RE: Manuscript NO.:81791

Title: Bone marrow mesenchymal stem cell-derived exosomal miRNAs target PI3K/Akt signaling pathway to potentially promote tendon-bone healing

JOURNAL EDITOR-IN-CHIEF (ASSOCIATE EDITOR) COMMENTS

Comment: The manuscript claimed, "MSC-derived exosomes promote fibroblast activation, possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing," solely based on the NIH3T3 cell model. The caveat was "Tendon is a relatively simple tissue, with one predominant cell type—fibroblasts, which in tendon are called tenocytes and embedded in an insoluble matrix of elongated collagen fibrils that are surrounded by a soluble compartment of glycoproteins including proteoglycans." Thus, tendon-bone-associated fibroblasts differ from the NIH3T3, with a modal chromosome number of 68 (Specific comment #15 below). Second, they did not specifically isolate MSCs-exomes on tenocytes, thus providing circumstantial data to claim the point. The authors need to address the following specific comments to improve clarity by thoroughly defining the limitations of the current version of the manuscript.

Reply: Thanks so much for the insightful comment the from editor-in-chief. We agree that "tendon-bone-associated fibroblasts differ from the NIH3T3". This study used bioinformatics analysis to obtain the miRNA data from three GSE datasets, all of which were derived from bone marrow MSC-exosomes. Then we used the NIH3T3 fibroblasts as an in vitro model to examine the effect of miRNAs. Thus, this study only provided evidence that exosomal miRNAs can activate fibroblasts. Our previous research demonstrated that conditioned medium of BMSCs can promote tendon-bone healing[1], and this study may suggest some possible mechanisms for that. In order to improve the clarity of this manuscript, we change the title to "Bone marrow mesenchymal stem cell-derived exosomal miRNAs target PI3K/Akt signaling pathway to promote the activation of fibroblasts". Following are responses to specific comments.

Specific comments:

1) The title overstated their data (refer to the above comment).

Reply: In order to improve the clarity of this manuscript, we have now changed the title to "Bone marrow mesenchymal stem cell-derived exosomal miRNAs target PI3K/Akt signaling pathway to promote the activation of fibroblasts".

2) Abstract: "the underlying mechanism is not comprehensive understood." Use [comprehensively].

Reply: We apologize for the error. The word "comprehensive" has been corrected to "comprehensively" (Line 6, Page 3).

3) Abstract: "Herein, this study aimed to identify the exosomal miRNAs universally carried by MSC-derived exosomes, and to verify their effects as well as mechanisms on fibroblasts." How did they define "universally?" As in the current version, neither its theory nor the experimental data justified such a term.

Reply: We apologize for the unclear description. We screened out the top 100 BMSC-derived exosomal miRNAs from three GSE datasets, and then obtained overlapped miRNAs through intersection. We have now changed the sentence to "Herein, this study aimed to identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets, and to verify their effects as well as mechanisms on fibroblasts" (Line 7 and 11, Page 3). We have also removed all terms such as "universally" from the manuscript.

4) "AIM: To identify the exosomal miRNAs universally carried by MSC-derived exosomes" – How did they define MSC-derived? MSCs are diversified in different contexts – what is their specific context? Refer to "doi: 10.1089/hum.2010.115 Bone marrow mesenchymal stem cells: historical overview and concepts. Please note that "In an attempt to standardize the

definition of an MSC, the International Society for Cellular Therapy (ISCT) proposed the concept of essential minimal criteria for MSCs in culture. The four minimal defining criteria for MSCs are: i) adherence to plastic under standard tissue culture conditions ii) expression of CD105, CD73, CD90 iii) lack of expression of CD45, CD34, CD14/CD11b, CD79/CD19 and HLA-DR surface markers and iv) differentiation into adipocytes, osteoblasts and chondroblasts in vitro [Dominici M, et al. Cytotherapy 8: 315-317, 2006]. Nevertheless, there is no consensus regarding the MSC phenotype, because of the broad variety of potential tissue sources and the differences in cell isolation and cell culture procedures used. In addition, differences in media formulations (FBS, platelet lysates, growth factor combinations), plating density and oxygen tension may affect the gene profile, epigenomic state and phenotype of the mesenchymal population" –[Roobrouck VD et al. Stem Cells 4: 583-589, 2011]. Along with these lines, what QC guidelines did they use in their MSCs?

