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

Geneva, October 20th 2020

Revision of the manuscript no. JoVE61957 "Induction and analysis of oxidative stress in Sleeping Beauty transposon-transfected human retinal pigment epithelial cells"

Dear editor,

Dear reviewers,

We appreciate the careful review of our manuscript, which we have modified based on your comments.

Below are detailed the changes and answers (in blue) to each comment. All comments are numbered followed by our answer. Similar comments or issues by more than one reviewer are answered once.

As suggested by the editor we will submit the modified version of the manuscript since the substantial changes made to the manuscript reduce readability significantly if shown in the track mode.

Thank you very much for your comments and for considering the revised manuscript for publication.

Sincerely,

Thais Bascuas, Ph.D.
Laboratoire d'ophtalmologie expérimentale
Hôpitaux Universitaires Genève / Université de Genève

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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.

We have proofread the manuscript and it was reviewed by an English native speaking individual. We believe that now the manuscript reads significantly better (see also Reviewer #1 comment 5 and 12, and Reviewer #3 comment 6).

2. Changes in the figures are requested:
   Figure 3: Please include a space between the number and the units: 50 uM instead of 50uM.
   Figure 4: Please use commas instead of apostrophes: 5,000 instead of 5'000

Both changes have been made throughout the manuscript.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Thank you for this valuable comment. We re-wrote the summary to clearly describe the method and its application (lines 28-31 of the revised manuscript).

4. Please revise the Introduction to also include the following… with citation:
   a) The advantages over alternative techniques with applicable references to previous studies

We have added a paragraph that describes methodological alternatives with appropriate references and the benefit of the presented protocol we have developed (lines 79-94 in the revised manuscript).

5. 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? For example, in 3.6, please mention methods of analysis (similar to how you have done for Western blot/ELISA in 4.6.1). Alternatively, add references to published material specifying how to perform the protocol action (e.g., for 4.3). 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.

Thank you for the detailed comment. We have modified the manuscript according to your comments and added appropriate references if applicable (e.g., reference for cell counting in Neubauer chamber [lines 198, 336 of the revised manuscript], button clicks for measuring luminescence [lines 308-317 of the revised manuscript], wavelength used in ELISA [line 80 of Supplementary Material]).

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6. 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. These should be removed from the Supplementary file as well. For example: ReliaPrep RNA Cell Miniprep System (Promega: Z6011)

We have removed all commercial symbols and ordering information is only referred to in the Table of Materials.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We highlighted the essential steps of the protocol considering a) these forms a cohesive narrative with b) at least one action in the imperative tense and c) highlighted all necessary sub-steps.

10. As we are a methods journal, please include the following as well in the Discussion section with citations:
    a) Any limitations of the technique
    b) The significance with respect to existing methods

We thank you for this important comment. We added a paragraph that discusses methodological limitations and a paragraph that describes the significance of the method (with respect to existing methods) (lines 577-601 of the revised manuscript).

11. Please consider adding the information in lines 24-25 in Supplementary material to the Acknowledgments section.

We agree and have transferred the sentence to the acknowledgments section (lines 628-632 of the revised manuscript).

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have uploaded to our Editorial Manager account the letter from the editor of Human Gene Therapy giving us explicit permission to reuse figures 3, 5, and 6 and have added the appropriate citation.

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Bascuas et al. present in this protocol a method to non-virally transduce Retinal Pigment Epithelial (RPE) cells using the Sleeping Beauty transposon system (SB100x) with plasmids encoding two growth factors: Pigment Epithelium Derived Factor (PEDF) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), known neuroprotective factors that mitigate damage induced by oxidative stress. The authors use a cell line derived from bovine RPE (ARPE-19) or primary human RPE cells obtained from postmortem donations. RPE cells are challenged with hydrogen peroxide to induce oxidative stress and their response to oxidative stress is measured using the following metrics:

(1) Expression of Glutathione (GSH) measured using a commercially available kit and following the manufacturer's protocol.
(2) Cellular viability measured using a commercially available kit and following the manufacturer's protocol or microscopic visual inspection of the cells.
(3) Analysis of the mitochondrial Uncoupling Protein 2 (UCP2) mRNA expression in RPE cells exposed to hydrogen peroxide by quantitative real-time PCR (QRTPCR).

