Hôpitaux Universitaires Genève
Hôpital Cantonal
Département des Neurosciences cliniques

Service d'Ophtalmologie
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UNIVERSITÉ DE GENÈVE

JoVE
Vineeta Bajaj
Review Editor
vineeta.bajaj@jove.com

Geneva, December 21st 2020

Revision of the manuscript no. JoVE62145 "Isolation, culture, and genetic engineering of primary pigment epithelial cells from small to large mammals for non-viral gene therapy studies"

Dear editor,

We appreciate the careful review of our manuscript, which we have revised according to the reviewers' comments. Changes and answers (in blue) to each comment are detailed below. Comments are numbered and are followed by our answer. Similar comments or issues by more than one reviewer are answered only once and referred appropriately. As suggested by the editor, changes are tracked within the revised manuscript to identify each edit, except for formatting changes.

Sincerely,

Thais Bascuas, Ph.D., corresponding author
Laboratoire d'Ophtalmologie Expérimentale
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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript; it was reviewed by an English native speaking individual and we verified that all abbreviations were defined at first use (see also Reviewer #2 comments item 30).

2. Please revise the title to "Isolation, Culture, and Genetic Engineering of Mammalian Primary Pigment Epithelial Cells for Non-viral Gene Therapy"

The title has been revised according to the suggestion.

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We revised the manuscript accordingly and deleted all personal pronouns.

4. Lines 80, 377: When citing authors in-text, please use either "Binder and colleagues" or 'Binder and co-workers" or "Binder et al." without the ampersand (&) and abbreviation (col.)

The in-text citations were modified accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Neon Transfection System; Tali image-based cytometry; Neon kit; 3P Pharmaceutical; CellTiter-Glo Luminescent Cell Viability Assay; ThermoFisher Scientific

All commercial symbols were removed and ordering information is referred only in the Table of Materials.

6. Being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text: a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

We agree and added a statement of ethics in the protocol (lines 153-160 of the revised manuscript).

b) Please specify the euthanasia method without highlighting it.

The euthanasia procedure has been specified (lines 157-158 of the revised manuscript).

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol was checked for references and specific details.

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8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

We revised the manuscript and reformatted the text accordingly.

9. Please include a scale bar for all images taken with a microscope (e.g., Figure 2) to provide context to the magnification used. Define the scale in the appropriate Figure Legends.

A scale bar has been added to all images shown (see also Reviewer #2 comments item 32).

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
    a) Critical steps within the protocol (points to be paid attention to)
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique

The items listed has been added in the discussion (a: lines 678-679, 706-708; b: lines 703-708; c: lines 696-699; d: lines 690-696; e: lines 669-670).

11. Please do not abbreviate journal names in the reference list.

The abbreviated Journal names have been replaced with the full name in the reference list.

12. Table alphabetically by the name of the material.

The table of materials has been modified to list materials alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors wrote a protocol to isolate RPE and IPE cells from the eyes of several animal species. The eyes that were used are donor eyes and enucleated. They isolate the cells, culture them and transfect them using Sleeping Beauty transposon-mediated transfection. In this case they used PEDF and GM-CSF. However, this system is applicable to other genes as well.

The protocol can be followed step by step. It is a nice addition to the protocols which have been published in Jove already.

Major Concerns:

13. The authors describe this procedure to be applicable to for example the transplantation of these cells in humans as treatment option for AMD. However, there are a lot of mountains to climb before this is possible. I am not sure whether the authors should make this a major goal of this protocol since we are not there yet. The transfection using the SB system; is the use of another system better? More feasible to use in the clinic?

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We agree with the reviewer that there are still obstacles to overcome before a therapy will become routine. However, our laboratory has specifically investigated the feasibility of treating neovascular age-related AMD by subretinal transplantation of genetically modified iris pigment epithelial cells and published the results of our investigations (refs. 17, 18, 19, 22, 24, 25, and 31 in manuscript). We have applied to Swissmedic for authorization to conduct a phase Ib/IIa clinical trial and have obtained approval for the trial by the ethical commission for research of the canton of Geneva (2019-00250). Swissmedic has requested some additional data that we are in process of providing. Our investigations have found that the *Sleeping Beauty* system is effective and safer than viral vectors as has also been shown by 12 clinical trials that are currently in progress (Gene Therapy Clinical Trials Worldwide, http://www.abedia.com/wiley/search.php (Accessed April 19, 2020), (n.d.); ref. 27 in manuscript). Information on the use of *Sleeping Beauty* transposon has been added to the introduction (lines 133-140 of the revised manuscript).