Reply: We apologize for the unclear description of MSC. The term "MSCs" in this manuscript refers to bone marrow mesenchymal stem cells (BMSCs). The exosomal miRNAs data we obtained from the three GSE datasets (GSE71241, GSE153752, and GSE85341) were all derived from BMSCs that have been shown to meet the criteria of ISCT[2]. We have now changed all "MSC" to "BMSC" in the manuscript.

5) Page 5: "Recent evidence indicates that conditioned medium, primarily contains [containing] exosomes of MSCs, can stimulate the activation of fibroblasts, thereby promoting tendon-bone healing[9-12]."

Reply: We apologize for the error. The word "contains" has been corrected to "containing" (Line 80, Page 5).

6) Page 6: "Three MSC-derived exosomal miRNA expression microarray datasets (GSE71241, GSE153752, and GSE85341) were retrieved from the GEO repository (https://www.ncbi.nlm.nih.gov/geo)." The day of access should be

provided.

Reply: Thank you for the comment. We have now added the day of access (Line 102, Page 6).

7) Figure 2. All the species of miRNAs should be supplemented in Excel files.

Reply: Thank you for the comment. We have now supplemented all the species of miRNAs in Excel files named "81791-All miRNAs".

8) Figures 3, 4, 5. All the species of genes should be supplemented in Excel files.

Reply: Thank you for the comment. We have now supplemented all the species of genes in Excel files named "81791-All genes".

9) Figure 6D, E, F, G. How did they titrate "The relative luciferase activity of WT-PTEN and MUT-PTEN in NIH3T3 cells co-transfected with miR-23b-3p mimics and pmirGLO-PTEN-3'UTR vector" to avoid squelching effects?

Reply: We apologize for the unclear description. NIH3T3 cells were co-transfected with luciferase vectors containing wild-type or mutant 3'-UTR of PTEN, and miR-23b-3p mimics or their control groups. Luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) after 48h transfection. The relative luciferase activities were determined by calculating the ratio of firefly luciferase activities over Renilla luciferase activities. We have conducted the Dual Luciferase reporting assay in a previous study[3], and the squelching effects did not occur, which is unlikely to affect the experimental results.

10) Fig7A, B: scale bars are needed.

Reply: Thank you for the comment. We have now added the scale bars.

11) Fig 7C. the scale bars seemed way off the number as marked.

Reply: Thank you for the comment. We have now adjusted the distance

between the scale bars and the number.

12) Fig 8. scale bars are needed.

Reply: Thank you for the comment. We have now added the scale bars.

13) Fig 7. “fibrosis of NIH3T3 fibroblasts” – How did they determine the fibrosis?

Reply: We apologize for the incorrect description. Collagen type I (Col 1) is a representative protein produced by myofibroblasts that form the extracellular matrix [Papaioannou I et al. Mol Biol Cell. 2018;29(2):84-95]. Alpha-smooth muscle actin (α-SMA) is currently considered a marker for myofibroblasts [Hand GC et al. J Bone Joint Surg Br. 2007;89(7):928-932]. The up-regulation of Col 1 and α-SMA indicated fibroblast-myofibroblast differentiation and collagen synthesis. We have now corrected “fibrosis” to “collagen synthesis” (Line 262, Page 12).

14) Fig 7. “COL I and α-smooth muscle actin positive expression.” How did they specify muscle actin?

Reply: Thank you for the comment. In healing tissues, fibroblasts acquire a contractile phenotype, characterized by formation of microfilament bundles and expression of alpha-smooth muscle actin (α-SMA), the actin isoform typical of vascular smooth muscle cells[4]. Increased α-SMA expression is sufficient to enhance fibroblast contractile activity[5]. Thus, we determined the collagen synthesis capacity of fibroblasts by detecting the expression of α-SMA and Col 1.

