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Induction and Analysis of Oxidative Stress in Sleeping Beauty Transposon-Transfected Human Retinal Pigment Epithelial Cells

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TITLE:

Induction and Analysis of Oxidative Stress in *Sleeping Beauty* Transposon-Transfected Human Retinal Pigment Epithelial Cells

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KEYWORDS:

ocular gene therapy, age-related macular degeneration, oxidative stress damage, *Sleeping Beauty* transposon, non-viral gene delivery, RPE cells, PEDF, GM-CSF

SUMMARY:

We present a protocol for the development and use of an oxidative stress-model by treating retinal pigment epithelial cells with H_2O_2, analyzing cell morphology, viability, density, glutathione, and UCP-2 level. It is a useful model to investigate the antioxidant effect of proteins secreted by transposon-transfected cells to treat neuroretinal degeneration.

ABSTRACT:

Oxidative stress plays a critical role in several degenerative diseases, including age-related macular degeneration (AMD), a pathology that affects ~30 million patients worldwide. It leads to a decrease in retinal pigment epithelium (RPE)-synthesized neuroprotective factors, e.g., pigment epithelium-derived factor (PEDF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), followed by the loss of RPE cells, and eventually photoreceptor and retinal ganglion cell (RGC) death. We hypothesize that the reconstitution of the neuroprotective and neurogenic retinal environment by the subretinal transplantation of transfected RPE cells overexpressing PEDF and GM-CSF has the potential to prevent retinal degeneration by mitigating the effects of oxidative stress, inhibiting inflammation, and supporting cell survival. Using the *Sleeping Beauty* transposon system (*SB100X*) human RPE cells have been transfected with the *PEDF* and *GM-CSF*

genes and shown stable gene integration, long-term gene expression, and protein secretion using qPCR, western blot, ELISA, and immunofluorescence. To confirm the functionality and the potency of the PEDF and GM-CSF secreted by the transfected RPE cells, we have developed an in vitro assay to quantify the reduction of H_2O_2-induced oxidative stress on RPE cells in culture. Cell protection was evaluated by analyzing cell morphology, density, intracellular level of glutathione, *UCP2* gene expression, and cell viability. Both, transfected RPE cells overexpressing PEDF and/or GM-CSF and cells non-transfected but pretreated with PEDF and/or GM-CSF (commercially available or purified from transfected cells) showed significant antioxidant cell protection compared to non-treated controls. The present H_2O_2-model is a simple and effective approach to evaluate the antioxidant effect of factors that may be effective to treat AMD or similar neurodegenerative diseases.

INTRODUCTION:

The model described here, offers a useful approach to evaluate the efficiency of biopharmaceutical agents for reducing oxidative stress in cells. We have used the model to investigate the protective effects of PEDF and GM-CSF on the H_2O_2-mediated oxidative stress on retinal pigment epithelial cells, which are exposed to high levels of O_2, and visible light, and the phagocytosis of photoreceptor outer segment membranes, generating significant levels of reactive oxygen species (ROS)[1,2]. They are considered a major contributor to the pathogenesis of avascular age-related macular degeneration (aAMD)[3–8]. Besides, there is a decrease in RPE-synthesized neuroprotective factors, specifically the pigment epithelium-derived factor (PEDF), insulin-like growth factors (IGFs), and granulocyte macrophage-colony-stimulating factor (GM-CSF) leading to the dysfunction and loss of RPE cells, followed by photoreceptor and retinal ganglion cell (RGC) death[3–5]. AMD is a complex disease that results from the interaction between metabolic, functional, genetic, and environmental factors[4]. The lack of treatments for aAMD is the major cause of blindness in patients older than 60 years of age in industrialized countries[9,10]. The reconstitution of the neuroprotective and neurogenic retinal environment by the subretinal transplantation of genetically modified RPE cells overexpressing PEDF and GM-CSF has the potential to prevent retinal degeneration by mitigating the effects of oxidative stress, inhibiting inflammation and supporting cell survival[11–16]. Even though there are several methodologies to deliver genes to cells, we have chosen the non-viral hyperactive *Sleeping Beauty* transposon system to deliver the *PEDF* and *GM-CSF* genes to RPE cells because of its safety profile, the integration of the genes into the host cells' genome, and its propensity to integrate the delivered genes in non-transcriptionally active sites as we have shown previously[17–19].

Cellular oxidative stress can be induced in cells cultured in vitro by several oxidative agents, including hydrogen peroxide (H_2O_2), 4-hydroynonenal (HNE), tertbutylhydroperoxide (tBH), high oxygen tensions, and visible light (full spectrum or UV irradiation)[20,21]. High oxygen tensions and light require special equipment and conditions, which limits transferability to other systems. Agents such as H_2O_2, HNE, and tBH induce overlapping oxidative stress molecular and cellular changes. We chose H_2O_2 to test the antioxidant activity of PEDF and GM-CSF because it is convenient and biologically relevant since it is produced by RPE cells as a reactive oxygen intermediate during photoreceptor outer segment phagocytosis[22] and it is found in ocular tissues in vivo[23]. Since the oxidation of glutathione may be partially responsible for the production of

H_2O_2 in the eye, we have analyzed the levels of GSH/glutathione in our studies, which are linked to H_2O_2-induced oxidative stress and the regenerative capacity of cells[21,22]. The analysis of glutathione levels is especially relevant since it participates in the anti-oxidative protective mechanisms in the eye[24]. Exposure to H_2O_2 is used frequently as a model to examine the oxidative stress susceptibility and antioxidant activity of RPE cells[1,25–30], and, additionally, it shows similarities to light-induced oxidative stress damage, a "physiological" source of oxidative stress[21].

To evaluate the functionality and the effectiveness of neuroprotective factors, we have developed an in vitro model that allows for the analysis to quantify the anti-oxidative effect of growth factors expressed by cells genetically modified to overexpress PEDF and GM-CSF. Here, we show that RPE cells transfected with the genes for PEDF and GM-CSF are more resistant to the harmful effects of H_2O_2 than are non-transfected control cells, as evidenced by cell morphology, density, viability, intracellular level of glutathione, and expression of *UCP2* gene, which codes for the mitochondrial uncoupling protein 2 that has been shown to reduce reactive oxygen species (ROS)[31].

PROTOCOL:

Procedures for the collection and use of human eyes were approved by the Cantonal Ethical Commission for Research (no. 2016-01726).

1. Cell isolation and culture conditions

1.1. Human ARPE-19 cell line

1.1.1. Culture 5 x 10^5 ARPE-19 cells, a human RPE cell line, in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/Ham´s F-12) supplemented with 10% fetal bovine serum (FBS), 80 U/mL penicillin, 80 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (complete medium) at 37 ˚C in a humidified atmosphere of 5% CO_2 and 95% air in a T75 flask (for other cell densities see Table 1).

1.1.2. Change the medium three times per week.

1.1.3. Once the cells are grown to approximately 90% confluence (evaluated qualitatively), aspirate the medium and wash the cells with sterile 1x PBS.

1.1.4. Incubate the cells with a 5% Trypsin-2% EDTA solution for 7–10 min at 37 °C (for volumes see **Table 1**). Monitor detachment visually.

1.1.5. Stop trypsinization by adding complete medium containing 10% FBS (for volumes see **Table 1**).

1.1.6. Transfect the cells (see step 2. of the protocol), sub-cultivate the cells at a ratio of 1:10

(once per week), or seed in a 96-well plate as detailed below (see steps 3.3 and 3.4 of the protocol).