Minor Concerns:
Summary

14. Maybe consider to add the word "primary" to line 33.

We added the word « primary » to line 33 of the Summary.

15. 34: add the species

The species has been added (line 34 of the revised manuscript).

16. 35: transferable to humans: is this the case? Is the transposon-mediated strategy transferable?

Please refer to Comment 13 above.

Introduction

17. 68: ATMP offers potential correction of gene defects in humans? or? Maybe consider to stretch that those are still experimental strategies

The reviewer is right that gene therapy approaches to treat AMD are still experimental; however, the potential should not be discounted as evidenced by the 13 gene therapies that have received marketing approval including a gene therapy (Luxturna) to treat the RPE65 mutation-associated retinal degeneration (lines 69-74 of the revised manuscript).

18. 73: not only age-associated risk factors. Many others as well.

We agree with the reviewer that age is only one of the multiple risk factors and we have so indicated in the revised manuscript (lines 77-78 of the revised manuscript).

19. 77: rewrite the las sentence. The authors probably mean that wet AMD only is treatable at the moment by monthly injections. There is no cure for dry AMD.

We modified the sentence to better explain that wet AMD is treatable and that there is no treatment for dry AMD (lines 80-83 of the revised manuscript).

20. 79-88: this strategy is not only applicable to wet AMD. Also for dry AMD.

We agree to the reviewer that similar strategies are applicable to dry AMD and have revised the manuscript to include dry AMD information (lines 94-101 of the revised manuscript).

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21. 98: the authors propose a "new" therapy. How new is this idea? There are similar strategies in clinical trials already.

The novelty of our treatment strategy is the use of autologous transplantation of ocular cells genetically modified by the addition of the *PEDF* gene using the hyperactive *Sleeping Beauty* transposon and the pFAR4 miniplasmid. The use of the pFAR4 plasmid prevents possible antibiotic resistance side effects, the use of the hyperactive *Sleeping Beauty* transposon will integrate the *PEDF* gene into the host cell genome for a life-long secretion of PEDF and thus a permanent solution (cure) to neovascular AMD delivery.

22. 102-104: Now dry AMD pops up again?

Based on the previous comment (item 20) we have introduced potential treatments for dry AMD.

23. 106: transplanted back as a suspension. It has been shown before that transplantation as a sheet might be a better strategy.

It is true that studies reported advantages in transplanting cell sheets; however, to isolate and transfect cell sheets is not feasible without culturing the cells. In addition, transplanting cell sheets would require a more invasive surgical procedure that could possibly cause additional retinal damage. In this special case, we are convinced the less invasive procedure of transplanting a suspension shows an optimal risk-benefit ratio. This issue was added to the discussion (lines 690-699 of the revised manuscript).

24. 118: mice are also mammals.

We modified the sentence accordingly (lines 131-133 of the revised manuscript).

25. Maybe the authors can make a statement about the purity of their cultures? Is the seeding density per species different? (/cm2)

The cultures are pure as evidenced by the lack of non-pigmented cells as well as by the expression of specific genes and proteins. Additionally, the culture conditions as specified in the protocol are specific for pigment epithelial cells and do not promote neural cell growth.

Discussion

26. 484: PE cell transplants: transfected as well?

"PE cell transplants" are transfected PE cells; we added the information to the discussion (line 659 of the revised manuscript).

27. 485: How many cells are necessary to perform a transplantation? You cannot remove the entire iris as has been done in this protocol.

We are isolating around 18,238±10,948 cells from an iridectomy, which is a routine surgical procedure that removes only a small section of the iris and not the entire iris. The procedure has been simulated with human donor eyes during the validation of the GMP-grade production of the cell product (not shown in the present manuscript).

28. 501: sufficient number of vital cells from donor eyes.

A sufficient number of viable cells (18,238±10,948) has been isolated from iridectomies from donor's eyes and the cells are viable following transfection using the *Sleeping Beauty* transposon system. During the GMP-grade production of the GTMP (PEDF-transfected primary human IPE cells) we have shown that cell viability was 33.4±18.02%.

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29. Table 2: seeding density: /cm2

The seeding density is written in Table 1 and has been added to the protocol (lines 511-512 of the revised manuscript).