15) Fig 9. “Figure 9 Inhibition of phosphatase and tensin homolog promoted fibroblastic, tenogenic, and chondrogenic potential of NIH3T3 cells” How did they alternatively verify these claims? Please note that “What does NIH3T3 stand for? They were obtained from desegregated NIH Swiss mouse embryo

fibroblasts by George Todaro and Howard Green. 3T3 stands for “3-day transfer, inoculum 3×105 cells” and is derived from the original cell transfer and inoculation protocol.” The spontaneously immortalized cells with stable growth rate were established after 20 to 30 generations in culture, and then named '3T3' cells. Since then, several cell lines have been established with this procotol:[3] 3T3-Swiss albino, the original 1962 cell line 3T3-J2, a subclone of 3T3-Swiss albino, commonly used as feeders for keratinocyte cultures[4] 3T3-Y, a subclone of 3T3-Swiss albino, used as a model of adipogenesis[5] NIH-3T3, also from Swiss albino mice BALB/c-3T3 clone 1, from BALB/c mice. “ “Cytogenetics 3T3 mouse cells are hypertriploid. The modal chromosome number is 68, which occurs in 30% of cells. Higher ploidies occur at a much lower rate of 2.4%.” Ideally, they must follow the model by Taylor, S.E., Vaughan-Thomas, A., Clements, D.N. et al. Gene expression markers of tendon fibroblasts in normal and diseased tissue compared to monolayer and three dimensional culture systems. BMC Musculoskelet Disord 10, 27 (2009). https://doi.org/10.1186/1471-2474-10-27. These experiments could be shown in Wang X. T., Liu P. Y., Xin K.-Q., Tang J. B. (2005). Tendon Healing In Vitro: bFGF Gene Transfer to Tenocytes by Adeno-Associated Viral Vectors Promotes Expression of Collagen Genes. J. Hand Surg. 30 (6), 1255–1261. doi:10.1016/j.jhsa.2005.06.001.

Reply: Thank you for the insightful comment. We apologize for the incorrect description. Normal tendon-bone insertion has a transitional structure consisting of four graduated layers including bone tissue, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon tissue[6], which suggests that fibrogenesis, tenogenesis and chondrogenesis are key characteristics of tendon-bone healing. In this study, we found that inhibition of PTEN changes the gene expression of fibroblastic-related factors (α-SMA and vimentin)[7], tenogenic-related factors (TNMD and Col I)[8] and chondrogenic-related factors (Col II and Sox-9)[9] in NIH3T3 cells. Thus, we have now corrected “Figure 9 Inhibition of phosphatase and tensin homolog

promoted fibroblastic, tenogenic, and chondrogenic potential of NIH3T3 cells" to "Figure 9 Inhibition of phosphatase and tensin homolog change the gene expression of fibroblastic-, tenogenic-, and chondrogenic-related factors in NIH3T3 cells".

16) Page 14: "Normal TBI has a transitional structure consisting of four gradated [? graduated] layers, including bone tissue, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer, and tendon tissue[27],"

Reply: Thank you for the comment. The word "gradated" has been changed to "graduated" (Line 318, Page 14).

References

[1] Sun Y, Chen W, Hao Y, et al. Stem Cell-Conditioned Medium Promotes Graft Remodeling of Midsubstance and Intratunnel Incorporation After Anterior Cruciate Ligament Reconstruction in a Rat Model [published correction appears in Am J Sports Med. 2021 Feb;49(2):NP16]. Am J Sports Med. 2019;47(10):2327-2337. doi:10.1177/0363546519859324

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Cell. 2001;12(9):2730-2741. doi:10.1091/mbc.12.9.2730

[6] Wu Y, Dong Y, Jiang J, Li H, Zhu T, Chen S. Evaluation of the Bone-ligament and tendon insertions based on Raman spectrum and its PCA and CLS analysis [published correction appears in Sci Rep. 2017 Mar 14;7:43974]. Sci Rep. 2017;7:38706. Published 2017 Jan 31. doi:10.1038/srep38706

[7] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. Am J Pathol. 2007;170(6):1807-1816. doi:10.2353/ajpath.2007.070112

[8] Younesi Soltani F, Javanshir S, Dowlati G, Parham A, Naderi-Meshkin H. Differentiation of human adipose-derived mesenchymal stem cells toward tenocyte by platelet-derived growth factor-BB and growth differentiation factor-6. Cell Tissue Bank. 2022;23(2):237-246. doi:10.1007/s10561-021-09935-7