[Place **Table 1** here]

1.2. Primary human RPE cells

1.2.1. Isolate primary human RPE cells as described by Thumann et al.[17], and culture cells in complete medium supplemented with 20% FBS.

1.2.2. Change the medium twice per week. Once the cells reach confluency (monitored visually), reduce FBS to 1% to avoid overgrowth.

1.2.3. Transfect the cells (see step 2 of the protocol), or seed in a 96-well plate as detailed below (see steps 3.3 and 3.4 of the protocol).

NOTE: Data presented here were obtained from the culture of RPE cells obtained from the eyes of four human donors. **Table 2** details the demographics of the donors from the Lions Gift of Sight Eye Bank (Saint Paul, MN). The eyes were enucleated 12.7 ± 5.7 h (mean ± SD) post-mortem after informed consent was obtained in accordance with the Declaration of Helsinki.

[Place **Table 2** here]

2. Electroporation of ARPE-19 and primary human RPE cells

2.1. Trypsinize ARPE-19 cells or primary human RPE cells as described in steps 1.1.3–1.1.5 of the protocol.

2.2. Perform electroporation with the commercially available transfection kit (see **Table of Materials**).

2.2.1. For transfection of ARPE-19 cells refer to Johnen et al.[32] and for primary hRPE to Thumann et al.[17]. Briefly, resuspend 1 x 10^5 ARPE-19 cells or 5 x 10^4 primary hRPE cells in 11 µL of R buffer and add 2 µL of plasmid mixture containing 0.03 µg pSB100X transposase[33] and 0.47 µg pT2-CMV-*PEDF*-His or pT2-CMV-*GMCSF*-His transposon (ratio transposase:transposon 1:16). For PEDF and GM-CSF double transfected cells, use a ratio of 1:16:16 (0.03 µg pSB100X, 0.47 µg pT2-CMV-*PEDF*-His, and 0.47 µg pT2-CMV-*GMCSF*-His). Use the following electroporation parameters: two pulses of 1,350 V for 20 ms (pulse width) for ARPE-19 cells; two pulses of 1,100 V for 20 ms for primary cells.

2.3. Seed 1 x 10^5 transfected ARPE-19 or 5 x 10^4 transfected primary hRPE cells in 6-well and 24-well plates, respectively, in medium supplemented with 10% FBS without antibiotics or antimycotics. Add penicillin (80 U/mL), streptomycin (80 µg/mL), and amphotericin B (2.5 µg/mL) with the first medium exchange 3 days after transfection.

2.4. Determine cell growth by weekly microscopical monitoring of the cells. Transfection efficiency is monitored by the analysis of gene expression by RT-PCR, and protein secretion by ELISA and WB (methods explained in **Supplementary Material**).

NOTE: Transfection efficiency can be evaluated for the first time once the cells reach confluency, i.e., at ~7 days and 4 weeks post-transfection for ARPE-19 cells and primary hRPE cells, respectively.

2.5. Seed cells in a 96-well plate as detailed below (see step 3.5 of the protocol).

3. Oxidative stress induction (H_2O_2 treatment) and neuroprotection (PEDF and/or GM-CSF treatment)

3.1. **Preparation of conditioned medium of transfected ARPE-19 cells**

3.1.1. Use ARPE-19 cells transfected with the genes *PEDF*, *GM-CSF,* or both (see step 2 of the protocol); culture cells for 28 days as described in step 1.1 of the protocol.

3.1.2. At 28 days post-transfection, trypsinize cells (see steps 1.1.3–1.1.5 of the protocol), count cells using a Neubauer chamber[34, 35], and seed 5 x 10^5 cells in T75 flasks in complete medium as described in step 1.1.1 of the protocol. Exchange the medium when the cell culture is approximately 80% confluent (approximately after 1 week; verified qualitatively). Collect the medium after 24 h.

3.1.3. Store the medium at -20 °C until use.

NOTE: Sufficient concentration of the recombinant PEDF and GM-CSF in the conditioned medium was verified by WB and quantified by ELISA as described in **Supplementary Material**.

3.2. **Purification of PEDF and GM-CSF from conditioned medium of transfected ARPE-19 cells**

3.2.1. Centrifuge the collected medium from step 3.1.2 at 10,000 x *g* for 15 min at 4 °C.

3.2.2. Use the Ni-NTA superflow (see **Table of Materials**) according to the manufacturer's protocols to purify His-tagged proteins as described below.

3.2.2.1. Pipette 30 µL of Ni-NTA mixture into a 1.5 mL tube and centrifuge at 2,600 x *g* for 30 s and discard the flow-through. Wash the pellet twice with 200 µL of 1x incubation buffer.

3.2.2.2. Centrifuge at 2,600 x *g* for 30 s and discard the flow-through. Add 40 µL of 4x Incubation buffer and resuspend.

3.2.2.3. Add 900 µL of centrifuged conditioned medium and incubate at 70 rpm (orbital shaker)

for 1 h at RT. Centrifuge at 2,600 x *g* for 1 min and the discard flow-through.

3.2.2.4. Wash the pellet twice with 175 µL of 1x incubation buffer. Centrifuge at 2,600 x *g* for 30 s and discard the flow-through.

3.2.2.5. To elute His-tagged PEDF and GM-CSF proteins, add 20 µL of Elution buffer and incubate at 70 rpm (orbital shaker) for 20 min at RT. Centrifuge at 2,600 x *g* for 30 s. Keep the supernatant containing recombinant PEDF or GM-CSF.

3.2.3. Quantify the total protein using the commercially available BCA protein assay kit (see **Table of Materials**) according to the manufacturer's instructions.

3.2.4. Store the protein solution at -20 °C until use.

NOTE: Incubation buffer (4x) contains 200 mM NaH_2PO_4, 1.2 M NaCl, and 40 mM Imidazol; Elution buffer contains 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM Imidazol.

3.3. **Treatment of non-transfected ARPE-19/primary hRPE cells with conditioned medium plus H_2O_2 (Figure 1A)**

3.3.1. Seed 3,000 non-transfected ARPE-19 (from step 1.1.6 of the protocol) or primary hRPE (from step 1.2.3 of the protocol) cells per well in 96-well plate and culture in 200 µL of conditioned medium from transfected ARPE-19 cells.

3.3.2. Culture the cells for 10 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Change the conditioned medium every day. Expose the cells to 350 µM H_2O_2 for 24 h.

3.3.3. Evaluate oxidative stress damage and determine the antioxidant effect of PEDF and GM-CSF by quantification of glutathione levels (see step 4.1 of the protocol), microscopy (see step 4.2 of the protocol), and cytotoxicity assay (see step 4.2 of the protocol).

NOTE: The duration of the experiment is 12 days. Clear flat bottom microwell plates are used to evaluate luminescence as well as cell morphology. To simultaneously perform the cytotoxicity and glutathione assay, two plates must be seeded with cells on the same day.

3.4. **Treatment of non-transfected ARPE-19/primary hRPE cells with PEDF and GM-CSF growth factors plus H_2O_2 (Figure 1B)**

3.4.1. Seed 3,000 non-transfected ARPE-19 (from step 1.1.6 of the protocol) or primary hRPE (from step 1.2.3 of the protocol) cells per well (96-well plates with a clear flat bottom) in 200 µL of complete culture medium containing 500 ng/mL recombinant PEDF and/or 50 ng/mL recombinant GM-CSF, purified from the medium of transfected ARPE-19 cells or commercially available. Culture cells for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Renew the medium including PEDF and GM-CSF growth factors daily.

