your valuable comment. We have made modifications according to your suggestion, specifying statistical tests in the statistical analysis section and adding p-values in the results section.

12.Response to comment: Strengths and Limitations: Emphasize the strengths of the study, such as the comprehensive exploration of various aspects of liver fibrosis and the use of multiple techniques to support the findings.

Response: Thank you for your valuable comment. We have added an exposition of the strengths of this study in the discussion section, as detailed on pages 18.

13.Response to comment: Clearly outline the limitations of the study, including the use of a mouse model, the need for further validation in human studies, and potential unexplored factors or mechanisms.

Response: Thank you very much for your suggestion. We have added an exposition of the limitations of this study in the discussion section, as detailed on pages 18.

14.Response to comment: Grammar and Language: The manuscript contains several grammar and punctuation errors throughout. Carefully proofread the entire paper to rectify these errors.

Response: Thank you for your careful reading of this manuscript. We have carefully checked the manuscript and rectified grammatical and punctuation errors. In addition, we have sent the revised manuscript to a professional English language editing company and completed further language polishing.

15.Response to comment: The writing style is somewhat convoluted in certain sections. Simplify the language to improve readability and comprehension for readers.

Response: Thank you for your helpful suggestion. We have revised the manuscript in detail with your suggestions.

Responses to Editorial Office's: Thank you very much for your suggestion and comments. Following suggestions and comments from the editorial department, we revised the manuscript and performed further language polishing. We have provided decomposable Figures in the PowerPoint file that are moveable and editable when opened with Photoshop software. We uploaded the revised manuscript and related materials on the submission system.

Thank you again for your helpful comments and valuable suggestions. We have tried our best to improve the manuscript. If any other modifications are still required, we would like very much to correct them. We appreciate for your warm work earnestly, and hope that the revised manuscript could be considered for publication in your journal.

Sincerely,

Ying Wu

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