[9] Chu Y, Huang L, Hao W, et al. Long-term stability, high strength, and 3D printable alginate hydrogel for cartilage tissue engineering application. Biomed Mater. 2021;16(6):10.1088/1748-605X/ac2595. Published 2021 Sep 28. doi:10.1088/1748-605X/ac2595

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Title: Bone marrow mesenchymal stem cell-derived exosomal miRNAs target PI3K/Akt signaling pathway to potentially promote tendon-bone healing

EDITORIAL OFFICE'S COMMENTS

(1) Science editor: The manuscript has been peer-reviewed, and it' s ready for the first decision. Language Quality: Grade B (Minor language polishing) Scientific Quality: Grade D (Fair)

Response to science editor: Thank you for your comments concerning our manuscript.

(2) Company editor-in-chief: I have reviewed the Peer-Review Report and the full text of the manuscript, all of which have met the basic publishing requirements of the World Journal of Stem Cells, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. In vivo studies must be added as suggested by the reviewer upon revision. The quality of the English language of the manuscript does not meet the requirements of the journal. Before final acceptance, the author(s) must provide the English Language Certificate issued by a professional English language editing company. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240. Before final acceptance, when revising the manuscript, the author must supplement and improve the highlights of the latest cutting-edge research results, thereby further improving the content of the manuscript. To this end, authors are advised to apply a new tool, the Reference Citation Analysis (RCA). RCA is an artificial intelligence technology-based open multidisciplinary citation analysis database. In it, upon obtaining search results from the keywords entered by the author, "Impact Index Per Article" under "Ranked by"

should be selected to find the latest highlight articles, which can then be used to further improve an article under preparation/peer-review/revision. Please visit our RCA database for more information at: https://www.referencecitationanalysis.com/. Uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Response to company editor-in-chief: Thank you for your letter and the reviewers' comments concerning our manuscript. These comments have been valuable in ensuring we produce a high-quality manuscript and have also been informative in guiding our research. We have reviewed the comments carefully and have made corrections with the hope that they meet the reviewers' standards. We have answered the reviewer's comments carefully and step by step as follows. We have answered the reviewer's comments carefully and step by step as follows. Revised portions are marked in **blue** throughout the paper, and changes to the manuscript are also highlighted with **red** text.

We understand that in vivo studies may better reveal the effect of exosomal miRNAs. However, in the present study, we mainly focused on identifying the exosomal miRNAs universally carried by exosomes derived from MSCs, and we think that bioinformatic analysis as well as in vitro studies may not be optimal, but should be sufficient to draw a conclusion that PTEN and PI3K/Akt signaling pathway may serve as potential targets to promote the activation of fibroblasts. Our previous studies (Sun Y, et al. Am J Sports Med. 2019;47(10):2327-2337.) have shown that activation of fibroblasts can promote tendon-bone healing. Our group is currently working on another paper

focusing on the in vivo studies about the effect of exosomal miRNAs. We hope to incorporate the in vivo part in this upcoming paper. We have also re-sent our article to a professional English language editing service again to improve its readability. We have also provided decomposable Figures (in which all components are movable and editable), and organized them into a single PowerPoint file. We appreciate the reviewers' and editor's careful review of our work and thank you for your comments and suggestions.

Reviewer #1:

