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June 24, 2022

Professor Lian-Sheng Ma, Editorial Office Director, Company Editor-in-Chief

Dear Prof. Ma,

Thank you very much for your email communication on June 11, 2022 regarding our manuscript "LncRNA SNHG16 promotes human placenta-derived mesenchymal stem cell proliferation capacity through the PI3K/AKT pathway under hypoxia" (Manuscript NO.: 77481, Basic Study). We are grateful for both you and the reviewers' very pertinent and constructive comments and suggestions, and would like to specifically address the points raised by them as follows:

Reviewers' Comments:

Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: In this manuscript, the authors have reported LncRNA SNHG16 promotes human placental-derived mesenchymal stem cells proliferation capacity through PI3K/AKT pathway under hypoxia. This manuscript suffers from several drawbacks in technical execution and data presentation, and, in my opinion, this manuscript in its current shape needs major revision. The main issues are as follows:

1. The manuscript must be carefully proofread for grammar, spelling, and punctuation issues.

Response: Thanks for the reviewer's comments. We have revised the grammar, layout,

etc. of the manuscript. The English in this document has also been checked by at least two professional editors, both native speakers of English (http://www.textcheck.com/certificate/MOdUfz). All changes to the contents are indicated in red in the revised manuscript.

2. The statements of this paper need improvement. The discussion part is not well written and needs a major rewrite. There is no balance between the different sections of the manuscript. The content of the introduction and results were too long.

Response: We fully agree with this suggestion and have made appropriate changes to Introduction, Results and Discussion sections. Modifications have been marked in red in the revised manuscript.

3. For P-value, please write the exact value.

Response: Following the reviewer's suggestion, we have added the exact P-value in Results section of the revised manuscript (marked in red). In addition, we have shown the P-value in the revised figures with the corresponding letters according to the publication requirements of the magazine, such as $^{a}P < 0.05$ and $^{b}P < 0.01$.

4. The method section was not well described and needed to be rewritten in more detail, such as the method of cell culture, cell transfection, etc.

Response: We thank the reviewer for pointing out this issue. Some experimental procedures have been well described in our previously published articles. To avoid repetition, we described some experimental methods in detail in the added supplementary material file.

We also show them as follows:

Cell culture

hP-MSCs were cultured in specialized medium (MesenCult® Human Basal Medium plus MesenCult® Human Supplement; STEMCELL Technologies Inc., Vancouver, Canada) in 25 cm^2 cell culture flasks (Nunc™ EasYFlask™; Thermo Fisher Scientific Inc., Waltham, MA). During the continuous culture period, the culture medium was

replaced every 3 days, and the cells were trypsinized and passaged upon reaching 70–80% confluence. The cells were stabilized in a standard humidified incubator (HERAcell150, Thermo Fisher Scientific Inc., Waltham, MA, United States) with a 21% O_2 and 5% CO_2 atmosphere. The hypoxic groups were placed in a humidified, water-jacketed CO_2 incubator with oxygen control (Forma™ Series II, Thermo Fisher Scientific Inc.) in an atmosphere containing 2.5% O_2 and 5% CO_2. The normoxia group continued to be incubated in the standard humidified incubator.

Colony-forming unit-fibroblast (CFU-F) assay

For the CFU-F assay, 1000 hP-MSCs were plated on six-well plates in triplicate and cultured in complete medium for 14 days under normoxic or hypoxic conditions with medium changes every 3 days. The culture dishes were rinsed with PBS, fixed with 4% paraformaldehyde and then stained with crystal violet. After rinsing with PBS, colonies consist of more than 50 stained cells were counted. Total colony numbers was determined.

Flow cytometry analysis of cell cycle

The cells were collected with trypsin and fixed with cold 70% ethanol for at least 2 h. After fixation, cells were transferred from 4 °C to the bench top and equilibrated to room temperature. The cells were washed with cold PBS and incubated in the dark with 400 μL staining buffer containing RNase A and propidium iodide at 37 °C for 30 min. A flow cytometer (BeamCyte-1026) was used to analyze the cell cycle distribution with ModFit LT ver. 5.0 (Verity Software House).