The authors present as supplementary material additional analyses
S1 Analysis of PEDF and GM-CSF mRNA expression by QRTPCR in transduced RPE cells
S2 Analysis of PEDF and GM-CSF expression by ELISA and immunocytochemistry in transduced RPE cells
S3 Western Blot Analysis of phosphorylated Akt, a signaling molecule associated with activation of survival pathways in GM-CSF transduced cells

We thank the reviewer for his thorough evaluation of the manuscript and before replying to the comments, we would like to clarify the objectives of the protocol and studies detailed in the manuscript.

The purpose of the protocol is not the transfection procedure, which has been published elsewhere (Johnen, S. *et al.* (2012) IOVS, 53 (8), 4787-4796; Thumann, G. *et al.* (2017) Mol Ther – Nucleic Acids, 302–314) and a separate manuscript detailing the protocol has been submitted to JoVE to be published as video (Johnen, S. *et al.* Electroporation-based genetic modification of primary human pigment epithelial cells using the Sleeping Beauty transposon system, under review). The cell isolation procedure has also been submitted to JoVE and is under review (Bascuas, T. and Kropp, M. *et al.* Isolation, culture, and genetic engineering of primary retinal and iris pigment epithelial cells from small to large mammals for *ex vivo* and *in vivo* non-viral gene therapy studies).

The objective of this manuscript is to provide in video format the protocol for determining and quantifying the anti-oxidative power of cells transfected with genes that express neuroprotective factors, i.e. the analysis of the antioxidant capacity of neuroprotective factors expressed by genetically modified cells using an H_2O_2 cellular stress model. The model is useful to determine the effect of proteins expressed by transfected cells to determine the potency of the transfected cells that are intended to be used as a gene therapy medicinal product. Our laboratory uses the protocol for the development of a gene therapeutic strategy for the treatment of geographic atrophy (aAMD) with human-derived ARPE-19 cells and primary human RPE cells transfected with the *PEDF* and *GM-CSF* genes.

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Besides, there is a 10-page limit (with proper formatting) for the amount of text written in the protocol section, and there is a 2.75-page limit on the amount of content that JoVE can film for a single video article. For that reason, it is not possible to include all the information asked by the reviewer in one JoVE video article.

Major Concerns:

The study has merit as it presents interesting data pertaining to an important field, generation of therapeutic cells for the treatment of Age related Macular degeneration. Regrettably it falls short, in presenting a protocol helpful for other scientists to learn the methods used herein. It suffers greatly from

1. lack of presentation of the overall goal of the manuscript,

We have modified the protocol and believe that it is sufficiently clear and detailed now that other scientists should be able to reproduce it and modify it to meet their specific objectives. The revised manuscript presents the goals in more detail.

2. lack of structure in the presentation of the experiments,

3. poor organization of sequential steps in the methods,

The revised manuscript presents the sequential steps with modification so that the protocol can be easily reproduced (see below).

PROTOCOL

1. **Materials**
2. **Cell isolation and culture conditions**
   - 2.1. Human ARPE-19 cell line
   - 2.2. Primary human RPE cells

Table 1. Cell culture volumes.

Table 2. Demographics of human donors for retinal pigment epithelial cells.

3. **Electroporation of ARPE-19 and primary human RPE cells** comment: this chapter has been shortened
4. **Oxidative stress induction (H_2O_2 treatment) and neuroprotection (PEDF and/or GM-CSF treatment) (Fig. 1)**
   - 4.1. Treatment of non-transfected ARPE-19/RPE cells with PEDF and GM-CSF growth factors recombinant proteins
   - 4.2. Purification of PEDF and GM-CSF from transfected ARPE-19 cells
   - 4.3. Treatment of non-transfected ARPE-19/RPE cells with conditioned medium
   - 4.4. Preparation of conditioned medium

Figure 1. Timelines of the H_2O_2 assay in the three different experimental approaches.

5. **Analysis of oxidative stress level and antioxidant capacity**
   - 5.1. Glutathione assay
   - 5.2. Cytotoxicity assay and microscopic analysis
   - 5.3. *UCP2* expression analysis by RT-qPCR
   - 5.4. Preparation of cell lysate for SDS-PAGE and WB analysis of pAkt (Ser473)

RESULTS

Induction of oxidative stress in human Retinal Pigment Epithelium cells

Figure 2. Oxidative stress level evidenced as glutathione level and cell viability, in human RPE cells dependent on the H_2O_2 treatment.

