Response to Reviewer Comments

This is a generally excellent paper in a highly significant field of research. This reviewer has several points following:

Query:

Use of the word "scaling" in the title and throughout the paper is questionable. Merriam Webster says that things can be scaled according to actual need, and they can be regulated, set or estimated, and the authors have tried to do that, but without testing how the various preparations compare in in vivo experiments, we do not know if the "scaling" has been successful. Here the authors showed very clearly that human umbilical stem cells (husc) can be separated, cultured and prepared for use in trials of regenerative medicine, but the cells were not "scaled" for a specific purpose, and there is no apparent previous use of this term in any of the papers referenced by the authors. It is recommended that the authors use a different, more functional term, perhaps like deriving or preparing.

Response:

The title of the manuscript is revised to "**Expansion of Human Umbilical Cord Derived Mesenchymal Stem Cells in Regenerative Medicine**".

Query:

It would have been very nice to see just the simplest in vivo experiment using one or more of the HUSC preparations. What form would the HUS cells take if they were injected into the peritoneal or pleural cavities of immunosuppressed mice?

Response:
In this study, we optimized the novel ex-vivo approach of reculturing human umbilical cord tissue and profiled the isolated MSCs. In future experiments, this study will be further explored for in vivo experiments. The in vivo experiments are out of the scope of this manuscript. However, we are working in the proposed are and we will report in vivo studies in future applications.

Query:

There are a number of papers, many of them referenced here that demonstrate the preparation of husc, e.g. Todtenhaupt, P., et al., A robust and standardized method

to isolate and expand mesenchymal stromal cells from human umbilical cord. 2023. How is this paper under review different, better, consistent or not with this 2023 paper? And this Todtenhaupt paper has an incomplete reference.

Response:

Todtenhaupt P et al., 2023, reported the isolation of MSCs from umbilical cord tissue, and they didn't report the reculturing of the human umbilical cord. Therefore, Todtenhaupt et al., approach is different than our manuscript.

In the current manuscript after a human umbilical cord culturing MSCs colonies reached a confluence of 80–90%, at this stage umbilical cord pieces were not discarded, but recultured in fresh cultured flask termed as "recultured tissue". The isolated MSC's were evaluated for stemness via genetic and phenotypic expression at every recultured group (recultured 1-10). Moreover, each recultured cord isolated MSCs were expanded until passage 15 to check population doublings. The number of cells obtained by reculturing greatly increased and showed consistent MSC stemness at every recultured number from every cord tissue. It rapidly increases the cell number *in vitro* to fulfill *in vivo* therapeutic cell doses. Since these MSCs were isolated from the same recultured hUC, they have persistent MSC stemness and may decrease tissue versus graft rejection due to the less rigorous HLA screening performed in allogenic transplantation, which could make it more cost-effective to uphold good manufacturing practices.

Query:

The use of English generally is quite good, but there are corrections that will need to be made throughout. For example, on page 3 in the last sentence of the page is the phrase "...will provide novel sight for cell-based...". Use of the word "sight" is not meaningful here, and on page 5 is the phrase "...immunological possessions..." that makes no sense.

Response:

The correction has been made in the revised version.

Query:

The data are nicely presented, but it seems that a number of the figures could be combined into sets to be tested statistically. In other words, the histograms in Fig. 4 show remarkable consistency among the various donors. Since the data are so robust, this entire figure could be reduced to a single statistically significant data point, thus saving considerable space. In addition, the data in figures 9 and 12

show no significant differences. Thus, they again could be condensed into a statistical data point with a few microphotographs of the salient points.

Response:

The figures are revised as suggested by the reviewers.

EIC Specific comments:

1) Abstract; pages 3-4: "Up until passage 15, the recultured hUC-MSC population continued to multiply and double in size."

   The statement "Up until passage 15, the recultured hUC-MSC population continued to multiply and double in size" lacks scientific justification and logical coherence (continued to multiply and double in size? What could be the size if up to 15 times?). It doesn't provide a clear understanding of the growth pattern of the population.

A more precise and scientifically valid statement could be:

"Up until passage 15, the recultured hUC-MSC population exhibited continued proliferation quantified by population doubling number and, time of [insert estimated size] based on [insert relevant growth measurements or data]."

This revised statement specifies the growth pattern without implying an exact doubling in size at each passage, which may not be scientifically accurate. Additionally, it encourages further clarification on the actual size reached by the population at passage 15, based on available data or measurements.

Response

"Up until passage 15, the recultured hUC-MSC population exhibited continued proliferation quantified by population doubling time 3-8 hours and, number 1.3 million cells.