Western blot assay

The cellular protein was harvested by solubilizing MSCs in RIPA lysis buffer (Beyotime) mixed with protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein concentration was determined using a BCA kit (Beyotime). A total of 20 μg of proteins were run on 4–15% Mini-PROTEAN® TGX™ gels (Bio-Rad Laboratories, Inc., Hercules, CA) and transferred onto a polyvinylidene difluoride

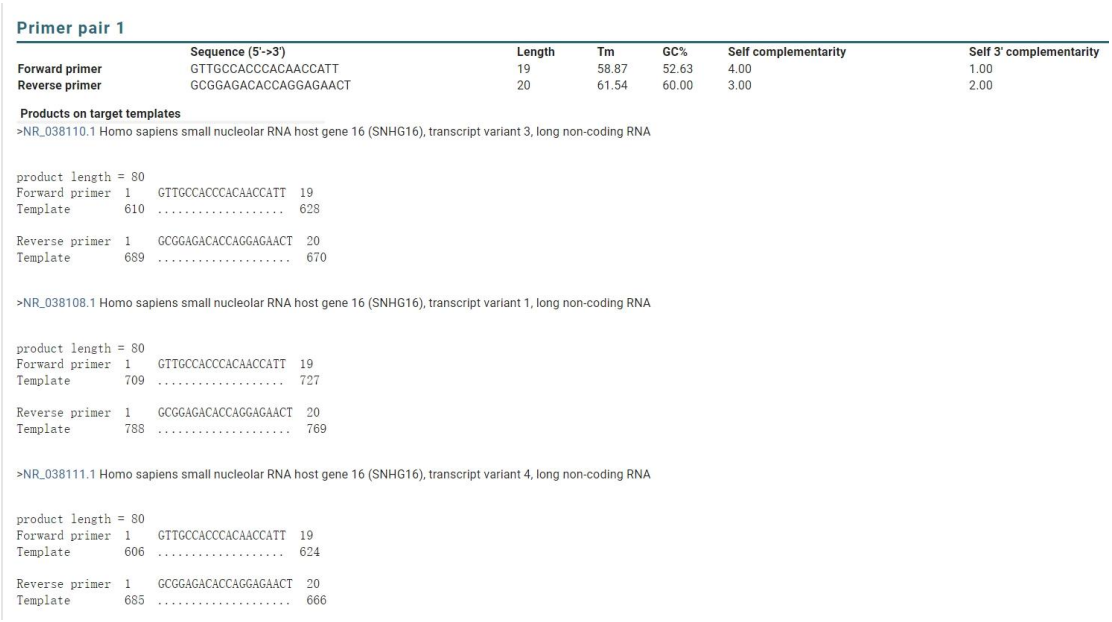
membrane (Merck KGaA, Darmstadt, Germany). Then, the membranes were incubated in QuickBlock™ Blocking Buffer (Beyotime Biotech Co., Ltd.) at room temperature. After blocking, the membranes were immunoblotted with specific primary antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4,000; Abcam, Cambridge, UK) for 1 hr at room temperature. After rinsing with Tris-buffered saline with 0.1% Tween, the membranes were incubated with Pierce™ ECL Western blot analysis Substrate (Thermo Fisher Scientific Inc.) for 1 min and detected with the ChemiScope Western Blot Imaging System (Clinx Science Instruments Co., Ltd., Shanghai, China). The primary antibodies were anti-β-actin (Abcam, Cambridge, UK), anti-GAPDH (Abcam), anti-HIF1α (Cell Signaling Technology, Danvers, MA, USA), anti-c-MYC (Abcam), anti-PCNA (Abcam), anti-CDK2 (Abcam), anti-CDK4 (Abcam), anti-CDK6 (Abcam), anti-CyclinD1 (Abcam), anti-CyclinE1 (Abcam), anti-AKT (Abcam), and anti-phospho-AKT (Abcam).

Cell transfection

Once the cells reached 50% confluence, the hPMSCs were distributed into four groups for different treatments to acquire distinct levels of SNHG16 expression. Recombinant lentiviruses for SNHG16 over-expression or the corresponding empty vectors (Genomeditech, Shanghai, China) were separately added to the medium to infect the respective groups of hPMSCs with an MOI of 50:1, with the aim of acquiring hPMSC that over-expressed SNHG16 and a corresponding negative control, empty vector. Similarly, two other groups of hPMSCs were individually infected with an SNHG16 shRNA for gene silencing or a scrambled shRNA for the negative control. The four groups of processed hPMSCs were cultured as usual for 48 hours, and the culture medium was replaced. Puromycin (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was mixed into each medium and filtered. After 2–3 days of extended culture, the medium was replaced and the filtering was repeated. Then, successful infection was confirmed by qRT-PCR.