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

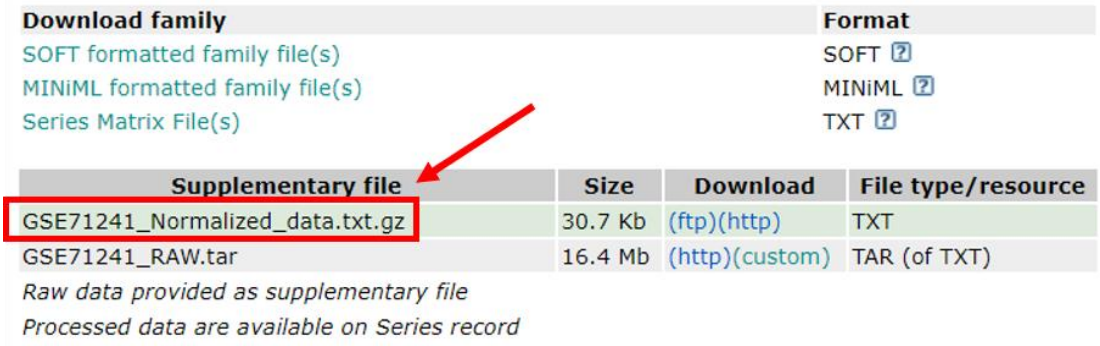
Conclusion: Minor revision

Specific Comments to Authors: This manuscript of review is acceptable after a few minor revisions. Li and co-workers are exploring the role of MSCs-derived exosomal miRNAs in tendon-bone healing. This is a traditional and quiet simple article. It is need to add some content before considering whether to accept. 1. In result "Screening and identification of candidate exosomal miRNAs and target genes", authors need to show the GSE calibration to reflect the completeness and correctness of the data. 2. Pictures of biometrics analysis concluding GO analysis and KEGG analysis are quiet simple and rough which are often shown in earlier years. Authors needs to further optimize pictures of biometrics analysis. 3. In result "Construction of target gene-PPI and miRNA-hub gene networks", Authors need to describe in detail how to get the hub gene, because there are many setting conditions in the STRING database. In sum, this is an acceptable manuscript of review paper. It is organized well with evident collections of previous work but need further improve relevant data.

Response to reviewer 1: Thank you very much for your positive feedback and meaningful suggestions for our manuscript, below are the point-by-point responses to each of your comments.

Q1. In result "Screening and identification of candidate exosomal miRNAs and target genes", authors need to show the GSE calibration to reflect the completeness and correctness of the data.

Response 1: Thank you for the comment. We apologize for the unclear description of the GSE data. GSE data (GSE71241, GSE153752, and GSE85341) we downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo) are all normalized data. The red arrow in the image below is the file we downloaded.



Download family			Format
SOFT formatted family file(s)			SOFT
MINiML formatted family file(s)			MINiML
Series Matrix File(s)			TXT

Supplementary file	Size	Download	File type/resource
GSE71241_Normalized_data.txt.gz	30.7 Kb	(ftp)(http)	TXT
GSE71241_RAW.tar	16.4 Mb	(http)(custom)	TAR (of TXT)

Raw data provided as supplementary file

Processed data are available on Series record

Q2. Pictures of biometrics analysis concluding GO analysis and KEGG analysis are quiet simple and rough which are often shown in earlier years. Authors needs to further optimize pictures of biometrics analysis.

Response 2: Per the reviewer's request, we have now optimized the pictures of biometrics analysis. We have also added the new Figure 3, 4, 5 here for your perusal.

