Da: BPG Editorial Office <editorialoffice@wjgnet.com>
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Oggetto: World Journal of Stem Cells Manuscript review of Manuscript NO: 67900
Dear Dr. Lo Furno,
Manuscript NO: 67900
Title: ARPE-19 conditioned medium promotes neural differentiation of adipose-derived mesenchymal stem cells Authors: Giuliana Mannino, Martina Cristaldi, Giovanni Giurdanella, Rosario Emanuele Perrotta, Debora Lo Furno, Rosario Giuffrida and Dario Rusciano
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Reviewer ID: 03448879

Reviewer #1: The study reported that the conditioned medium of ARPE-19 could promote neural differentiation of ASCs. The study design is logical. Experimental methods and statistical methods are appropriate. Questions raised:

Q1. The company and product number of the culture medium should be added.
A1. Product codes have been indicated in the revised manuscript.

Q2. The neural differentiation of ASCs should be proven from multiple aspects. Several markers were used in this study, including western blotting and immunofluorescence imaging. However, it would be better if functional indicators are added.
A2. In this study, only the modification of ASC immunophenotype was investigated. Further experiments will be designed to explore functional characteristics, such as membrane potential properties.

Q3. Language editing is required.
A3. The manuscript was now revised by a native English teacher.

Reviewer #2: The aim of the present study is to demonstrate that ARPE-19 conditioned medium is able to upregulate neural specific genes in adipose MSCs. The data presented goes part way towards supporting this hypothesis by showing differences in expression using immunofluorescence (Figs 2-5) and Western blots.

Q1. The authors point out that PGP9.5 expression is similar across all conditions at D1. However, immunofluorescent data (Fig 3) clearly show higher intensity levels between ASC vs CM/ASC at D1, but not by Western analysis.

A1. Bright immunofluorescent cells reported at day 1 (fig.4; PGP 9.5; ASC vs CM/ASC at D1) were just sporadic samples that were shown for their appearance. Instead, results were deduced from western blot data, which refer to measurements of larger samples. Moreover, western blot data are corroborated by immunofluorescence quantification, which is now added in an additional figure (now figure 6) in the manuscript.

Q2. Although these fluorescence intensities remain similar within conditions (ie. D1 vs D8, it is not clear if the difference seen between is necessarily due only to upregulation of expression per cell, or if there is some degree of selection occurring, whereby the growth of PGP9.5 high cells is favoured by conditioned medium. This possibility has not been addressed in the context of differential cell growth and viability between ASC controls and CM/ASCs across time points (Fig 1).

A2. Further analyses have now been carried out (percentages of immunopositive cells and quantitative immunofluorescence). Some considerations on cell proliferation rate have been added in the discussion.

Q3. The effect at D1 seen across conditions is not addressed in the manuscript, however, this observation that 24 hours is sufficient to increase levels of neural markers, (with little opportunity for the effects of selective growth) supports their interpretation that ARPE-19 CM does indeed cause upregulation of neural markers, rather than favour their growth.
A3. Although some differences were noted already at day 1, they were more clearly appreciable at day 8. This observation has been added in the discussion.

Q4. I suggest direct analysis using flow cytometry to determine changes in cell MSC population markers, as well as to measure the proportion and intensity of neural markers tested here by immunostaining.
A4. Flow cytometry was not carried out in this study and it is not easy to plan for the time being. However, quantitative estimates were carried out to indicate the percentages of immunopositive cells and fluorescence intensity. We hope that quantitative data now included can answer this question.

Q5. Related literature missed: 1.
The increased cell viability (inhibition of apoptosis) in CM/ASCs vs serum free cultures should be mentioned from previous studies. It is understood that the cells are not exactly the same, however, the mechanism may be. Pigment epithelium-derived factor from ARPE19 promotes proliferation and inhibits apoptosis of human umbilical mesenchymal stem cells in serum-free medium.
Ding DC, Wen YT, Tsai RK. Exp Mol Med. 2017 Dec 15;49(12):e411. doi: 10.1038/emm.2017.219.