NOTE: Add the growth factors fresh to the medium.

3.4.2. After 48 h of treating the cells with the growth factors, remove the medium and add complete medium containing 350 µM H_2O_2 plus 500 ng/mL PEDF and/or 50 ng/mL GM-CSF.

3.4.3. Evaluate oxidative stress damage and determine the antioxidant effect of PEDF and GM-CSF by quantification of glutathione levels (see step 4.1 of the protocol), microscopy (see step 4.2 of the protocol), and cytotoxicity assay (see step 4.2 of the protocol).

NOTE: The duration of the experiment is 3 days.

3.5. **Treatment of transfected ARPE-19/primary hRPE cells with H_2O_2 (Figure 1C)**

3.5.1. Verify sufficient gene expression and protein secretion of transfected cells by WB and ELISA as described in the **Supplementary Material**.

3.5.2. Remove the medium from the wells containing the transfected cells (see step 2 of the protocol).

3.5.3. Trypsinize cells as described in steps 1.1.3–1.1.5 of the protocol. Count the cells using a Neubauer chamber[34, 35].

3.5.4. Seed 5,000 transfected cells/well in 96-well plate in 200 µL of complete medium. Culture cells for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, expose the cells to 350 µM H_2O_2 for 24 h.

3.5.5. Evaluate oxidative stress damage and determine the antioxidant effect of PEDF and GM-CSF by quantification of glutathione levels (see step 4.1 of the protocol), microscopy (see step 4.2 of the protocol), cytotoxicity assay (see step 4.2 of the protocol), and determination of *UCP2* gene expression (see step 4.3 of the protocol).

NOTE: The duration of the experiment is 2 days.

[Place **Figure 1** here]

4. Analysis of oxidative stress level and antioxidant capacity

4.1. **Glutathione assay**

4.1.1. Measure the Glutathione (GSH) levels using the commercially available kit (see **Table of Materials**) following the manufacturer's instructions. Briefly, prepare and appropriate volume of 1x Reagent mix (100 µL reagent/well): Luciferin-NT substrate and Glutathione S-Transferase diluted 1:100 in Reaction Buffer.

NOTE: A 96-well plate requires 10 mL of 1x Reagent mix, which is prepared by adding 100 µL of Luciferin-NT substrate and 100 µL of Glutathione S-Transferase to 10 mL of Reaction buffer. Prepare the 1x Reagent mix immediately before use. Do not store prepared Reagent mix for future use.

4.1.2. Prepare the Luciferin Detection Reagent by transferring one bottle of Reconstitution buffer to the lyophilized Luciferin Detection Reagent.

4.1.3. Prepare a standard curve using a Glutathione (GSH) standard solution (5 mM). Dilute 5 mM GSH solution 1:100 with dH_2O (add 10 µL of 5 mM GSH solution to 990 µL of dH_2O). Perform 7 serial 1:1 dilution in 500 µL of dH_2O. Transfer 10 µL of each diluted standard to an appropriate well in duplicate.

NOTE: The final concentration of glutathione will range from 0.039 µM to 5 µM.

4.1.4. Prepare the blank (1x Reagent mix) and transfer 10 µL (duplicates) to the appropriate wells.

4.1.5. Remove the H_2O_2-treated cells from the incubator.

NOTE: Document the morphology of the H_2O_2-treated cells by brightfield microscopy (40x). When the cells are oxidated, they look more rounded and less spread.

4.1.6. Carefully aspirate the culture medium. Add 100 µL of prepared 1x Reagent mix to each well. Mix the cells with the reagent for 15 s at 500 rpm on an orbital shaker.

4.1.7. Incubate the plate at RT for 30 min. Add 100 µL of reconstituted Luciferin Detection Reagent to each well.

4.1.8. Mix the solution for 15 s at 500 rpm on an orbital shaker. Incubate the plate for 15 min at RT.

4.1.9. Determine luminescence using a plate reader using a pre-installed program ADP-Glo.

NOTE: Put the plate inside the plate reader without the lid.

4.1.9.1. Click on **Change Layout** and choose the following settings in **Basic Parameters**: Costar 96-well plate; top optic; positioning delay: 0.1; measurement start time: 0.0; measurement interval time: 1.0; time to normalize the results: 0.0; the gain is adjusted automatically by the device. Define blanks, standards, and samples. Click on **Start Measurement**.

4.1.9.2. Export the data as an Excel file. Calculate the concentration of GSH in each sample by interpolation of the standard curve.

4.2. **Cytotoxicity assay and microscopic analysis**

4.2.1. Aspirate the medium from the cells and add 100 µL of complete medium containing 1% FBS to each well. Return the cells to the incubator.

NOTE: 1% FBS is used because Higher percentages of FBS can interfere with the measurement of the luminescence, therefore 1% FBS is used in this case.

4.2.2. Measure cell viability using the commercially available cytotoxicity assay kit (see **Table of Materials**) following the manufacturer's instructions. Briefly, prepare the Reagent mix adding the Assay buffer to the lyophilized Substrate. Prepare the Lysis Reagent by adding 33 µL Digitonin to 5 mL Assay buffer (for one 96-well plate). Mix well by pipetting up and down to ensure homogeneity.

NOTE: For optimal results, use freshly prepared Reagent mix. Use within 12 h if stored at RT. Reagent mix can be stored at 4 °C for up to 7 days and may be stored in single-use aliquots for up to 4 months at -70 °C. Freezing and thawing must be avoided. The Lysis Reagent can be stored at 4 °C for up to 7 days.

4.2.3. Prepare a standard curve with untreated ARPE-19 cells.

4.2.3.1. Trypsinize the cells as described in steps 1.1.3–1.1.5 of the protocol and count the cells using a Neubauer chamber[34, 35]. Centrifuge the cells at 120 *g* for 10 min at RT. Aspirate the supernatant and resuspend the cell pellet in DMEM/Ham's F12 medium containing 1% FBS to a final concentration of 1 x 10^5 cells/mL.

4.2.3.2. Prepare 7 serial 1:1 dilutions in 200 µL medium containing 1% FBS. Transfer 100 µL of each standard to the appropriate wells (duplicates). Add 50 µL of Reagent mix to all the wells.

4.2.4. Mix the cells with the reagent for 15 s at 500 rpm on an orbital shaker. Incubate the plate for 15 min at RT. Measure luminescence using the plate reader as described in step 4.1.9 of the protocol. Add 50 µL of the lysis reagent and incubate for 15 min. Measure luminescence using the plate reader as described in step 4.1.9 of the protocol.

4.2.5. Calculate the percentage of viable cells: (100 - % dead cells) and the percentage of dead cells = [1st luminescence measurement ((dead cells in the sample))/ 2nd luminescence measurement (all cells dead after digitonin treatment)] x 100.

4.3. ***UCP2*** **expression analysis by RT-qPCR**

4.3.1. Trypsinize transfected cells as described above (steps 1.1.3–1.1.5 of the protocol).

4.3.2. Count the cells using a Neubauer chamber[34, 35].

4.3.3. Seed 5,000 transfected ARPE-19 cells/well in 96-well plates.

4.3.4. After 24 h of culture, treat the cells with 350 µM H_2O_2 for 24 h.

4.3.5. Isolate total RNA using a commercial kit for isolation of RNA from low number of cells (see **Table of Materials**) following the manufacturer's instruction.

