Author's response to reviews

Title: Therapeutic potential of placental mesenchymal stem cells after transplantation through portal vein into Chinese miniature pigs with acute liver failure

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Version: 2 Date: 13 January 2012

Author's response to reviews: see over
Dear Dr. Lee,

In response to your email communication on October 31, 2011 regarding our manuscript (MS: 1537165508601985), we have completed all the experiments suggested by the reviewers and would like to address the reviewers’ comments point-by-point as follows:

**Reviewer: Dr. Maurizio Parola**

1. In order to improve and complete their characterization, Authors should further analyze the hepatocyte-like phenotype of hPMSCs by investigating critical functional parameters like urea synthesis, LDL uptake and glycogen storage; Authors should also offer data on whether these cells may operate drug metabolism by investigating mRNA or protein levels or enzymatic activity of selected CYP-450 isoforms.

   **Response:** We fully agree with these suggestions and conducted all the proposed experiments. Our results demonstrated that the hepatocyte-like cells differentiated from hPMSCs exhibited characteristics typical of hepatocytes, such as LDL uptake, glycogen storage, urea synthesis, and CYP-450 enzymatic activity. We added the corresponding content to the ‘Methods’ (p.14–15) and ‘Results’ (p.23–24) sections, and Fig. 6 in the revised manuscript.

2. It would be of interest to have more direct and time-dependent morphological informations concerning engraftment/localization of transplanted cells into injured liver; this could be accomplished (just by looking at the two relevant groups, that are Gal only and Gal plus hPMSCs via portal vein) by means of either immune-histochemistry or, even better, multiple indirect immune-fluorescence analysis by employing antibodies versus human antigens.

   **Response:** This is a very important and constructive suggestion. To determine whether some hPMSCs were localized to injured pig livers and differentiated into hepatocyte-like cells in Group IV (GalN plus hPMSCs via the portal vein),
expression of human hepatocyte-specific markers, such as ALB, AFP, and CK18, were investigated by immunohistochemistry and RT-PCR. Our results revealed that ALB$^+$ or CK18$^+$ cells were detected in the vast majority of recipient pig livers five months post-transplantation (in three of four pig samples). ALB, but not AFP, was detected by immunohistochemistry and RT-PCR in one liver biopsy specimen six weeks post-transplantation. This may be due to a limitation of the sampling method: because only a small quantity of biopsied liver samples was attainable, the probability of detecting colonized hPMSCs-derived cells in these samples was low. Nevertheless, detection of some ALB$^+$ or CK18$^+$ cells throughout recipient livers demonstrated the engraftment/localization of transplanted cells into the injured liver. We have added these data to the ‘Results’ (p.28-30) and ‘Discussion’ (p. 31-32) sections, and Figs. 13 and 14 in the revised manuscript.

Given the importance of this issue, as indicated in our email response to Dr. Lee on September 20, 2011, we attempted to track the fate of transplanted hPMSCs in vivo using a green fluorescent protein (GFP) reporter system. However, the GFP transfection efficiency was extremely low and cell proliferation ceased after transfection, making the task extremely difficult. In addition to the above experiments, we also employed X-ray irradiated hPMSCs as a transplantation control. In our opinion, this control provides strong evidence that the survival effect was caused by the transplanted hPMSCs, and not by the transplantation procedure per se, or other processes.

**Reviewer: Dr. Tamara Vanhaecke**

**Main concerns:**

**Major remarks concerned with the methodologies used:**

1. -positive control cells should have been included for IC and PCR experiments with respect to osteogenic and adipocyte differentiation; so in Figure
both for the pictures and the PCR data results of human adipocytes and osteocytes should be shown as well.

Response: This is a good suggestion. We have performed these additional experiments for IC and PCR and revised Fig. 4. Human intraoperative abandoned adipose tissue and immortalized human fetal osteoblastic cells served as positive controls, respectively. The appropriate changes have been made to the ‘Methods’ (p. 11–12) and ‘Results’ (p. 21–22) sections, and Fig. 4 in the revised manuscript.

2. in analogy to the previous point, in Figure 5 also the data for human hepatocytes should be included in the PCR data.

Response: Good suggestion. Human liver tissues were now used as positive controls. The results showed that human liver tissue was positive for ALB and CK18, and negative for AFP and CK19. Fig. 5 has been revised to this effect.

3. quantitative RT-PCR should have been used taking into account the MIQE guidelines

Response: We agree that qRT-PCR would be the optimal method to quantify the mRNAs of the genes of interest. However, because we use those genes as biomarkers and our purpose was only to verify their expression, but not to quantify the levels accurately, we feel that routine RT-PCR adequately serves this function.

4. an MTT assay measures cell viability, but not cell proliferation. So, to know the effect of irradiation on proliferation of hPMSCs, an assay specifically measuring DNA synthesis (e.g. thymidine incorporation, BrdU labeling) should have been used, or the authors have to talk about cell viability instead of proliferation in the text.

Response: We agree with this suggestion. We have changed “proliferation” to “cell viability” in the text, according to the reviewer’s advice.

5. based upon positive expression of ALB, AFP and CK18, the authors claim to be able to differentiate the hPMSC into ‘hepatocyte-like cells’. However, it
would have been much more convincing if they could also show some expression of CYPs, and even better CYP activity (vs. human hepatocytes). In addition, as they also have expression of CK19, which is a marker for cholangiocytes, it is obvious that the obtained cells are in an early, hepatoblast state rather than being ‘hepatocyte-like cells’.