Reviewer #2:

Manuscript Summary:

In this manuscript, author have shown the protocol for isolation, culture, and SB100X-mediatedtransfection of RPE and IPE cells from various species including bovine, pig, rabbit, rat and mouse. They used non-viral gene delivery system encoded with pigmented epithelium-derived factor (PEDF) and/or the granulocyte macrophage-colony stimulating factor (GM-CSF) and shown the efficiency of non-viral vector system on retinal and iris pigmented epithelial cells.

Major Concerns:

30. This manuscript should be rewritten carefully, there are many points which need to be considered to improve the manuscript.

Please, refer to Editorial comments, item 1.

31. In Figure 2. It's really hard to compare the transfection efficiency of pFAR4-CMV-PEDF transposon plasmid in bovine IPE cells as compared to control. Is there any quantitative data for this experiment?

We have added the mean percentage of transfected cells (quantified by image-based cytometry) (lines 560-565 of the revised manuscript).

32. Author did not put any scalebar in the figure?

Please, refer to Editorial comments, item 9.

33. In Figure 3, author did viability assay to check the toxicity level of the buffer, Author did not mention anytime point, after how many hrs/days of cell transfection they measured the cell viability? Is there any control group for this experiment? Author can also add a Relative Luminescence unit?

We have added the information including units (arbitrary units) to the figure legend (line 612 of the revised manuscript). The viability assays were done 3±1 days post-transfection. The assays were always performed with a control without power (Co-P) (not electroporated) (lines 549-550, 612 of the revised manuscript).

34. In Figure 4: Author has not shown any positive transfection agent? They could have compared the transfection efficiency with known positive control? Why have they mentioned day 21 and 64, Have they done transfection after 21 and 64days of cell culture? Please rewrite this part because it's very confusing in the text?

As a positive control, we have added to the figure images of transfected ARPE-19 cells, which have shown have a reproducible transfection efficiency of 90-100% (ref. 30 in the manuscript). The cultures are monitored microscopically over the whole culture time; the randomly chosen, representative images shown were captured at day 21 and 64 post-transfection. The figure, caption, and manuscript have been modified for clarity (lines 557- 558, 566-569, 614-623 of the revised manuscript).

35. Did the author perform any kind of tight junction analysis to show the maturity of cells with time?

ZO-1 expression, as evidence of tight junctions, has been published by our laboratory (ref. 31 in the manuscript).

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36. Generally, for RPE cells are hard to transfect when they achieve the maturity level? Is there any difference in transfection efficiency at day 21 and 64?

The 21 and 64 days in figure 4 refer to days post-transfection and not to the number of days cells were cultured before transfection. However, we have not found any difficulty in transfecting "mature cell cultures". Transfections are done when cells reach confluence, which in our laboratory is approximately 4 weeks for RPE and 5-6 weeks for IPE cells. We added this information to the protocol (lines 456-457 of the revised manuscript).

37. Did the author perform any experiment in dividing RPE and IPE cells, to check efficiency of pFAR4-CMV-Venus and pT2-CAGGS-Venus vector?

RPE and IPE cells are isolated and cultured separately. We have transfected both IPE and RPE cells using the pFAR4-plasmids as well as pT2-plasmids. Transfection efficiency with the pFAR4 plasmid is usually somewhat higher but not statistically different from the transfection efficiency using pT2 plasmids (lines 701-703 of the revised manuscript).

38. Similarly, for figure 5, why any positive control is not selected for the experiment? How did author compare the transfection efficiency? And why has this experiment been performed only on RPE, not in IPE cells? Author should have mentioned the donor in the graph and figure legends?

ARPE-19 transfected cells were used as the positive control (see answer to comment 34). Additionally, transfection efficiency is always compared to two negative controls: "control without power (C-P)" (cells were not electroporated), and "control with power (C+P)" (cells were electroporated without addition of plasmids). We have modified the text to add the information (lines 566-569, 631-633 of the revised manuscript). We have done the experiment both with RPE and IPE; however, we had not mentioned in the manuscript or figure providing only essential information.

39. Why did the author did transfection on Rabbit IPE and RPE with pFAR4-CMV-Venus vector only 21 days while in other cases they have also included 64 days, is there any specific reason?

As mentioned in the answer to comment 34, we usually transfect cells once the cultures reach confluence; 21 and 64 days refer to post-transfection time. All cultures are monitored for the whole culture time and we have micrographs from both younger and older cultures, however, we tried to limit the representative results.

40. Why WB analysis for therapeutic genes PEDF and GM-CSF is not done with rabbit PE? is there any reason?

See Reviewer comments, items 38 and 39.