4.3.6. Perform Real-Time quantitative PCR (RT-qPCR) as described in **Supplementary Material**. Briefly, generate cDNA by retrotranscription using a commercially available mix containing an optimized M-MLV Reverse Transcriptase (see **Table of Materials**).

4.3.7. For qPCR employ a ready-to-use reaction cocktail containing all components (including SYBR Green) except primers (see **Table S1** of **Supplementary Material**) and DNA template. Use the following thermocycling conditions: initial denaturation at 95 °C for 10 min, 40 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 32 s.

4.3.8. Use 2^(-ΔΔCT) method for analysis[36].

4.4. **Preparation of cell lysate for SDS-PAGE and WB analysis of pAkt (Ser473)**

4.4.1. Seed 3 x 10^5 GM-CSF-transfected ARPE-19 cells/well in 6-well plates (≥21 days post transfection) to determine whether GM-CSF protects RPE cells from damage by H_2O_2 through the activation of the Akt survival pathway[15].

4.4.2. After 24 h of culture cells are exposed to 350 µM H_2O_2 for 24 h.

4.4.3. Mix 1 mL of RIPA buffer with 10 µL of protease phosphatase inhibitor cocktail, 10 µL of 0.5 M EDTA, and 25 µL of 8 M urea (volumes used for one well).

4.4.4. Carefully aspirate medium and wash the cells with 1x PBS.

4.4.5. Add the entire volume of RIPA buffer mix to the cells.

4.4.6. Pipette up and down.

4.4.7. Collect the lysate in 1.5 mL tubes.

4.4.8. Centrifuge at 20,000 x *g* for 30 min at 4 °C.

4.4.9. Transfer the supernatant to a new 1.5 mL tube.

4.4.10. Determine the levels of pAkt in 15 µL of undiluted cell lysate by WB as described in **Supplementary Material**.

REPRESENTATIVE RESULTS:

Induction of oxidative stress in human Retinal Pigment Epithelial cells

ARPE-19 and primary hRPE cells were treated with varying concentrations of H_2O_2 for 24 h and the intracellular level of the antioxidant glutathione was quantified (**Figure 2A**,**B**). H_2O_2 at 50 µM and 100 µM did not affect glutathione production, whereas at 350 µM there was a significant decrease of glutathione in ARPE-19 and primary hRPE cells. Analysis of cytotoxicity showed that 350 µM is the lowest concentration of H_2O_2 that causes a significant decrease in cell viability (**Figure 2C**). Morphologically, ARPE-19 cells treated with H_2O_2 appear less spread and more rounded, characteristics that become more obvious with increasing H_2O_2 concentration (**Figure 3**). The effect was less prominent for PEDF- and GM-CSF-transfected cells treated with H_2O_2 (**Figure 3**). To demonstrate the effect of cell number on H_2O_2-mediated oxidative stress, 5,000 and 10,000 ARPE-19 cells per well were seeded in a white 96-well plate; the day after, cells were treated with 350 µM H_2O_2 for 24 h and the levels of glutathione were determined. **Figure 4** shows that the level of glutathione was decreased only in the wells (n = 3) seeded with 5,000 cells. 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.

[Place **Figure 2** here]

[Place **Figure 3** here]

[Place **Figure 4** here]

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

As positive controls, ARPE-19 and primary human RPE cells were treated with 5, 50, or 500 ng/mL commercially available PEDF or GM-CSF for 2 days before and during the 24 h H_2O_2 treatment. ARPE-19 cells treated with 500 ng/mL PEDF or 50 ng/mL GM-CSF produced significantly more glutathione compared to untreated controls under oxidative conditions (H_2O_2-treated) (**Figure 5A**); comparable PEDF and GM-CSF purified from culture media of transfected ARPE-19 cells showed a similar effect (**Figure 5B**). In primary hRPE cells, the addition of 500 ng/mL PEDF, 50 ng/mL GM-CSF, or 500 ng/mL PEDF plus 50 ng/mL GM-CSF whether commercial or purified from media conditioned by PEDF- or GM-CSF transfected ARPE-19 cells reduced cell damage as reflected by a significant increase in glutathione levels (**Figure 5C**). Primary hRPE cells treated for 10 days with conditioned medium from transfected ARPE-19 cells also showed higher glutathione levels compared to control cells (**Figure 5D**). Based on these results, further experiments have been done with 500 ng/mL for PEDF and 50 ng/mL for GM-CSF.

ARPE-19 and primary hRPE cells were transfected with the genes coding for PEDF and/or GM-CSF using the *Sleeping Beauty* transposon system combined with electroporation. Following

transfection and analysis of gene expression by RT-qPCR, WB, ELISA, and immunohistochemistry (see **Supplementary Material**, **Figure S1**, and **Figure S2**), transfected ARPE-19 cells exposed to 350 µM H_2O_2 for 24 h showed significant higher glutathione levels than non-transfected H_2O_2-treated cells (**Figure 6A**). For primary hRPE cells, there is a significant increase in glutathione levels in PEDF-transfected cells compared with non-transfected cells treated with H_2O_2 when all donors were included in the analysis. Moreover, donors 2 and 3 show a significant increase in glutathione levels for all transfected groups (PEDF, GM-CSF, PEDF, and GM-CSF) (data not shown).

The study of the *UCP2* gene expression completed the analysis by examination of mitochondrial oxidative stress. A proof-of-concept series was carried out in transfected ARPE-19 cells treated with 350 µM H_2O_2 for 24 h. As shown in **Figure 7**, in transfected ARPE-19 cells, the levels of *UCP2* gene expression after H_2O_2 treatment are increased but the increase is not statistically significant. **Figure 8** shows a WB of phosphorylated Akt (pAkt) from a lysate of GM-CSF-transfected cells exposed to H_2O_2; the normalized data shows only a small decrease compared with the untreated control, indicating that GM-CSF can protect the cells from oxidative stress damage.

[Place **Figure 5** here]

[Place **Figure 6** here]

[Place **Figure 7** here]

[Place **Figure 8** here]

FIGURE AND TABLE LEGENDS:

Table 1: Cell culture volumes. Recommended media volumes for cell culture plates and flasks for the culture of ARPE-19 and primary human RPE cells.

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

Figure 1: Timelines of the H_2O_2 assay in the three different experimental approaches. 3,000 non-transfected cells treated with the conditioned medium/recombinant proteins or 5,000 transfected cells were seeded in 96-well plates for treatment with H_2O_2. To determine the effect of conditioned medium, cells were cultured in 100% cultured medium for 10 consecutive days, changing medium every day. To determine the effect of recombinant growth factors, cells were cultured by adding the appropriate amount of growth factors each day for 3 consecutive days. Note that non-transfected cells were seeded at 3,000 cells per well to avoid overgrowth during the longer culture duration compared to transfected cells.