**Response:** We concur with this point, and have added the hepatic differentiation experiments the reviewer suggests. Our results revealed that the hepatocyte-like cells differentiated from hPMSCs possess characteristics typical of hepatocytes, such as LDL uptake, glycogen storage, urea synthesis, and CYP-450 enzymatic activity. More details are provided above (Reviewer 1, major critique 1).

CK19, expressed by immature hepatocyte or biliary epithelial lineage, is a biomarker of bipotential progenitor cells. In our study, because cells were collected on days 7, 14, 21, 28, and 35 after induction, their development was continuous from immature to mature, and detection of CK19 by RT-PCR and IC indicated the presence of the phenotype intermediate between hepatoblasts and mature hepatocytes.

6. – with regard to osteogenic differentiation, it is better to use Alizarin Red S to stain for Ca$^{2+}$-deposits (bone mineralization) instead of ALP expression as the latter is also present in cartilage (chondrocytes) and thus is not specific enough

**Response:** We fully agree with this suggestion, and repeated the osteogenic differentiation experiment using Alizarin Red S to stain for Ca$^{2+}$-deposits. The results were satisfactory and are included in the revised Fig.4.

**Major remarks concerned with the conclusion:**

7. - in the discussion part, 4 possible explanations are pointed out as why non-irradiated hPMSCs transplantation is preferred over irradiated hPMSCs and no cell transplantsations. Yet, real data are not included in the paper to support these 4 hypotheses. Moreover, in the ‘conclusion’ part, it is stated that the underlying mechanisms still need to be determined. Therefore, the authors should either clearly state in the discussion that it involves hypotheses for which no
evidence is yet provided or provide evidence to support the hypotheses. Therefore, it is highly recommended to perform for example stainings of anti-human ALB, AFP and CK18 on liver slices obtained from sacrificed miniature pigs after transplantation (at least 1 month after successful engraftment) to show the successful in vivo conversion of hPMSCs into ‘hepatocyte-like cells’. Furthermore, the immunomodulating properties of engrafted hPMSCs can be evidenced by performing ELISA assays for immunomodulating agents such as LIF.

**Response:** We thank the reviewer for her very constructive suggestions. We have addressed the points regarding the detection of human ALB, AFP, and CK18 in recipient pig liver slices by immunohistochemistry and RT-PCR assay in detail above (reviewer 1, major critique 2).

Furthermore, we examined human LIF by ELISA and IFN-γ by ELISPOT assay. Our results indicated that the transplanted hPMSCs may indeed exert certain immunoregulatory effects on the ALF pigs. We thank the reviewer for drawing out attention to the importance of cytokines, which we will look into in our future study. We have now added the corresponding details to the ‘Methods’ (p. 18–19), ‘Results’ (p. 26-27), and ‘Discussion’ (p. 32) sections, and Fig. 11 in the revised manuscript.

**Minor points:**

8. - the list of abbreviations is not complete (e.g. MTT, Tri are missing)

**Response:** We have now completed the abbreviation list.

9. - p7: are references 24, 25, 26 and 27 correct?

**Response:** Yes, the references were mistakenly placed. We thank the reviewer for pointing out this important issue which we overlooked when inserting references using EndNote. We have re-organized the references.

10. - concerning the methods used: in general much more details need to be presented with respect to the isolation and cultivation of the hMPSCs (e.g. collagen concentration used, initial cell density, type of plates used,…).
Response: Again, this is a good point, and we have added further details regarding the isolation and cultivation of hMPSCs (p. 10).

11. - always specify what is meant by % when describing the methods: v/v, w/v?
Response: We agree with this suggestion, and have added the appropriate details to the ‘Methods’ section.

12. - give the concentration of the used
Response: Except for the AFP and CK 19 antibodies, the concentrations of which are not available from the manual or catalog, we have added details on antibody concentrations and dilution factors.

13. - radiation should be replaced by irradiation
Response: Agree. All mentions of “radiation” had been replaced by “irradiation” in the revised manuscript.

14. - p11: cells were ‘processed’: indicate how
Response: Again, this is a good point, and we have added the procedure to the ‘Methods’ section (p. 16), “the cells were fixed with pre-cooled absolute ethanol (-20°C).”

15. - p14: 3000rpm should be converted into g force
Response: We agree. 3000 rpm was converted to 1751 × g (p. 18).

16. - p<0.05: indicate what has been compared to what exactly
Response: Thank you for the suggestion. We have clarified this on p. 25–26 of the revised manuscript.

17. - Figure 9: indicate clearly on all figures the onset of the injury with D-galactosamine and the start of the transplantation with the hPMSCs
Response: The original Fig. 9 has been re-numbered as Fig. 10 in the revised manuscript. In the revised figure, we have clearly labeled the pre-D-galN, post-D-galN treatment, and post-cell transplantation time points.
Given all these additional experiments and revisions based on extremely valuable comments provided by you and the two reviewers, we feel that the quality of our manuscript has been substantially improved, and therefore would like to re-submit it for your consideration. Thank you very much again for your attention and great help.

Sincerely,

Lanjuan Li

January 12, 2012