Peer Review File

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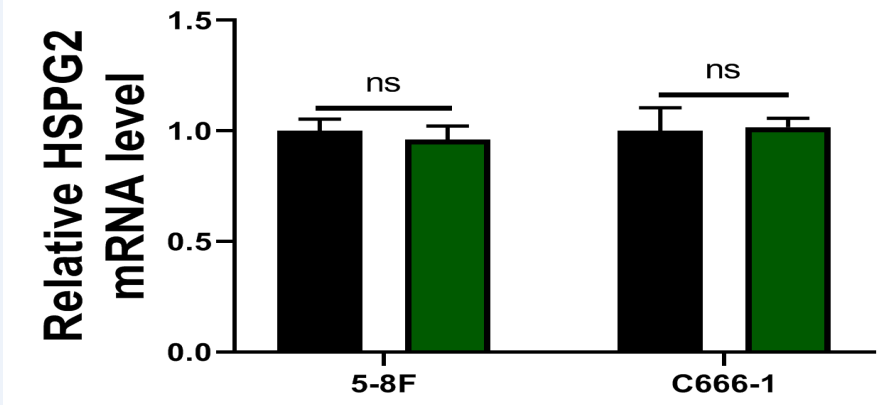
Reviewer A:

The authors in this interesting study show that circular-RNA, HSPG2 interacts with miR-25-3p to regulate cell death and proliferation in cardiomyocytes through its interaction with PAWR. The interactions described here are interesting and significant and have implications in therapy as well as understanding the role of circular RNAs.

1. Figure 1: It is unclear how the authors obtained non-circular HSPG2mRNA. Is this done by linearizing circHSPG2 mRNA or is this available naturally. In this case, does non-circular HSPG2 also get regulated by hypoxia? I am curious about the occurrence, role of non-circular HSPG2 as it can also be modulated by almost all the experiments described in this paper. Does it get silenced by siRNA aimed towards circular HSPG2? Does it also bind miR-25-3p?

Reply 1: miR-25-3p overexpression had no effect on HSPG2 mRNA.



2. When cells are transfected with cicular-HSPG2, how is the circular form of HSPG2 confirmed?

Reply 2: Transfection efficiency of the oe-cicHSPG2 vector was demonstrated by RT-qPCR analysis in combination with divergent primers. See Page 13, lines 276-278

3. Since circ-HSPG2 possibly serves as a binding site for numerous miR-25-3p, authors should use the plasmid they made mut-cir-HSPG2 to understand how the specific interaction between circ HSPG2 and miR-25-3p is in determining its effects on cell viability, Edu Expression, number of colonies and apoptosis etc. (Results in figure 4)

Reply 3: We constructed WT/MUT-circHSPG2 and WT/MUT-PAWR 3'UTR reporter plasmids to verify the targeting relationship between circHSPG2 or PAWR and miR-25-3p.

3. Similarly, since other miRs are likely able to bind to PAWR mRNA, this reviewer suggests that in evaluating cell activity for viability etc, the authors use the mutated form of PAWR mRNA to determine if this interaction is enough to influence cell outcomes

Reply 3: PAWR silencing weakened hypoxia-mediated cell dysfunction in AC-16 cells. See Page 15, lines 306-309.

All comments are in the comments to authors section. The specificity of the interactions described by the authors is not evident based on the experiments described by the authors. Further experiments using some of the engineered plasmids would be able to further explain the concerns raised.

Reviewer B:

Zhao et al. investigated the role of circHSPG2 (circular HSPG2) in the induction of the cellular damage and decline of the viability in AC-16 cells under hypoxic condition. The authors showed that circHSPG2 expression is induced by hypoxia, and the suppression of circHSPG2 by siRNA abolishes hypoxia-induced cell death and reduction in proliferation. They also presented some data suggesting that miR-25-3p is directly silenced by circHSPG2 upon hypoxia, which results in activation of PAWR, a candidate gene of miR-25-3p target. Overall, their findings including circHSPG2 and its downstream target are interesting, and may help establish a new therapeutic strategy for the MI heart in future. However, there are several issues in the text and statistical analyses. Also because of the lack of several critical experiments, the authors' conclusion, especially regarding specific roles of circHSPG2, not that of HSPG2 mRNA, has not been supported by data. In addition, several additional experiments with regard to circHSPG2-miR-25-3p-PAWR axis would give additional support to their conclusion.