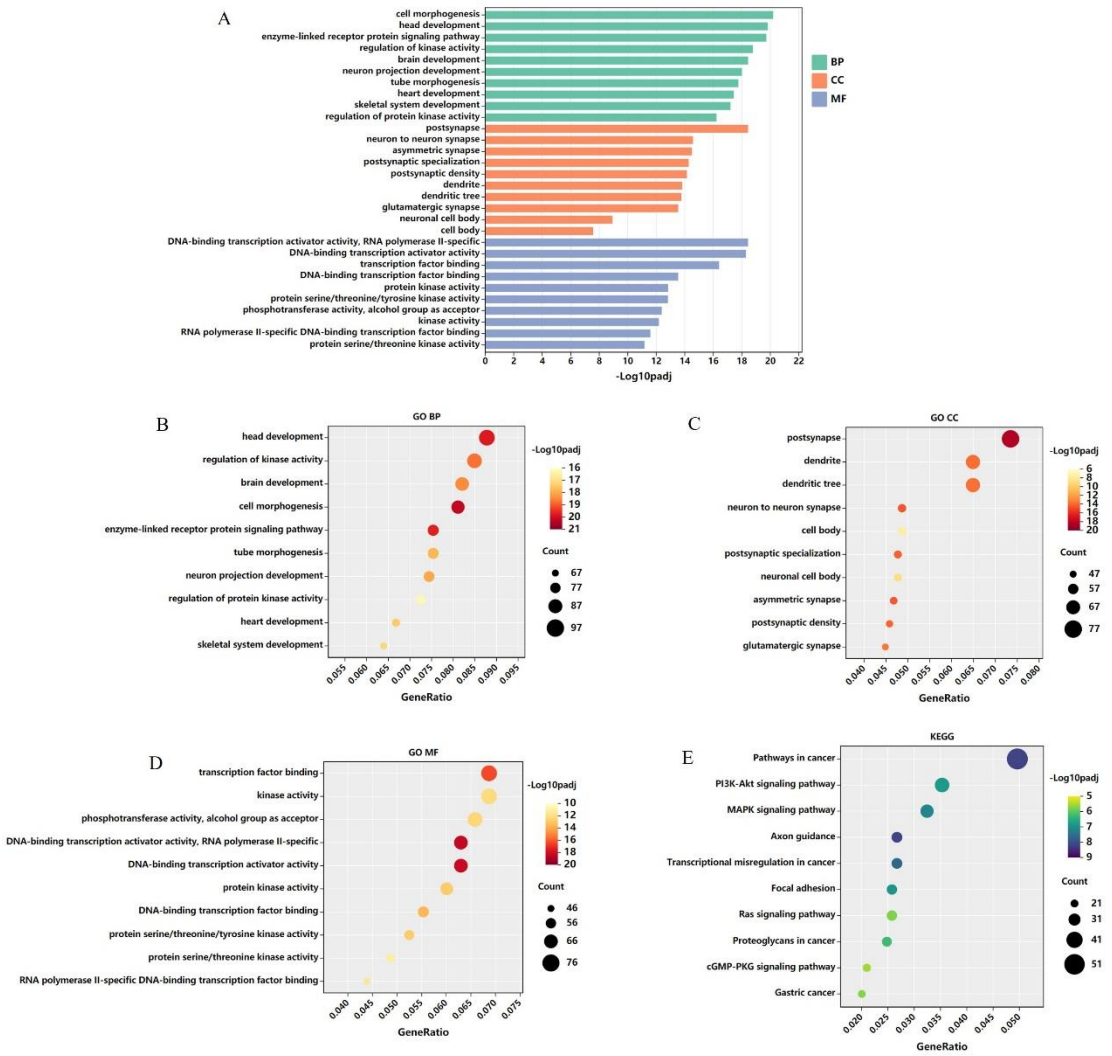


Figure 3 GO and KEGG analysis of genes targeted by hsa-miR-144-3p. (A–D) Top 10 GO biological process (BP), cellular component (CC), and molecular function (MF) terms enriched in target genes of hsa-miR-144-3p. (E) Top 10 KEGG pathways enriched in target genes of hsa-miR-144-3p.