2) Page 4: "paired-box 6, bone morphogenetic protein 2, and transforming growth factor β1 " - please use standardized gene name abbreviations: Pax6, BMP2, TGFb1. Please follow the rules across the page (e.g., page 3, “octamer-binding transcription factor, sex-determining region Y-box 2”), according to WJSC standardized publications for gene nomenclature.

Response

Standard abbreviations were added throughout the manuscript.

3) Page 4: "The quality of recultured hUC-MSCs was maintained and showed negative expression of mycoplasma, cytomegalovirus, and endotoxin." To be accurate and logical, please change to "The quality control assessment of recultured hUC-MSCs remained consistent, indicating negative expression for

mycoplasma, cytomegalovirus, and endotoxin. However, there was no indication of mycoplasma
contamination."

Response

We have revised the text as suggested by the editor.

4) Page 4 "Delayed cellular senescence was observed ($P < 0.01$) by increased expression of hTERT at recultured numbers 8-10." (not clear, which contradicts to " Up until passage 15, the recultured hUC-MSC population continued to multiply and double in size." First, use the same language as "passage number" – 8-10 or 15? Which is correct? Fig 3 shows up to R12; Fig 4, R10. Fig 7, R10.
Response

Response

The sentence is corrected from "Delayed cellular senescence".
We have added clarification for Figure 3 in the results section, that reculture 11 and 12 take more time for cell proliferation, therefore we selected to perform all the experiments till reculture 10.

5) Page 3: "trilineage differentiation," where was the data for each passage? What was the passage number in Fig 6?

Response

The trilineage differentiation represents qualitative data, showing that isolated cells have the potential to differentiate into osteocytes, adipocytes, and chondrocytes.
We check the isolated cells from all three groups (i,e. reculture 1, 5, and 8) at passage 6. However, we present this data only once in Figure 6, as it is qualitative data to prove only that isolated cells are MSCs in nature.

6) Page 3: "quantitative expansion of MSCs" – What did they define it?

Response

We have added the clarification in the aim at page 3. The sentence is now " To optimize a novel protocol to achive qualitative and quantitative expansion of MSCs to achieve the desirable number of cells for cellular transplantation and minimize the limitation in stem cell therapy protocol.

Page 4: "CONCLUSION

This study advocates the development of a cutting-edge protocol for scaling the stem cell population that can meet rapidly with the increased necessary demand of the in-vitro cell doses, required for in-vivo implantation. Since these MSCs were isolated from the same recultured hUC, they have persistent MSC stemness, as indicated by the International Society of Cellular Therapy, which could make it more cost-effective to uphold good manufacturing practices."

This statement overstated their data. Neither in vivo nor dosing data nor manufactory process was provided; instead, a descriptive MSC culture scheme was provided.

Response

We have revised the conclusion, which is now "This study proposes the development of a novel protocol for efficiently expanding stem cells population. This would address the growing demand for larger stem cell doses needed for cellular transplantation, and will significantly improve the feasibility of stem cell based therapies."

7) Fig 5: quantifications?

Response

We performed immunostaining on the isolated cells from all three groups (i,e. reculture 1, 5, and 8) at passage 6. However, we present this data only once in Figure 5, as it is qualitative data to prove only that isolated cells are MSCs in nature. If we added the immunostaining figure at different recultures it would only increase the figure number. Quantification of immunostaining for positive and negative cells will provide the data for percent MSCs markers expressing cells which is already quantified on larger population in immunophenotyping and data is provided in Figure 4.

8) Fig 6: quantifications? "trilineage differentiation," where was the data for each passage? What was the passage number in Fig 6?

Response

The trilineage differentiation represents qualitative data, showing that isolated cells have the potential to differentiate into osteocytes, adipocytes, and chondrocytes.
We check the isolated cells from all three groups (i,e. reculture 1, 5, and 8) at passage 6. However, we present this data only once in Figure 6, as it is qualitative data to prove only that isolated cells are MSCs in nature.

9) Fig 8B, C, D: the figure legends are unclear. For example, in Fig8C, R10 changed from 3, 5, 6, 7, to 4. How did they explain the fluctuation of doubling times? The similarity in the fluctuation patterns existed in other panels if they claimed novel and stable QC.

Response

The figure legend is correct and improved. Clarification is added.

10) Many errors were manifested in crawling around the page. E.g., Page 49: "was confirmed by Alcian blue stained cells" is not the same as page 11 "Alizarin red staining solution"

Response

We have reviewed pages 11 and 49, and corrected the trilineage staining procedure. Now these statements are uniform on page 11, and 49.