Major comments

1. Introduction: there are some shortages, errors and inadequate citations in the references. (1) the authors should provide a reference for AC-16 cells (JMCC 2005; 39: 133-147). (2) Reference #8 (Mol Cell Biochem. 2019;462:185-194.) has been retracted. (3) Reference #11 (Sci Rep. 2018;8:724.) relates to dilated cardiomyopathy, rather than ischemic cardiomyopathy.

Reply 1: (1) We have added references for AC-16 cells (JMCC 2005; 39: 133-147). (2) Reference #8 has been replaced. (3) For reference #11, we have corrected it to dilated cardiomyopathy. See Page 5, line 66, Page 4 line 64, and Page 4, line 72.

2. Materials and methods: Overall, this section needs a more detailed description. (1) Please provide the formal name of pcDNA (line 123). (2) The catalog number of all materials should be provided. (3) The dilution ratio of antibodies used for western blot analysis should be provided.

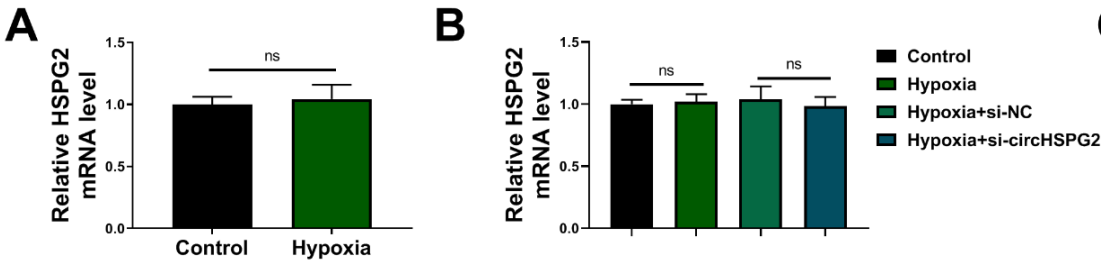
Reply 2: We have provided the official name of the pcDNA, the catalog number of all materials, and the dilution ratio of the antibody used for the western blot analysis. See the section marked yellow in Materials and Methods.

3. Statistical analysis: (1) Rather than standard deviation, standard error should be used for experiments with technical repetitions. (2) Which data were analyzed by one-way ANOVA, and was multiple comparison test performed? The way to present asterisks in for examples all graphs in figure 1, and many others, makes me wonder the authors might perform t-test, rather than one-way ANOVA. In addition, two-way ANOVA is better to assess the combinatorial effect of hypoxia and other factors such as si-RNA, oe-RNA, and inhibitors.

Reply 3: Values for this study are from three biological replicates, so standard deviations are used. We have added the statistical analysis of each experiment to the legends. See the section marked yellow in figure legends.

4. Results: Overall, the authors need to clarify more about the experimental design, especially how specifically the function of circHSPG2 was assessed without affecting HSPG2 mRNA. For example, was siRNA against circHSPG2 designed to specifically target HSPG2 mRNA? Does luciferase activity represent only the expression of circHSPG2, rather than HSPG2 mRNA? And even if these experiments were designed to specifically target circHSPG2, the authors still need to assess the level of HSPG2 mRNA in figures 1, 2, 3, and 4.

Reply 4: siRNA targeting circHSPG2 had no effect on HSPG2 mRNA expression under hypoxic conditions.



5. Fig. 2C: to make sure only healthy and mature cardiomyocytes were analyzed, co-staining of EdU with cardiomyocyte markers such as cardiac troponin, sarcomeric actinin, or myosin heavy chain is needed. The same is applied to Figure 4C and Figure 6C.

Reply 5: Due to the limitations of experimental conditions, we cannot provide these data temporarily.

6. Fig. 2G: TUNEL staining or co-staining with apoptosis markers (e.g. cleaved -caspase3) would give an additional support for the induction of apoptosis. The same is true for Figure 4H and Figure 6H.

Reply 6: Due to time constraints, we are temporarily unable to provide these data.

7. Fig. 5: FBXWJ is reported as another target of miR-25 to promote the cell proliferation in human ES cell derived cardiomyocytes and the zebrafish heart (Mol Ther Nucleic Acids 2020; 19: 1299-1308). Does circHSPG2/miR-25-3p also regulate proliferation in AC-16 cells in parallel with PAWR?