5. The results of Primer Blast for gene SNHG16 primers showed a large number of nonspecific targets and no binding to the main target, which needs further investigation.

Response: Thank you for carefully examining our data. Before the experiment proper starts, we blasted the primers in Pubmed Primer Blast. Here is the searching result:



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTTGCCACCCACAACCATT	19	58.87	52.63	4.00	1.00
Reverse primer	GCGGAGACACCAGGAGAACT	20	61.54	60.00	3.00	2.00

Products on target templates

>NR_038110.1 Homo sapiens small nucleolar RNA host gene 16 (SNHG16), transcript variant 3, long non-coding RNA

```
product length = 80
Forward primer  1    GTTGCCACCCACAACCATT  19
Template        610  ..................  628

Reverse primer  1    GCGGAGACACCAGGAGAACT  20
Template        689  ..................  670
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>NR_038108.1 Homo sapiens small nucleolar RNA host gene 16 (SNHG16), transcript variant 1, long non-coding RNA

```
product length = 80
Forward primer  1    GTTGCCACCCACAACCATT  19
Template        709  ..................  727

Reverse primer  1    GCGGAGACACCAGGAGAACT  20
Template        788  ..................  769
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>NR_038111.1 Homo sapiens small nucleolar RNA host gene 16 (SNHG16), transcript variant 4, long non-coding RNA

```
product length = 80
Forward primer  1    GTTGCCACCCACAACCATT  19
Template        606  ..................  624

Reverse primer  1    GCGGAGACACCAGGAGAACT  20
Template        685  ..................  666
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It can be seen from the above results that the specificity of this primers is acceptable. During the alignment process, it should be noted that the database should be selected as Refseq RNA, not Refseq mRNA. The default database, Refseq mRNA, may not be suitable for alignment of lncRNA primers, as it only contains coding RNA.

6. It is recommended to mention the statistical analysis results of cell cycle comparisons.

Response: We concur with the point and we have supplemented the statistical analysis results of cell cycle comparisons both in revised figure 4, 5, 6 and corresponding texts (marked in red).

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: In this manuscript, the authors described that LncRNA SNHG16 promotes human placental-derived mesenchymal stem cells proliferation capacity through PI3K/AKT pathway under hypoxia. I suggest accepting this manuscript after authors address the following concerns.

1. In Figure 1A&E, WB images were poor quality, so I suggest supplementing immunofluorescence staining for evidence.

Response: We appreciate the reviewer's professional suggest. Actually, we tried to do immunofluorescence staining initially but it was difficult to detect the signal because of the lower stability and higher degradability of HIF-1α under normoxia. These probably relate to a bit blurry of the band of HIF-1α in Figure 1A, though, it can show the increased expression of HIF-1α under hypoxia. In addition, in other related literatures, we rarely see the use of immunofluorescence methods to detect the expression of HIF-1α protein. In revised Figure 1E, we replaced the previously submitted poor-quality GAPDH band with a higher quality band.

2. In Figure 1D, I suggest repeating the experiment with a better quality diagram.

Response: We fully agree with this suggestion. In fact, in the course of the experiment, we have repeated this experiment. Since histograms did not represent individual values well, we now use a scatter plot with bar in the revised Figure 1D to better present the results.

3. In Figure 4F, RNA FISH should be supplemented to provide the spatial location of lncRNA SNHG16.

Response: We fully agree with this professional advice. However, due to the lack of experimental resources and the impact of the epidemic, we cannot complete the RNA FISH experiment now. But, SNHG16 was predicted by the bioinformatics tools in lncATLAS to be located mainly in the cytoplasm [1]. Some studies have also identified a characterization of spatial localization of SNHG16, it is certain that SNHG16 is predominantly located in the cytoplasm [1-4].

Reference:

[1] Ni C, Fang QQ, Chen WZ, Jiang JX, Jiang Z, Ye J, Zhang T, Yang L, Meng FB, Xia WJ, Zhong M, Huang J. Breast cancer-derived exosomes transmit lncRNA SNHG16 to induce CD73+γδ1 Treg cells. Signal Transduct Target Ther. 2020 Apr 29;5(1):41.

[2] Asila A, Yang X, Kaisaer Y, Ma L. SNHG16/miR-485-5p/BMP7 axis modulates osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. J Gene Med. 2021 Mar;23(3):e3296.

[3] Bu J, Guo R, Xu XZ, Luo Y, Liu JF. LncRNA SNHG16 promotes epithelial-mesenchymal transition by upregulating ITGA6 through miR-488 inhibition in osteosarcoma. J Bone Oncol. 2021 Jan 19;27:100348.