Figure 2: Oxidative stress level evidenced as glutathione level and cell viability, in human RPE cells treated with H_2O_2. (**A**) ARPE-19 cells exposed to several concentrations of H_2O_2 showed significantly decreased glutathione levels (in brackets) at 350 µM (0.66 µM), 500 µM (0.022 µM),

and 700 µM (0.002 µM) compared to H_2O_2-non-treated cells (2.9 µM) ($p < 0.0001$ for 350, 500, and 700 µM H_2O_2). (**B**) Primary human RPE cells showed decreased levels of glutathione; however, the effect was less prominent than for ARPE-19 but still statistically significant compared to the controls at 350, 500, and 700 µM H_2O_2. 350 µM was the lowest H_2O_2 concentration that produced significant oxidative damage as shown by decreased glutathione levels compared with non-treated control cells ($p = 0.0022$). Glutathione levels decreased with increasing H_2O_2 concentrations (500 µM: $p = 0.022$; 700 µM: $p = 0.0005$). (**C**) Cytotoxicity analysis showed that 350 µM H_2O_2 was the lowest concentration that produced a significant decrease in the percentage of viable cells ($p < 0.0001$ for 350, 500, and 700 µM). Data is presented as mean ± SD (n = 3 replicates) and significant differences are indicated with (*); post-hoc calculations of the ANOVA were performed using Tukey's multi-comparison test comparing C- with the H_2O_2-treatment groups. C-: H_2O_2 non-treated cells. This figure has been modified from Bascuas et al.[37].

Figure 3: Morphology of non-transfected and PEDF- or GM-CSF-transfected ARPE-19 cells treated with H_2O_2. Cells treated with increasing concentrations of H_2O_2 show fewer cells in the culture wells and display a more rounded, less spread morphology, a known sign of cellular stress. Note that for PEDF- or GM-CSF-transfected cells, cellular stress is less prominent and grow similar to non-treated control cells. C-: non-treated control cells.

Figure 4: Influence of cell number on the effect of H_2O_2-induced oxidative stress. 5,000 and 10,000 ARPE-19 cells/well were seeded in 96-well plates. After 24 h, cells were treated with 350 µM H_2O_2 for 24 h. Significant differences in glutathione levels were observed in the wells seeded with 5,000 cells ($p = 0.031$, *t*-test) but not in the wells seeded with 10,000 cells. C-: non-treated cells.

Figure 5: Glutathione level as a marker of the antioxidant capacity of PEDF and GM-CSF. (**A**) Treatment of ARPE-19 cells with 500 ng/mL PEDF or 50 ng/mL GM-CSF for 3 days before and during 24 h H_2O_2 exposure increased the level of glutathione from 0.83 µM (C) to 1.83 µM (PEDF) and 1.3 µM (GM-CSF), $p = 0.026$ and $p = 0.031$, respectively. At a concentration of 5 ng/mL no increase in glutathione was observed; the difference in the level of glutathione between 50 and 500 ng/mL was not significant for either PEDF or GM-CSF. (**B**) PEDF (500 ng/mL) and GM-CSF (50 ng/mL) purified from conditioned media of transfected ARPE-19 cells showed an effect similar to commercially available PEDF or GM-CSF ($p = 0.018$, ANOVA). (**C**) The addition of 500 ng/mL PEDF, 50 ng/mL GM-CSF, or 500 ng/mL PEDF plus 50 ng/mL GM-CSF for 3 days before and during 24 h H_2O_2 treatment to the culture medium of primary hRPE cells significantly increased the levels of glutathione in cells treated with PEDF (2.6 µM [commercial], 2.5 µM [purified]), GM-CSF (2.9 µM [commercial], 3.3 µM [purified]), and PEDF plus GM-CSF (3.0 µM [commercial], 2.9 µM [purified]) compared to non-treated cells (1.9 µM) ($p = 0.006$, Kruskal-Wallis test). (**D**) A significant increase in glutathione levels was observed for hRPE cells cultured for 10 days in conditioned medium from PEDF-, GM-CSF-, or PEDF-GM-CSF-transfected ARPE-19 cells before the cells were treated with H_2O_2 ($p = 0.003$, Kruskal-Wallis test) (data showed for one donor). Data are expressed as mean ± SD (n = 3 replicates). Significant differences are indicated with (*); post-hoc calculations of the analyses of variance were performed by calculating Tukey's or Dunnett's multi-comparison tests comparing "C" with the PEDF-/GM-CSF-treated groups. C: cells treated only with H_2O_2, P:

cells treated with PEDF, G: cells treated with GM-CSF, P+G: cells treated with PEDF plus GM-CSF. This figure has been modified from Bascuas et al.[37].

Figure 6: Glutathione level as a marker of the antioxidant capacity of PEDF- and GM-CSF-transfected human RPE cells. (**A**) The levels of glutathione of transfected ARPE-19 cells exposed to 350 µM H_2O_2 for 24 h (56 days post-transfection) were significantly higher compared to non-transfected cells (1.9 µM), i.e., 3.0 µM for PEDF- and GM-CSF-transfected cells, and 3.4 µM for double transfected cells (p = 0.0001, ANOVA). Data is expressed as mean ± SD (n = 3 replicates). (**B**) The dot plot shows the mean glutathione values for four different donors (C: 0.77 µM; P: 1.45 µM; G: 1.16 µM; P+G: 1.2 µM), which differs significantly between non-transfected and PEDF-transfected cells (p = 0.028, post-hoc calculations of the ANOVA were performed using Tukey's multi-comparison tests comparing "C" with the PEDF-/GM-CSF-treated groups). When the donors are analyzed separately, donor N°2 and N°3 (see **Table 2** for symbol in the graph) show significant differences for all transfected groups compared to the non-transfected control (significances are not shown) treated with H_2O_2. C: non-transfected cells, P: PEDF-transfected cells, G: GM-CSF-transfected cells, P+G: PEDF- and GM-CSF-transfected cells. This figure has been modified from Bascuas et al.[37].

Figure 7: *UCP2* gene expression in transfected ARPE-19 cells treated with H_2O_2. Since *UCP2* gene expression can be used to examine mitochondrial oxidative damage, we examined the effect of the overexpression of PEDF and GM-CSF by transfected ARPE-19 cells. Transfected ARPE-19 cells treated with H_2O_2, even though not statistically significant, show increased *UCP2* gene expression compared with the non-transfected control indicating oxidative stress reduction, the fold-increase was 1.57 for PEDF-, 1.51 for GM-CSF-, and 2.36 for PEDF- plus GM-CSF-transfected cells compared with the non-transfected control.

Figure 8: Western Blot of phosphorylated Akt (Ser473) from a cell lysate of GM-CSF-transfected ARPE-19 cells. The WB demonstrated that GM-CSF enhances the phosphorylation of Akt in both, untreated and H_2O_2-treated cultures (UT: 3.32; H_2O_2: 2.69). The values are normalized to non-transfected non-H_2O_2-treated cells (C/UT). C: non-transfected, G: GM-CSF-transfected cells, UT: cells non-treated with H_2O_2, H_2O_2: cells treated with H_2O_2.

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

Figure S1: *PEDF* and *GM-CSF* gene expression analysis in transfected hRPE cells. The RT-qPCR verified that transfected primary hRPE cells showed a significant increase in *PEDF* (p = 0.003, Kruskal-Wallis test) and *GM-CSF* (p = 0.013, Kruskal-Wallis test) gene expression compared with non-transfected cells. 2^(-ΔΔCT) method was used in this case[36]. Data is expressed as mean ± SD (n = 4 donors). Each dot represents the average of three replicates. This figure has been modified from Bascuas et al.[37].

Figure S2: Protein secretion in transfected primary hRPE and ARPE-19 cells. (**A**) The quantification of secreted proteins by ELISA showed that transfected hRPE cells secreted significantly more PEDF and GM-CSF than non-transfected cells (p = 0.014 for PEDF, and p = 0.006

for GM-CSF, Kruskal-Wallis test). Data is presented as mean ± SD (n = 4 donors). Each dot represents the average of three replicates. (**B**) The PEDF-GM-CSF double staining confirmed the co-secretion of PEDF and GM-CSF in double-transfected ARPE-19 cells (merged figure). This figure has been modified from Bascuas et al.[37].