Reply 7: The regulatory mechanism of the circRNA/miRNA/mRNA network is complex, and whether FBXWJ is regulated by the circHSPG2/miR-25-3p axis can be explored in the future. We have added this to the discussion. See Page 18, lines 388-390.

8. Additional experiments elucidating circHSPG2-miR25-PAWR axis would further strengthen the authors' conclusion. (1) Figure 1: is the expression level of circHSPG2 directly regulated by the oxygen level? Is it upregulated under the hyperoxic condition?

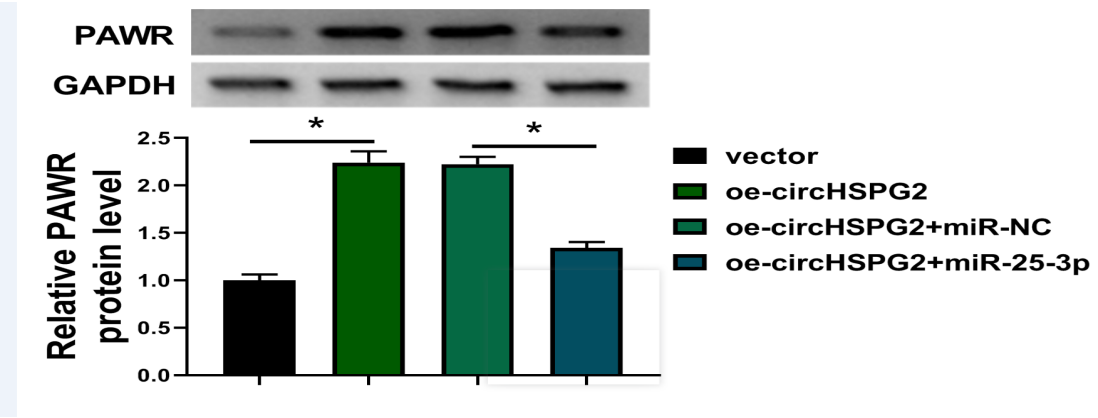
Reply 8: A previous report showed that hyperoxia gradually increases cellular inflammation and cytotoxicity [1], and whether hyperoxia affects the level of circHSPG2 in cardiomyocytes can be further explored in the future. See Page 18, lines 354-357.

(2) Figure 1: in situ hybridization analysis with AC-16 cells would give additional support for the localization of circHSPG2.

Reply 8: Sorry, we are temporarily unable to provide this data.

(3) Figure 5-7: does the overexpression of circHSPG2 induce the same effect under normoxic condition? And is it rescued by co-transfection with miR-25-3p?

Reply 8: Under normoxic condition, circHSPG2 overexpression elevated PAWR expression, but this elevation was weakened after miR-25-3p introduction.



(4) Immunostaining with anti-PAWR antibody would help elucidate the direct role of PAWR in AC16 cells.

Reply 8: Sorry, we are temporarily unable to provide this data.

Minor comments:

1. Fig. 2E: please indicate the population of apoptotic cells on the scatter plot or add some description to the figure legend (Annexin and PI double positive or including Annexin single positive?).

Reply 1: Q1LL: Annexin V-/PI-: viable cells; Q1LR: Annexin V+/PI-: early apoptotic cells; Q1UR: Annexin V+/PI+: late apoptotic cells; Q1UL: Annexin V-/PI+: necrotic cells. We have added these to the figure legend. See Page 22, lines 519-521.

2. Fig. 4D: please show representative pictures of colony assay like Fig. 2D.

Reply 2: we have showed representative pictures of colony assay for Fig. 4D. See Fig. 4D.

3. Fig. 4E: please show the scatter plot like Fig. 2E.

Reply 3: We have made changes to the figure as suggested. See Fig. 4E.

4. Color codes in some bar graphs and Fig. 3A are difficult to distinguish from each other. Authors should change the color, if possible according to the color universal design.

Reply 4: We have made changes to the figure as suggested.

Reviewer C:

Would the authors also provide in vivo/animal study data to support their conclusion?

Reply 4: Sorry, due to time constraints we cannot provide these data to support the conclusion at this time.