[4] Gong CY, Tang R, Nan W, Zhou KS, Zhang HH. Role of SNHG16 in human cancer. Clin Chim Acta. 2020 Apr;503:175-180. doi: 10.1016/j.cca.2019.12.023.

4. Please explain why the results of control groups do not match in Figure 5C and Figure 4A.

Response: We thank the reviewer for carefully examining our data. When we first observed the results, we were also surprised and wondered why the results of control groups do not match. After conducting a review of the experimental operation, we speculate that the reason for this result is the influence of lentiviral transfection. The control in Figure 5C was transfected with scrambled shRNA and subjected to a screening step with puromycin compared to the control group in Figure 4C. But we think this difference does not affect our conclusions, after all, variables are controlled for comparison between groups in a single experiment.

5. The title contains a word "under hypoxia". In Figure 5, why the normal and hypoxia groups were not set after SNHG16 was knocked down. The proliferation phenotypes should be further confirmed by WB, IF and colony formation. In addition, the expression of SNHG16 should be restored after knockdown to confirm whether the phenotype is consistent.

Response: We appreciate your kindly comments. Indeed, we should simultaneously demonstrate whether SNHG16 regulates proliferation under hypoxia, as the title contains a word "under hypoxia". So, following your suggestion, we added the results of the proliferative phenotype after SNHG16 knockdown and overexpression under hypoxia in the supplementary material. However, due to limited experimental resources, we regret that we can only provide the results of CCK8 and colony formation. It is hoped that these results will shed light on the effect of SNHG16 on proliferation under hypoxia.

6. In Figure 6, WB images were poor quality in CDK6 and Cyclin E1, so I suggest repeating them and adding immunofluorescence or ELISA evidence, as well as detecting the related RNA level of this pathway.

Response: Thank you for your suggestion. The reason for the low quality of the bands may be that the expression levels of CDK6 and Cyclin E1 are relatively low. We have repeated the experiments in the previous experiments and found that the bands of CDK6 and Cyclin E1 are extremely difficult to display with high quality, but we can see a trend of increased expression. Considering that the detection limit of WB is generally lower than that of immunofluorescence or ELISA, we feel that immunofluorescence or ELISA experiments may be of limited help. It is undeniable that your advice is very worthy of recognition and very professional.

7. Both MYC and PI3K/AKT pathways can promote cell proliferation. Please explain why the MYC pathway was not detected later.

Response: Thank you for your comments. According to the results of our bioinformatics analysis in this study, SNHG16 is found related to the expression of the subunit of PI3K, so we speculated that SNHG16 may be related to the PI3K/AKT pathway and verified it. SNHG16 may also be related to the MYC pathway, but it is not the focus of our study. The overall logic of the article may be incomplete if we focus on the MYC pathway.

Reviewer #3:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: The authors studied the role of lncRNA in regulating of hypoxia on hP-MSCs. Hypoxia lead to activation of AKT pathway.

1. The author should demonstrate that whether SNHG16 alter the activation of AKT in response to hypoxia.

Response: We concur with the points raised. It is a pity that there is no directly related experimental evidence in this experiment, but according to the fact that SNHG16 can significantly affect the phosphorylation of AKT under normoxia, we speculate that the increase of SNHG16 expression under hypoxia can cause the phosphorylation of AKT to promote proliferation. The relationship between SNHG16 and AKT is widely studied. It is reported that SNHG16 may activate phosphorylation of AKT and upregulate the expression of MMP9 to promote cell proliferation, invasion and migration of ovarian cancer [1]. Zhou et al. revealed that lncRNA SNHG16 promotes the tumorigenicity of glioma through miR-373/EGFR axis by PI3K/AKT pathway [2].

Reference:

[1] Yang XS, Wang GX, Luo L. Long non-coding RNA SNHG16 promotes cell growth and metastasis in ovarian cancer. Eur Rev Med Pharmacol Sci. 2018 Feb;22(3):616-622.

[2] Zhou XY, Liu H, Ding ZB, Xi HP, Wang GW. lncRNA SNHG16 promotes glioma tumorigenicity through miR-373/EGFR axis by activating PI3K/AKT pathway. Genomics. 2020 Jan;112(1):1021-1029.

