Peer Review File

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Comment 1: In this study the authors have compared subretinal versus intravitreal administration of human CD34+ bone marrow stem cells in a rat model of inherited retinal degeneration. The authors report that both subretinal and intravitreal injection of human CD34+ BMSCs can provide functional rescue of degenerating retina and that this may be sustained longer after subretinal injection. Unfortunately, the study suffers multiple weaknesses, many of which are highlighted by the authors in the penultimate paragraph of the discussion.

Reply 1: Thank you very much for valuable comments and suggestions, which have certainly helped us to improve the manuscript. Followings are point-to-point responses to the reviewer comments.

Comment 2: The number of animals used in these studies is inadequate for robust comparative statistical analyses. As can be seen in table 1, 3 or 4 animals per group for the 4 week time point and 2 or less animals for the other time points. A minimum of 5 animals per group is essential for robust statistical analysis. Similarly, for the comparative histological and immunohistochemical studies it is not possible to make valid comparison at the 2 and 7 weeks time points where there are only 1 or 2 animals per group. Furthermore, reference to “trends” or “potentially lasting longer” should be omitted since this is clearly non-significant.

Reply 2: We appreciate the reviewer’s comment. Since a larger sample size would yield a more robust statistical analysis and since we did not have data for intravitreal CD34+ cell alone group, we have performed an additional experiment since initial submission of the manuscript. The additional data are included in the revised manuscript. Specifically, 10 newly bred RCS rats were used for this additional study (2 for subretinal CD34+/exosome, 2 for subretinal CD34+ alone, 2 for intravitreal CD34+/exosome, and 4 for intravitreal CD34+ alone). The revised Table summarizes the updated sample size for the various studies conducted for the various study groups. All animals underwent ERG examination at every time-points until they were sacrificed. Although some groups still have 3 or 4 animals, the additional data enabled a more robust analysis. Nonetheless, the main study results did not change.

The histological examination at 2 or 7 weeks was for qualitative analysis to evaluate the early or late effects; we did not use the data for quantitative statistical analysis. In addition, according to the comment we deleted the expression including "potentially lasting longer" in the abstract and Discussion.

Comment 3: Since the aim of the study is to compare subretinal with intravitreal injection the failure to perform intravitreal injection of CD34+ cells alone is a major omission and such data must be included. Furthermore, there is no intravitreal PBS control.

Reply 3: Thank you for the valuable comments. As described above, we performed additional studies and included the intravitreal CD34+ cell alone group in this revision. This group showed comparable ERG signal and outer nuclear layer thickness to the group treated with intravitreal CD34+ combined with exosome. Figure 1 and 2 were revised to include the results of this group. We did not have enough animals to include a control group of intravitreal PBS, and we included this point as a limitation of this study in the Discussion (Page 19, line 423-425).

Comment 4: The ERG data is unconvincing in that: A) Even the untreated controls do not show a robust ERG trace at 2weeks. B) A control trace should be included from a WT group to show a normal ERG trace so that the degree of ERG protection/improvement can be assessed. C) The b-wave amplitude is reduced by >75% in the CD34+ treated groups indicating that any improvement is very small even at the 2- and 4-week time points.

Reply 4: Thank you for the comments. Followings are our individual responses to the above three comments/concerns:

A) At 2 weeks after injection, which corresponds to the age of 5 weeks, the b-wave amplitude of RCS rat is decreased to about 50% of that at the age of 3 weeks when retina begin to degenerate (ref 27; Adachi et al). Nevertheless, a representative ERG trace of untreated control at 2 weeks after injection in the Figure 1 still shows all components of ERG, including the a-wave, b-wave, and oscillatory potential.

B) As commented, a wild-type animal as a control (i.e. Wistar rat) would be helpful

to contrast with RCS rats with retinal degeneration. Since prior studies have shown that the ERG recording of WT animals is similar to that of RCS rats at the age of 3 weeks and remains stable thereafter (ref 26; Rosch et al, ref 27; Adachi et al), we added instead the ERG traces of 3-week-old RCS rats on the day prior to injection in this updated manuscript. This information is included in the updated Figure 1 and discussion. We also included the lack of WT ERG as a possible limitation of the present study in the updated Discussion (page 19-20, line 425-430).

C) In prior studies, the b-wave ERG amplitude of RCS rat is reported to be basically extinguished at the age of 50 days (ref 27; Adachi et al), which corresponds to 4 weeks after injection in our study. As commented, the b-wave amplitudes in the CD34+ treated groups at 4 weeks after injection were decreased compared to 2 weeks in this study but still measurable. Whether this apparent waning protective effect of CD34+ cells in RCS rats results from rejection of human cells or true transient effect of CD34+ cells is unknown at this time as mentioned in the Discussion (page 17, line 356-364).

Comment 5: Less than 1% of the CD34+cells are likely to be stem cells with the majority being progenitors, thus the authors should not refer to bone marrow stem cells throughout the paper. “Bone marrow-derived” would seem more appropriate.
Reply 5: Thank you for the comment. We changed the description to “bone marrow-derived” throughout the updated title and manuscript as recommended. Since adult CD34+ cells are believed to be multipotent rather than pluripotent, there is some controversy whether we should call them “stem cells” or “progenitor cells”. Both terminologies have been used in publications.

Comment 6: Some aspect of the methodology need clarification: What was the efficiency of infection with EGFP and why was this necessary since a) it could affect cell behavior and b) immunohistochemistry was performed using anti-human antibodies? Some characterization of the exosomes would have been helpful in respect to purity, homogeneity and characterization (i.e. do the exosomes generated under the authors’ conditions have net neurotropic properties.
Reply 6: We labeled the human CD34+ cells with EGFP to help identify the cells in

the retina following intraocular injection. Since immunohistochemical analysis is not 100% sensitive in detecting the cells of interest, having dual markers to identify these human cells would be used. Our prior studies have shown that some of the cells incorporated into the retina can be identified using human cell markers while others were identified by EGFP while still others could be identified using either markers. We did not perform flow cytometry to determine the exact rate of transfection with EGFP in these cells. However, direct visualization of the CD34+ cells after transfection showed that a majority of the cells appeared to be labeled with EGFP.

Regarding exosomes used in this study, the exosomes were harvested using a standard method developed by the authors (ref 18; Anderson et al.). The resulting exosomes have been characterized in detail and shown to contain almost 2000 different proteins including various growth factors and NF-kß signaling pathway proteins. This information has been added to the discussion (page 19, line 413-415).