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Figure 3. Morphology of non-transfected PEDF- or GM-CSF-transfected ARPE-19 cells treated with H_2O_2. Comment: pictures for PEDF- and GM-CSF-transfected cells treated with H_2O_2 were added.
Figure 4. Influence of cell number on the effect of H_2O_2-induced oxidative stress.

Analysis of the antioxidant effect of PEDF and GM-CSF delivered by *SB100X*-transfected human RPE cells in oxidative stress conditions

Figure 5. Glutathione level as a marker of the antioxidant capacity of PEDF and GM-CSF.
Figure 6. Glutathione level as a marker of the antioxidant capacity of PEDF- and GM-CSF-transfected human RPE cells.
Figure 7. *UCP2* gene expression in transfected ARPE-19 cells treated with H_2O_2.
Former **Figure S3** now **Figure 8.** Western Blot of phosphorylated Akt (Ser473) from a cell lysate of GM-CSF-transfected ARPE-19 cells.

SUPPLEMENTARY MATERIALS
(The Supplementary Materials present methods and results that are necessary for cell preparation but are not in the scope of the main protocol)
MATERIALS AND METHODS
1. **Real-Time quantitative PCR**

Table S1. Primer pair sequences and annealing time/temperature used for RT-qPCR.

2. **SDS-PAGE and Western Blot (WB) analysis**
3. **Immunohistological anti-PEDF and anti-GM-CSF staining**
4. **ELISA**
5. **Statistical analysis**

REPRESENTATIVE RESULTS
1. ***PEDF* and *GM-CSF* gene expression**

Figure S1. *PEDF* and *GM-CSF* gene expression analysis in transfected hRPE cells.

2. **Secretion of PEDF and GM-CSF by transfected cells**

Figure S2. Protein secretion in transfected hRPE and ARPE-19 cells.
Comment: increased pAkt in RPE cells under oxidative stress conditions moved to the main manuscript

4. missing details,

See Editorial comments item 5.

5. poor writing,

See Editorial comment item 1.

6. lack of rigor and specificity, and confusing repetitions of steps and interpretation.

The three critical elements of the protocol, which are 1) non-transfected cells treated with recombinant proteins, 2) non-transfected cells treated with conditioned medium, and 3) secretion of recombinant proteins by transfected cells, do create some repetitions. We have condensed and modified the protocol steps such that its specificity and organization are more clearly defined.

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To illustrate just a few examples:

7. Table 2 presents a list of 8 human donors that provided the group with specimens for generation of primary RPE cells. The authors mention that RPE cells from 4 donors were used and that the eyes were enucleated 12.7 hrs. post mortem. None of the donors listed in table 2 fit that description. Why list 8 when only 4 were used?

We have modified the figure to reflect that showing only the 4 donors used for the studies are reported in the manuscript.

8. For most experiments it is difficult to ascertain what tissue culture plates and what cells were used and at what density of plating. One needs to go back and forth from the beginning to the end of the protocol and to the figure legends or supplementary data to try to figure that out. The information in Table 1 is not necessary. What would be necessary is to specify the number of cells and the plate type used for the propagation of cells and individual experiments.

We revised the protocol to ensure that information regarding culture conditions, i.e. cell used, plating density, etc., are stated for each experiment. We have revised Table 1 to include details of what plate and density is used when for what.

9. It is unclear why the Supplementary data is presented. QPCR expression of the transduced RPE cells should be the first representative results figure, as it pertains to the efficiency of transduction in this protocol. It could be discussed how the DNA ratio for the different plasmids was established and what differences in expression of the transduced cells were found at other ratios. ELISA and immunohistochemistry data should also be presented in the body of the protocol as they validate the expression of the transduced proteins.

We are sorry that the objectives have not been clearly described. We have modified the protocol and transferred the preparation of cell lysate for SDS-PAGE and WB analysis of pAkt from the Supplementary Material to the manuscript. See also answer for Reviewer #1 "The objective of the protocol is not the transfection procedure, which has been published elsewhere (Johnen, S. *et al.* (2012) IOVS, 53 (8), 4787-4796; Thumann, G. *et al.* (2017) Mol Ther – Nucleic Acids, 302–314…."

10. It is often unclear what cells were used for the experiments presented in the figures.

We have made sure that this information is either given in the figure legend or title.