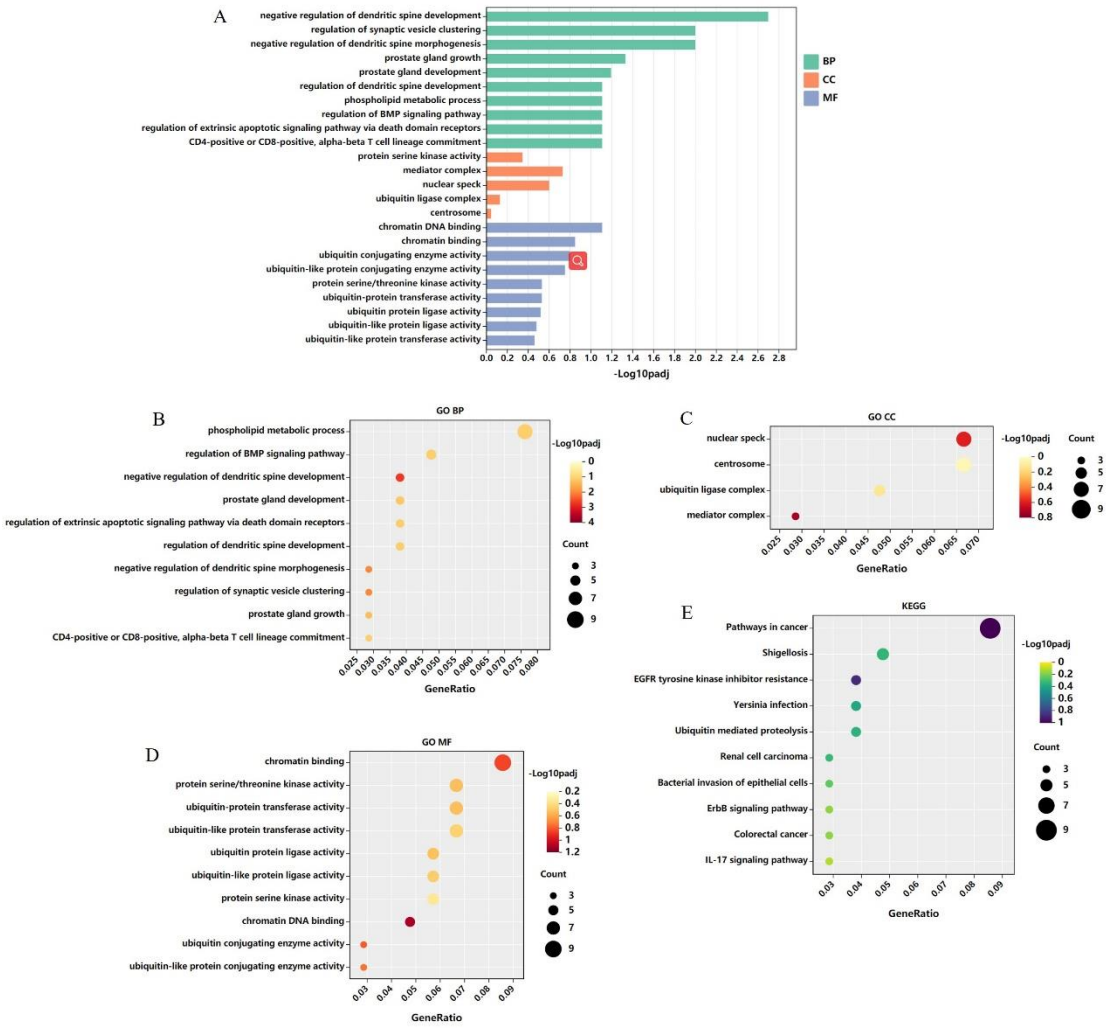


Figure 4 GO and KEGG analysis of genes targeted by hsa-miR-23b-3p. (A–D) Top 10 GO biological process (BP), cellular component (CC), and molecular function (MF) terms enriched in target genes of hsa-miR-23b-3p. (E) Top 10 KEGG pathways enriched in target genes of hsa-miR-23b-3p.