A5. We thank Reviewer #2 for his/her suggestion. Possible PEDF-related effects on cell proliferation have been now included in the discussion: "PEDF-related effects may explain some our results on the proliferation rate observed in the present study. In fact, as already reported for human umbilical cord MSCs, the addition of PEDF significantly reduced apoptosis when cells were cultured in serum-free medium. In particular, the authors show that this PEDF-induced apoptosis reduction was due to a decreased p53 expression". The reference (Ding et al., 2017) has been added in the list.

Q6. Final note, authors should be careful not to describe upregulation of neural markers as neural differentiation itself. For example, hypoxia can upregulate OCT4 (pluripotent marker) in MSCs, but this does not make the cells human induced pluripotent stem cells.
A6. A neural-like differentiation was deduced by the increased expression of all the four markers tested. However, to address this concern, the word "likely" was added in the results about "ASC neural differentiation"

Q7. Suggest adjustment of language.
A7. The manuscript was now revised by a native English teacher.

Reviewer #3: In this paper, Mannino, et al. reported that the conditioned medium from ARPE-19 cells promoted ASCs differentiate to a neural cell-like phenotype, the authors isolated human ASCs from healthy donors and compared their differentiation under four culture conditions, they found that neural cell markers and ASC proliferation/viability were upregulated when the cells were cultured in ARPE-19 conditioned medium compared to other serum-free mediums. This paper is interesting and the results are of potential medical relevance, however, several problems including scientific questions throughout the manuscript need to be solved before final acceptance:

Q1. There are some mistakes in English grammar throughout the manuscript, please check with a native speaker or a language editor before submission.
A1. The manuscript was now revised by a native English teacher.

Q2. Type setting of the manuscript needs to be carefully checked and normalized, for example, the reference part appears two times in the manuscript, one consists 37 papers (page 15), while one consists 36 papers (page 24), the figures also appear two times, one consists 5 pictures (page 19) and one consists 6 pictures (page 29), this should not happen in a submitted manuscript.

A2. We are deeply sorry for that. Something wrong must have happened while we were uploading the document. Special attention will be paid for the revised version.

Q3. As the ASCs were primary cells isolated from human tissues, specific markers of ASCs shall be checked to confirm the purity of the cells.

A3. As we report in the Methods, the MSC nature of ASCs used in the present study was verified in previous recent works, where cells of the same stock were investigated. Virtually the entire population (above 98% of cells) was immunopositive for typical MSC markers (CD44, CD73, CD90, and CD105), whereas only a few cells (less than 1%) was immunostained for typical hematopoietic stem cell markers (CD14, CD34, and CD45). These percentages are now included in the text.

Q4. In the method part of immunofluorescence and western blot analysis, product code of the indicated antibodies shall be provided to ensure the reproducibility of the research, only dilution information is not enough, as a company always has several different antibodies for one kind of protein.
A4. Product codes have been specified in the revised manuscript.

Q5. The authors should compare cell differentiation when ASCs are cultured in complete conditioned medium (including FBS) from ARPE-19 cells, as Figure 1 showed that control ASCs cultured in basal DMEM have better cell viability compared to ASCs cultured in serum-free conditioned medium from ARPE-19, while the differentiation markers (Figure 2-6) were more obvious in serum-free conditioned medium from ARPE-19, it is important to exclude the influence of FBS.

A5. The use of serum-free culture medium was also intended to set a protocol that can be further developed for future clinical applications. Indeed, the use of ARPE-19 serum-free conditioned medium reduced ASC apoptosis, even promoting their differentiation ability. Moreover, the presence of FBS may interfere with ASC differentiation ability (Discussion)

Q6. In Figure 2-5, the pictures are only representative images, how is the status of other cells? In order to make the results more convincible, statistical results of different pictures from biologically independent experiments shall be performed and added in the manuscript.

A6. A quantitative estimate of the immunopositivity was carried out in a large number of pictures, counting immunostained cells and DAPI-stained nuclei. These data are now reported in the Results.