11. Data is presented when cells were treated with conditioned medium from transduced cells, however this method is not presented in the protocol, but merely in a very large figure legend.

We added details of the preparation of conditioned medium (lines 251-262 of the revised manuscript).

As mentioned before, the data presented is important and interesting. The manuscript could be greatly improved by creating a logical plan for the sequence of protocols presented, careful and rigorous attention to methodological details, increased clarity of presented data, improved quality of the writing with better structure of the sequence of paragraphs and proper English grammar. The manuscript mentions collection of RPE cells from

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human donors. This method would be interesting to present in detail, and would likely yield an exciting video production

Minor Concerns:

12. Writing could be greatly improved with editorial help from someone proficient in English.

See Editorial comments item 1.

Reviewer #2:

The authors demonstrated an H_2O_2-model for investigation of gene therapy of eye diseases. The model might be interesting to readers working in different fields. Here are some specific comments.

Main text:

1. Line 162: please mention the number of cells per well

We have added the information (3,000 or 5,000 cells) (lines 201, 244, 266 of the revised manuscript).

2. Line 168: what is the volume of the medium in each well?

We have added the information (200 µL medium) (lines 202, 245, 266 of the revised manuscript).

3. Line 180: what's the volume of the medium in each well for non-transfected cells?

We have added the information (200 µL medium) (line 245 of the revised manuscript).

4. Line 182: what are the cell culture conditions during the 28 days?

We have added all details in the protocol (lines 251-259 of the revised manuscript)

5. Line 193: what is the volume of the medium in each well?

We have added the information (200 µL medium) (line 266 of the revised manuscript).

6. Line 225: how is the luminescence measured?

We added a detailed description in the manuscript (lines 308-319 of the revised manuscript).

7. Line 246: what does "Step 2" mean?

Step 2 means point 2 of the protocol; we detailed the information as “steps 2.1.3.1.-2.1.3.4. of the protocol”) (line 334 of the revised manuscript).

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8. Line 247: what is the volume of the medium?

We added the volume of the medium (200 µL) (lines 340-342 of the revised manuscript).

9. Line 252: how is the luminescence measured?

10. Line 255: how is the luminescence measured?

See comments Reviewer#2 item 6.

Supplementary Materials:

11. Line 5: please show the maps/sequences of the final constructs

We have deleted the description of the final construct since both plasmids are described in detail in the manuscript Bascuas *et al.* (2020) Bioelectrochemistry (in preparation), both plasmids were constructed using the existing pT2-CMV-PEDF-EGFP plasmid reported in Johnen, S. *et al.* (2012) IOVS, 53 (8), 4787-4796.

12. Line 50: how many GM-CSF-transfected ARPE-19 cells were seeded in 6-well plates?

We transferred the paragraph from Supplementary Material to the body of the manuscript and added the missing information (lines 369-383 of the revised manuscript).

13. Line 73: is the confluence of cell monolayer close to 100%?

We have added the information that the monolayer was about 80% confluent (line 64 of the revised Supplementary Material).

Reviewer #3:

Manuscript Summary:
In this manuscript, Bascuas et al. analyzed protective effects of introduction of growth factor genes (pigment epithelium-derived factor (PEDF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) against oxidative stress in human retinal pigment epithelial (RPE) cells. Oxidative stress is known to play a crucial role in pathogenesis of degenerative retinal diseases, such as age-related macular degeneration. The authors have previously reported a non-viral cell-based gene therapy approach for treatment of AMD. In this study, they introduced PEDF and/or GM-CSF expression constructs into human RPE cells by using the Sleeping Beauty transposon system and electroporation, and examined their effects on prevention of cellular damage caused by oxidative stress in in vitro assays. The authors found that introduction of PEDF and/or GM-CSF provided human RPEs with protection against oxidative stress. Their experimental system would provide an approach to evaluate the effects of potential therapeutic reagents for degenerative retinal diseases.
Major Concerns:
This is an interesting study that describes introduction of growth factor genes into human RPEs by using a transposon system and in vitro electroporation and analyzes its antioxidative effects. The following issues need to be addressed before the publication.

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1. Statistical analysis
   The manuscript does not include the statement on statistical analysis. In many figures, different numbers of asterisk ("*", "**", "****"etc.) are used to indicate "significant difference", but no explanations regarding their meanings are provided. The authors should state which statistical methods they employed in this study and what statistical differences (p values) they found in each experiment. In figures 5, 6, S1, S2, it is not clear which data were compared for statistical analysis.