DISCUSSION:

The protocol presented here offers an approach to analyze the anti-oxidative and protective function of PEDF and GM-CSF produced by transfected cells, which can be applied to cells transfected with any putative beneficial gene. In gene therapeutic strategies that have the objective to deliver proteins to tissue by transplanting genetically modified cells, it is critical to obtain information as to the level of protein expression, the longevity of expression, and the effectiveness of the expressed protein in a model of the disease. In our laboratory, the protocol presented here has been useful to define the effectiveness of PEDF and GM-CSF on oxidative stress, which has been hypothesized as an important element in the pathogenesis of aAMD[6,7]. Specifically, we have used the protocol to define the anti-oxidative effect of *SB100X*-mediated PEDF/GM-CSF-transfected primary hRPE cells. Several investigators have shown that H_2O_2 induces significant symptoms of oxidative stress but still allows cell regeneration[28,29,38], similar to the results of our experiments that have shown that 350 µM for 24 h induces effective oxidative stress in human ARPE-19 and primary RPE cells that can be used to analyze the protective effect of the PEDF and GM-CSF. H_2O_2 as oxidative agent has been chosen for the study because of its physiological presence in the eye and corresponding defense mechanisms, e.g., glutathione metabolism[20,21]. Our laboratory has examined other models of oxidative stress such as treatment of cells with tBH, which initiates lipid peroxidation in the presence of redox-active metal ions[1]; however, oxidative stress was negligible. In the experiments presented here, cells were treated with H_2O_2 for 24 h because we found that shorter treatment times of 2–6 h is sufficient to induce changes in gene expression[20], but subsequent consequences, e.g., cell proliferation, cell viability, and glutathione levels, might not be visible yet. Otherwise, the small size of the wells, necessary for the cytotoxicity and glutathione assays, rapidly leads to a confluent culture well; this might lead to contact inhibition and a masking of the effect of the oxidative agent. Therefore, a long incubation with H_2O_2 seems not useful, though the degeneration seen in aAMD is caused by chronic oxidative stress[6,7].

A limitation of the experiments presented here is that the number of cells seeded influences the oxidative effect of H_2O_2, i.e., for the same H_2O_2 treatment, significant differences in the glutathione levels between H_2O_2-treated and non-treated cells were observed when 5,000 cells but not when 10,000 cells were seeded (**Figure 4**). The protocol we present requires seeding a low number of cells, i.e., 3,000 when cells are cultured for 3 days and 5,000 when cells are cultured for 2 days (**Figure 1**). Another limitation is that the concentration of H_2O_2 is depleted with time; Kaczara et al.[39] have shown depletion of H_2O_2 over a few hours in ARPE-19 cell cultures, which affects the development of chronic oxidative stress models. These investigators have proposed an alternative method for sustained H_2O_2 treatment, specifically continuously generating H_2O_2 from glucose in the medium using the glucose oxidase, but a standardized concentration of H_2O_2 cannot be guaranteed. On the other hand, the protocol we established with delivery of the oxidant agent in one single pulse, has the advantage of being faster and

simpler to perform compared with chronic models in which the H_2O_2 treatment has to be repeated for several days[38].

The ability of cells to counteract the oxidative damage is determined by the balance between ROS production and the capacity to generate antioxidants. In the cell, the tripeptide glutathione (GSH) is the predominant reducing agent, which can be oxidized to glutathione disulfide (GSSG) and regenerated by glutathione reductase utilizing NADPH[40]. In healthy cells, more than 90% of the total glutathione pool is present in the reduced form. When cells are exposed to an increased level of oxidative stress, GSSG accumulates and the ratio of GSSG to GSH increases. Consequently, monitoring the glutathione redox state in biological samples is essential for the evaluation of the detoxification status of cells and tissues from free radicals generated during oxidative stress and cell injury. The protocol detailed here for the quantification of glutathione is sensitive enough to detect the antioxidant effect of PEDF and GM-CSF expressed by RPE cells genetically modified.

Since oxidative stress affects mitochondrial activities[40], it is particularly interesting that the control of ROS levels by PEDF is related to the regulation of the mitochondrial uncoupling protein 2 (UCP2), and PEDF attenuates the effects of oxidative stress by increasing UCP2 expression[11,41]. The main function of UCP2 is controlling mitochondria-derived ROS and acting as a sensor of mitochondrial oxidative stress[41,42]. Here, in addition to examining the effect of PEDF and GM-CSF on glutathione levels, we have the gene expression of *UCP2* tend to increase (**Figure 7**); additional studies are necessary to establish the role of PEDF and GM-CSF on *UCP2* gene expression.

Overall, the present H_2O_2-model offers a comprehensive approach to investigate the beneficial effect of transposon-based gene therapies that aim to deliver antioxidant therapeutic genes to the patient's cells to treat neurodegenerative disease as AMD.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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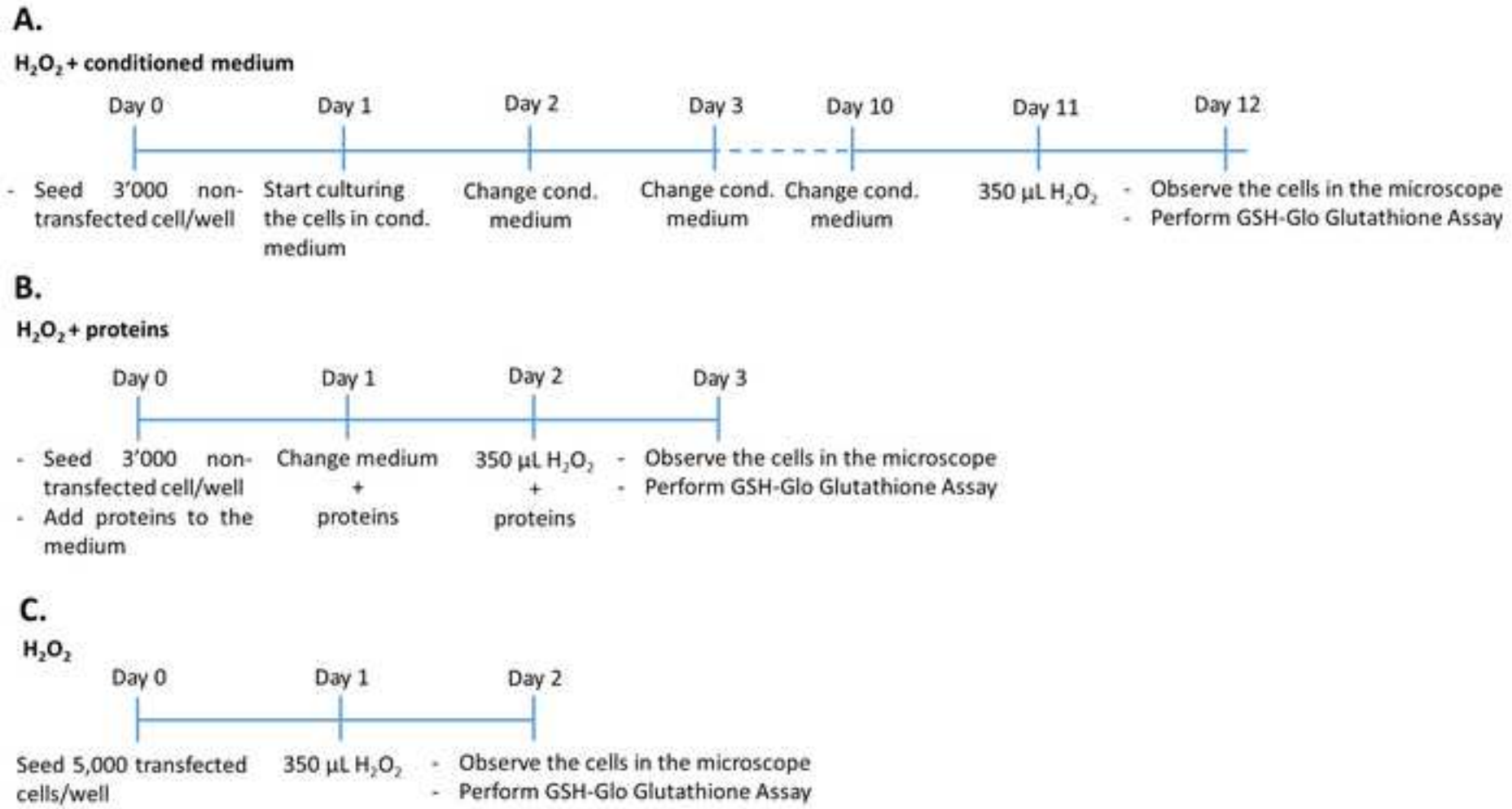

