Responses to Reviewer's Comments

We are thankful for the reviewers' constructive comments. We have responded to each of the reviewer's comments in a point-by-point manner. In addition, we have provided a revised version of the manuscript with changes highlighted.

Reviewer 1: Specific Comments to Authors:

Comments to Editor I recommend a major revision before accept. Comments to the Author In this manuscript, Ullah and colleagues investigated the effect of heart shock protein 20 (HSP20) on proliferation of human iPSCs. In their experiment, HSP20 was overexpressed in iPSCs and the results showed that the cell viability was significantly increased in the HSP20-overexpressing cells. They found that HSP20 acts in a SIRT1-dependent pathway to drive cellular proliferation. This is a very interesting study because the authors mentioned that HSP20 has been implicated in cellular proliferation but conflicting studies have shown that it can either promote or suppress proliferation. This manuscript is generally well-written and easy to follow. However, the paper suffers from some low-quality data and several issues need to be addressed. Major concerns:

1. Why did the authors choose iPSC as a cell model, because this kind of cell itself is a highly proliferative type of cell?

Response: Thanks for the comments. We have added the rational that why we choose iPSC as a cell model, we used iPSCs because of their proliferation. This study aimed to enhance the proliferation of iPSCs by HSP20 expression, as it is essential for regenerative and healing purposes. The concurrent application of HSP20 could provide a favorable condition for culturing iPSCs to be used in clinical applications associated with tissue engineering, due to the enhancement of cellular proliferation and regenerative potential.

2. The cell morphology in figure 1B shows that iPSCs cells may differentiated after HSP20 expression. So, the state of pluripotency should be further identification.

Response: Thanks. Based on our data it does not change cell morphology [1-4]. We did one additional experiment to confirm the state of pluripotency genes. HSP20 upregulation enhanced the expression of SOX2, NANOG, KLF4, and SSEA4 (Figure-4), which validates that after HSP20-expression, the cells are more pluripotent and not differentiated phenotype.

3. What phenotype will appear if HSP20 was knock down in IPSCs? Minor concerns: 1. This manuscript is missing page and line numbers. 2. In figure2a, it is not clear about vertical axis. In addition, the pentagram should be replaced with a snowflake symbol. 3. The authors were also encouraged to check typos error (e.g., in fig1 and fig3, "βactine" should be "β-actin").

Response: Thanks. We speculate that aging phenotype may appear after HSP20 knock down. Because after HSP20-expression, the cells are more pluripotent and not differentiated phenotype. After HSP20 overexpression, the cells are not differentiated as shown by the higher expression of NANOG, KLF4, and SSEA4 in Figure-4, which means that cells are in more pluripotent stage compared to their control iPSCs. Previously we studied that HSP20 knockdown induced cell senescence [5, 6]. Following the reviewer's comments, we have corrected the typos and grammar throughout the manuscript.
We have corrected the β-actin, axis of the figures, and the spelling throughout the manuscript.

Reviewer 2: Specific Comments to Authors:

It is an interesting study exploring the role of heat shock protein 20 on cellular proliferation in induced pluripotent stem cells. The study has a rational and sound study design. The following should be considered when available:

1. As the authors used iPSCs in their experiments, they should give more consideration to this issue in the introduction section. Likely as how it is derived, their uses in regenerative medicine, blah, blah……

Response: We mentioned the purpose of using iPSCs in the introduction results and discussion sections to reinforce the rationale behind using the cell type. Statements of purchased cells added to the method section [2].

2. The hypothesis of the study should also be considered at the end of the introduction section.

Response: Thanks, we added the hypothesis at the end of the introduction section.

3. Statements of the ethical approval and checklist for reporting in-vitro studies should be added in the methodology section.

Response: we included the statements of the ethical approval in the method section.

4. The authors mentioned that "SIRT1, a member of the sirtuin family of NAD-dependent deacetylase, is known to induce proliferation and inhibit apoptosis", why they didn't use certain markers for apoptosis in their study.

Response: Following the reviewer's suggestion, we have added more details about the induced pluripotent stem cells (iPSCs). We consistently used HSP20 and SIRT1 in the revised version. We studied proliferation instead of apoptosis. Because this study aimed to enhance the proliferation of iPSCs by HSP20 expression, as it is essential for regenerative applications.

5. The abbreviation of Heat shock protein and Sirtuin-1 should be consistent throughout the manuscript (HSP/Hsp), and also for sirtuin(SIRT-1/Sirt-1). 6. How they obtain iPSC, purchased as a cell line or it was

prepared inside their labs.

Response: Thanks, we consistently used HSP20 and SIRT1 in the revised version. We studied proliferation but it is worth to consider apoptosis. We described the sources of iPSC origin in the method section of the manuscript.

References:

1. Ullah, M., R. Feng, and Z. Sun, *Induced Pluripotent Stem Cells (iPS)-Derived Extracellular Vesicles Improves Immune Dysfunction and Attenuates Splenomegaly in Aged Mice.* The FASEB Journal, 2018. **32**: p. 753.6-753.6.
2. Ullah, M., et al., *iPS-derived MSCs from an expandable bank to deliver a prodrug-converting enzyme that limits growth and metastases of human breast cancers.* Cell death discovery, 2017. **3**(1): p. 1-10.
3. Ullah, M., et al., *Microbubbles versus Extracellular Vesicles as Therapeutic Cargo for Targeting Drug Delivery.* ACS Nano, 2021. **15**(3): p. 3612-3620.
4. Ullah, M., *Need for specialized therapeutic stem cells banks equipped with tumor regression enzymes and anti-tumor genes.* 2020.
5. Ullah, M., et al., *Reversing acute kidney injury using pulsed focused ultrasound and MSC therapy: a role for HSP-mediated PI3K/AKT signaling.* Molecular Therapy-Methods & Clinical Development, 2020. **17**: p. 683-694.
6. Ullah, M., et al., *HSP70-Mediated NLRP3 Inflammasome Suppression Underlies Reversal of Acute Kidney Injury Following Extracellular Vesicle and Focused Ultrasound Combination Therapy.* Int J Mol Sci, 2020. **21**(11): p. 4085.