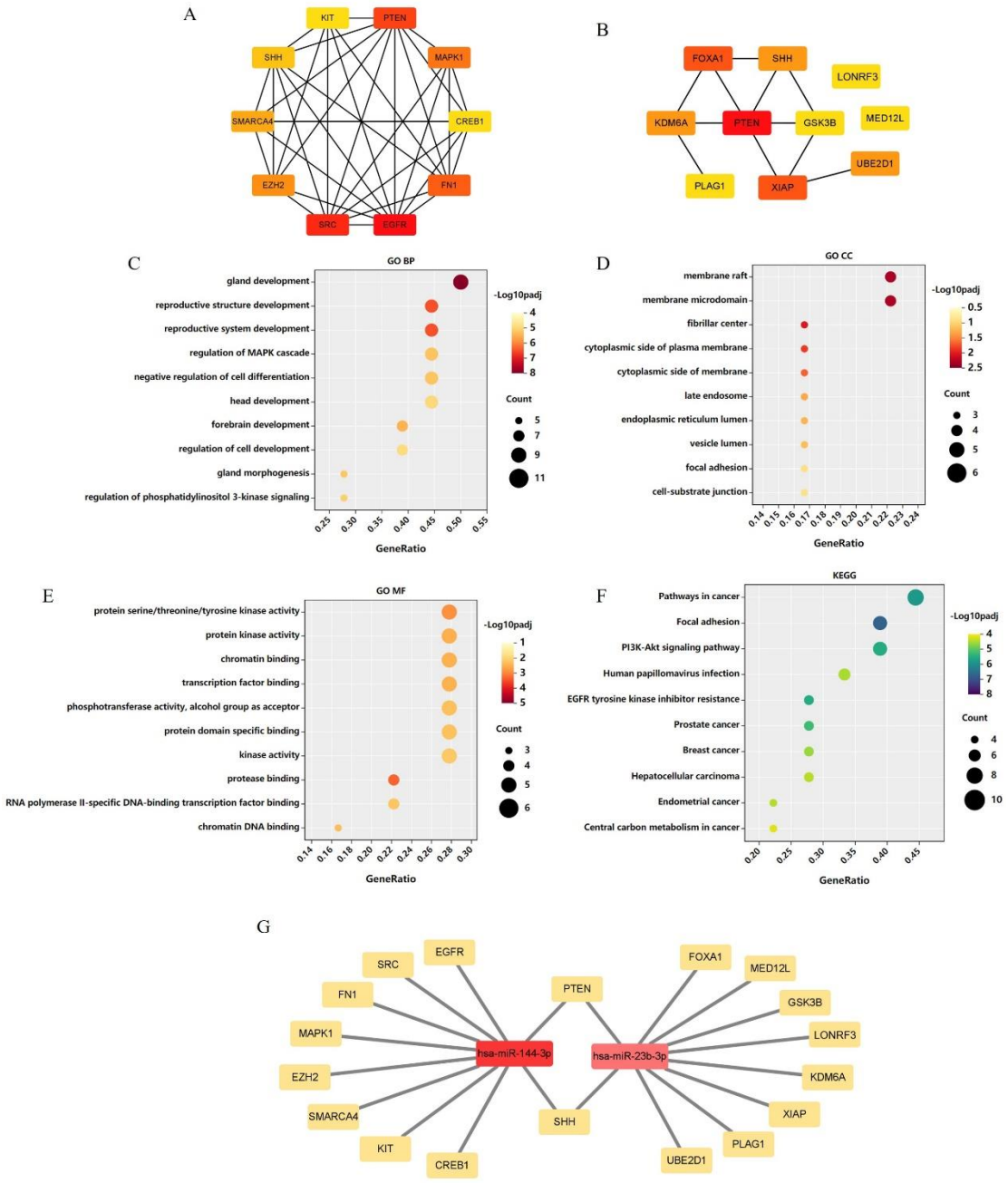


Figure 5 GO and KEGG analysis of hub-gene targets. (A) Top 10 hub-gene targets for hsa-miR-144-3p. (B) Top 10 hub-gene targets for hsa-miR-23b-3p. (C–E) Top 10 GO BP, CC, and MF terms enriched in the top 20 hub-gene targets. (F) Top 10 KEGG pathways enriched in the 20 hub-gene targets. (G) Interaction network of the two miRNAs and their hub-gene targets.

Q3. In result "Construction of target gene-PPI and miRNA-hub gene networks", Authors need to describe in detail how to get the hub gene, because there are many setting conditions in the STRING database.

Response 3: Thank you for your comment. We uploaded target gene data to the STRING database. Interactions with a combined score > 0.4 were considered significant. A PPI network was constructed using the STRING database. The network was further processed with Cytoscape software (version 3.9.1) to visualize the interaction among genes more intuitively. Then, the cytoHubba plugin in Cytoscape was used to identify the top 10 hub genes using the Degree algorithm. We have now added this description to the results.

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: The manuscript entitled "A comprehensive analysis of bone marrow mesenchymal stem cells-derived exosomal miRNAs reveals PI3K/Akt signaling pathway as a potential target to promote tendon-bone healing" by Fangqi Li, et al. use bioinformatis analyzing found two most important miRNAs which may lead to tendon-bone healing promotion effect by mesenchymal stem cells-derived exosome. They are miR-144 and miR-23b. Furthermore, the two exosomal miRNAs can promote fibroblast cell lines proliferation, migration and so on. It may through PTEN-AKT pathway. However, they did not do in vivo test. The in vivo effect of two exosomal miRNAs should be verified in animal models.

Response to reviewer 2: Thank you for your kind comments. We understand that in vivo studies may better reveal the effect of two exosomal miRNAs. However, in the present study, we mainly focused on identifying the exosomal miRNAs universally carried by exosomes derived from MSCs, and we think that bioinformatic analysis as well as in vitro studies may not be optimal, but

should be sufficient to draw a conclusion that PTEN and PI3K/Akt signaling pathway may serve as potential targets to promote the activation of fibroblasts. Our previous in vivo studies (Sun Y, et al. Am J Sports Med. 2019;47(10):2327-2337.) have shown that activation of fibroblasts can promote tendon-bone healing. Our group is currently working on another paper focusing on the in vivo studies about the effect of exosomal miRNAs. We hope to incorporate this part in this upcoming paper.