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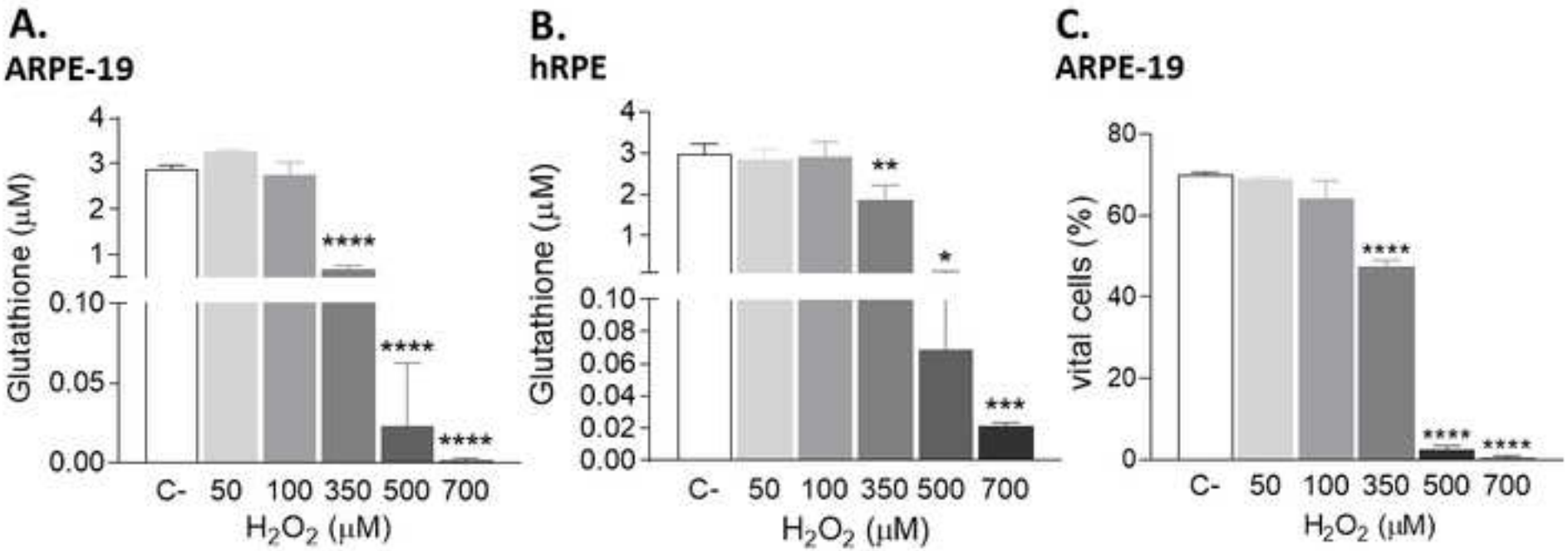

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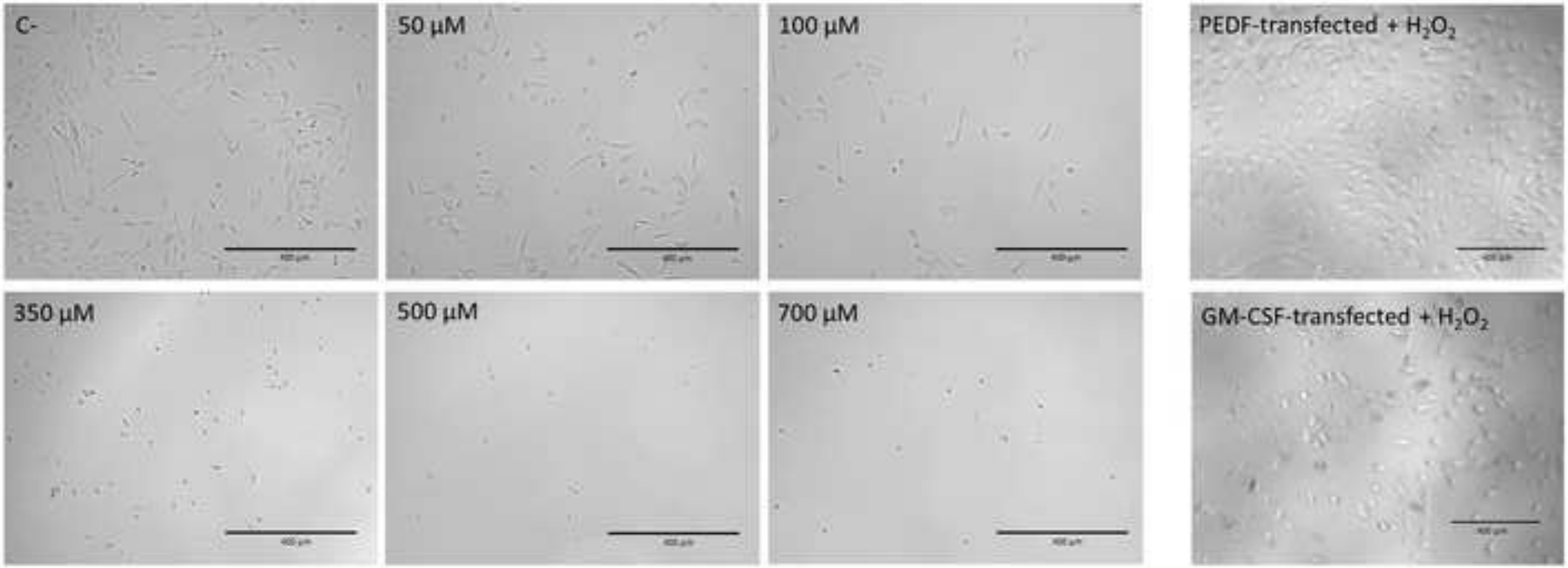