We have added a chapter in the Supplementary Materials and added the statistical information in the figure legends.

2. Section 4.6 "Treatment of non-transfected ARPE-19/RPE cells with conditioned medium."
   It is not clear how the conditioned medium was prepared. Additional information on preparation methods should be provided. Were the ARPE-/RPE cells incubated with 100% conditioned medium?

See comments Reviewer#1 item 11.

3. The authors used only two cell densities (5,000 and 10,000 cells/well) and one time point (24 hrs) for analysis of the effects of H_2O_2 treatment (Fig. 4) and concluded, "the optimal experimental parameters for the induction of oxidative stress in human RPE cells are an incubation of 3,000-5,000 cells for 24h". How could they reach this conclusion, if 3,000 cells/well was not used in their experiments and no other time points were tested? More detailed analysis should be conducted before determining the optimal condition.

We re-wrote the sentence as follows: "For experiments to determine the effect of antioxidants of H_2O_2-generated ROS, it is essential to consider the number of cells; for the specific protocol presented in this report 3,000-5,000 cells/well (96-well plates) treated for 24 h with 350 µM H_2O_2 are appropriate to show significant cell damage while retaining the capacity to recover mimicking a sub-acute response to oxidative stress-induced cell damage" (lines 399-404 of the revised manuscript). Additionally, the choice of these parameters has been discussed in more detail in the revised article (lines 588-593 of the revised manuscript).

4. Plasmid constructs (Supplementary Materials, Section 1.1)
   It is not entirely clear how pT2-CMV-BMCSF-His plasmid was constructed. The terms and phrases used in this section do not appear to be standard ones used in regular molecular biology papers and are somewhat confusing. For example:
   * What is the pT2-CMV-//-His construct? Is it an empty vector obtained from another lab or an intermediate product made in the authors' lab (linearized vector without an insert)?
   * How were a consensus Kozak sequence and His tag added?
   * What does the "cutting sequence" mean (restriction enzyme site?)?

See comments Reviewer#2 item 11.

5. In Figure 3, the authors stated, "Cells treated with increasing concentration of H_2O_2 present less confluent culture wells and a round morphology". Whereas the figure seems to show that it was the case, the authors did not show quantitative data. In addition, what was the effect of growth factor gene expression on the number and morphology of H_2O_2-treated human RPE cells?

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We did not analyze the microscopic images quantitatively since such an analysis would not give accurate information due to the variability in cell shape between treatments, e.g. round versus spread cells, variation in cell spread, difficulty in deciding whether rounded cell were alive or dead. However, the quantification of cell viability and glutathione levels, offer more significant parameters of the effect of treatment.
To also document the anti-oxidative effect of PEDF and GM-CSF in terms of cell morphology and cell proliferation, we have added micrographs of PEDF and GM-CSF-transfected cells to Figure 3.

Minor Concerns:

6. I would recommend that the paper be revised by a native English speaker to improve the grammar and readability. For example,
   * The authors use "trypsin" as a verb (e.g. lines 112, 139, etc.). It should be "trypsinize".
   * The authors call PEDF, GM-CSF, etc. "the proteins", which should be more properly referred to (e.g. the growth factors?).

Regarding the general wording, please see the Editorial comments item 1. Additionally, as suggested by the reviewer, to better define and describe PEDF and GM-CSF we have used the term "growth factors".

7. Are the units of growth factors concentrations in line 167 (500 ng/µl of PEDF and 50 ng/µl GM-CSF) correct? Their units are "ng/ml" in line 307.

Indeed there was a typing error, the correct unit is ng/mL and has been modified in the text (lines 202-203 of the revised manuscript).

8. Table 1

What does "Volume medium to trypsin" mean?

We revised the table including the title that has been modified to read "medium (mL) to stop trypsinization"

9. Figure S3

The authors stated that the values were normalized by C- (non-transfected cells without H2O2 treatment) in the text (lines 425-426), whereas they were normalized by C (non-transfected cells with H_2O_2 treatment).

We understand that our labeling was ambiguous. We have modified figure and legend accordingly. All samples are normalized to "C/UT". Notice that this figure was transferred from the Supplementary Material to the manuscript.