2. lncRNA have several mechanisms to regulate gene expression. The authors should make clear or discuss how SNHG16 regulate AKT pathway.

Response: We concur with this point. In this study, we focus on the discovery and

functional verification of hypoxia-responsive lncRNAs, and the exploration of the exact mechanism is still superficial, which is a limitation of this study. However, how SNHG16 regulates the AKT pathway has been reported in some related studies. For example, SNHG16 could regulate PLK4 expression by sponging miR-338-3p and SNHG16/miR-338-3p/PLK4 axis could affect the activation of PI3K/AKT pathway in cisplatin-resistant neuroblastoma cells [1]. Moreover, SNHG16 may exert oncogenic function as a competing endogenous RNA (ceRNA) to regulate EGFR by sponging of miR-373-3p through activating PI3K/AKT pathway [2]. Upregulation of SNHG16 in HG-stimulated hRMECs facilitates proliferative DR-related abnormalities in cell proliferation, migration, and angiogenesis through regulating miR-146a-5p/IRAK1 and miR-7-5p/IRS1 to activate NF-κB and PI3K/AKT signaling pathways [3]. It can be seen that SNHG16 mainly acts as a competing endogenous RNA to participate in the regulation of PI3K/AKT signaling pathway. Our follow-up studies will take this as a starting point to elucidate the detailed mechanism of SNHG16 regulation of PI3K/AKT pathway in this study. Relevant discussion was added to the Discussion section, as follows:

However, how SNHG16 becomes integrated in the PI3K/AKT signaling pathway in the study remains unknown. There are some related articles on the mechanism by which SNHG16 regulates the AKT pathway in other models. For example, SNHG16 could activate the PI3K/AKT pathway through SNHG16/miR-338-3p/PLK4 axis in cisplatin-resistant neuroblastoma cells. Moreover, SNHG16 was found to facilitate proliferative DR-related abnormalities in cell proliferation through regulating miR-7-5p/IRS1 to activate PI3K/AKT pathway in HG-stimulated hRMECs. It can be seen that SNHG16 mainly acts as a competing endogenous RNA to participate in the regulation of PI3K/AKT signaling pathway. Our follow-up studies will take this as a starting point to elucidate the detailed mechanism of SNHG16 regulation of PI3K/AKT pathway.

Reference:

[1] Xu Z, Sun Y, Wang D, Sun H, Liu X. SNHG16 promotes tumorigenesis and

cisplatin resistance by regulating miR-338-3p/PLK4 pathway in neuroblastoma cells. Cancer Cell Int. 2020 Jun 12;20:236.

[2] Zhou XY, Liu H, Ding ZB, Xi HP, Wang GW. lncRNA SNHG16 promotes glioma tumorigenicity through miR-373/EGFR axis by activating PI3K/AKT pathway. Genomics. 2020 Jan;112(1):1021-1029.

[3] Cai F, Jiang H, Li Y, Li Q, Yang C. Upregulation of long non-coding RNA SNHG16 promotes diabetes-related RMEC dysfunction via activating NF-κB and PI3K/AKT pathways. Mol Ther Nucleic Acids. 2021 Feb 4;24:512-527.

Editorial Office's Comments

(1) Science editor:

− The study is interesting; however, it would require a major overhaul before further consideration. The overall writing should be greatly improved, for example, the introduction section should be shortened. Method section should include all essential details for others to repeat experiment. Author should seek professional English editing help. In terms of data presentation. Author should add numerous experiments suggested by the reviewers. For example, the Figure 1 WB image should be improved with a higher quality one, and add IF. Figure 4F, add RNA FISH. The proliferation phenotypes should be further confirmed by WB, IF and colony formation. Please demonstrate that whether SNHG16 alter the activation of AKT in response to hypoxia.

Response: We appreciate the Science editor's suggestions and comments. We have made point-by-point responses to each of the issues raised in the peer review report and consequential revisions have also been made based on your comments.

− Language evaluation: The English-language grammatical presentation needs to be improved to a certain extent. There are many errors in grammar and format, throughout the entire manuscript. Before final acceptance, the authors 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.

Response: We agree with the Science editor's point. We have revised the grammar, layout, etc. of the manuscript. The English in this document has also been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/MOdUfz. We have also uploaded the English Language Certificate issued by Textcheck onto the system.

− Medical ethics: The authors described the Institutional Review Board approval, but not the informed consent. Specific comments: Informed consent statement is missing.

Response: The informed consent may not be required for this basic research.

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 red 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