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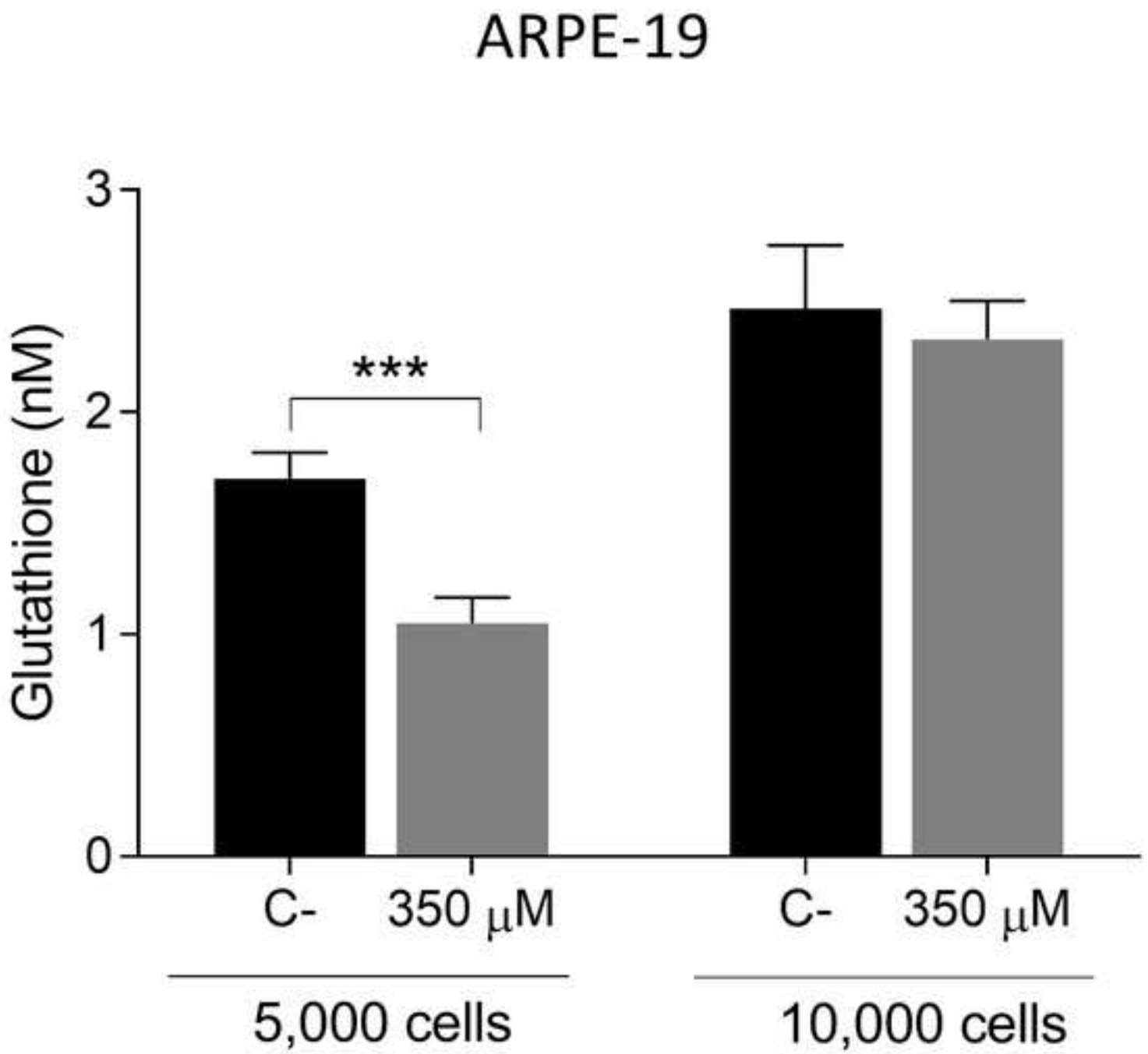

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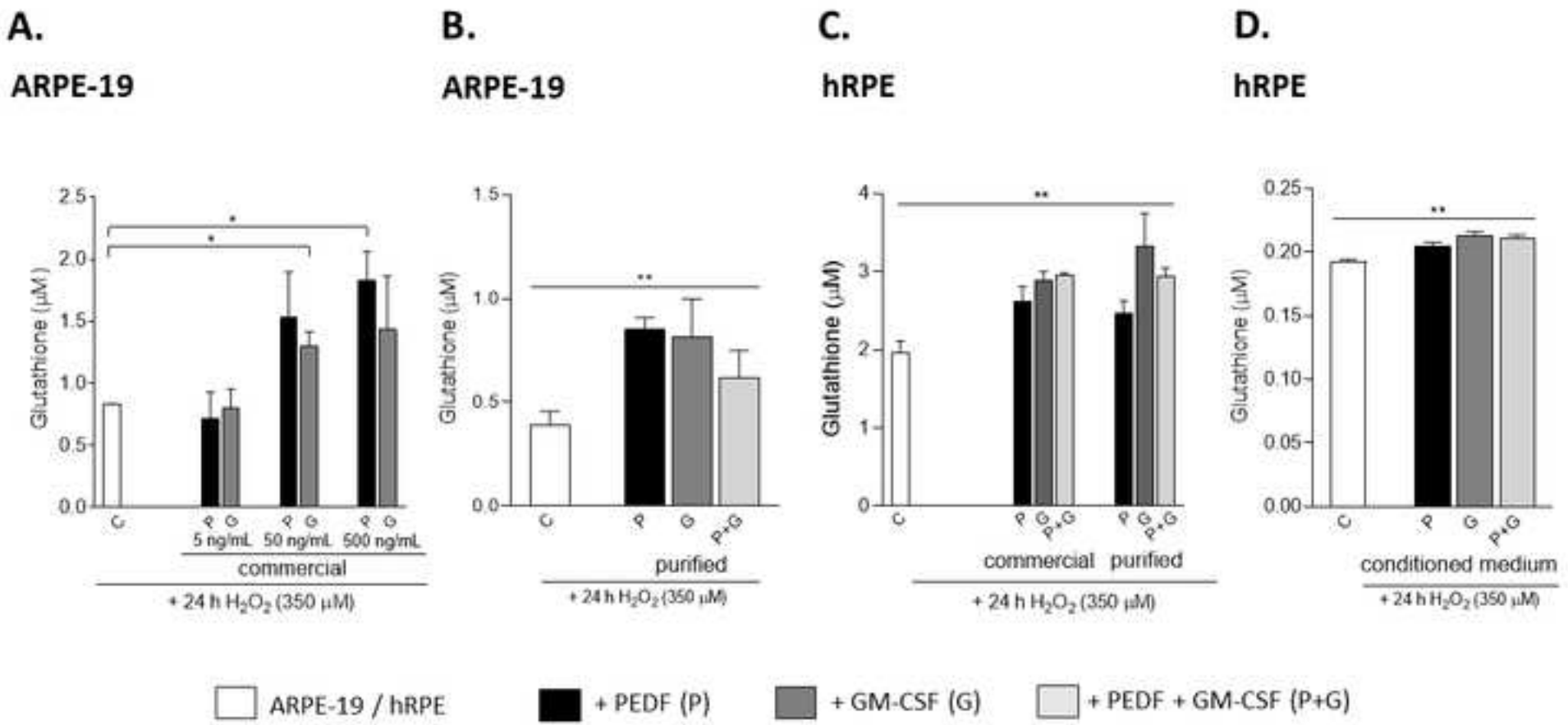

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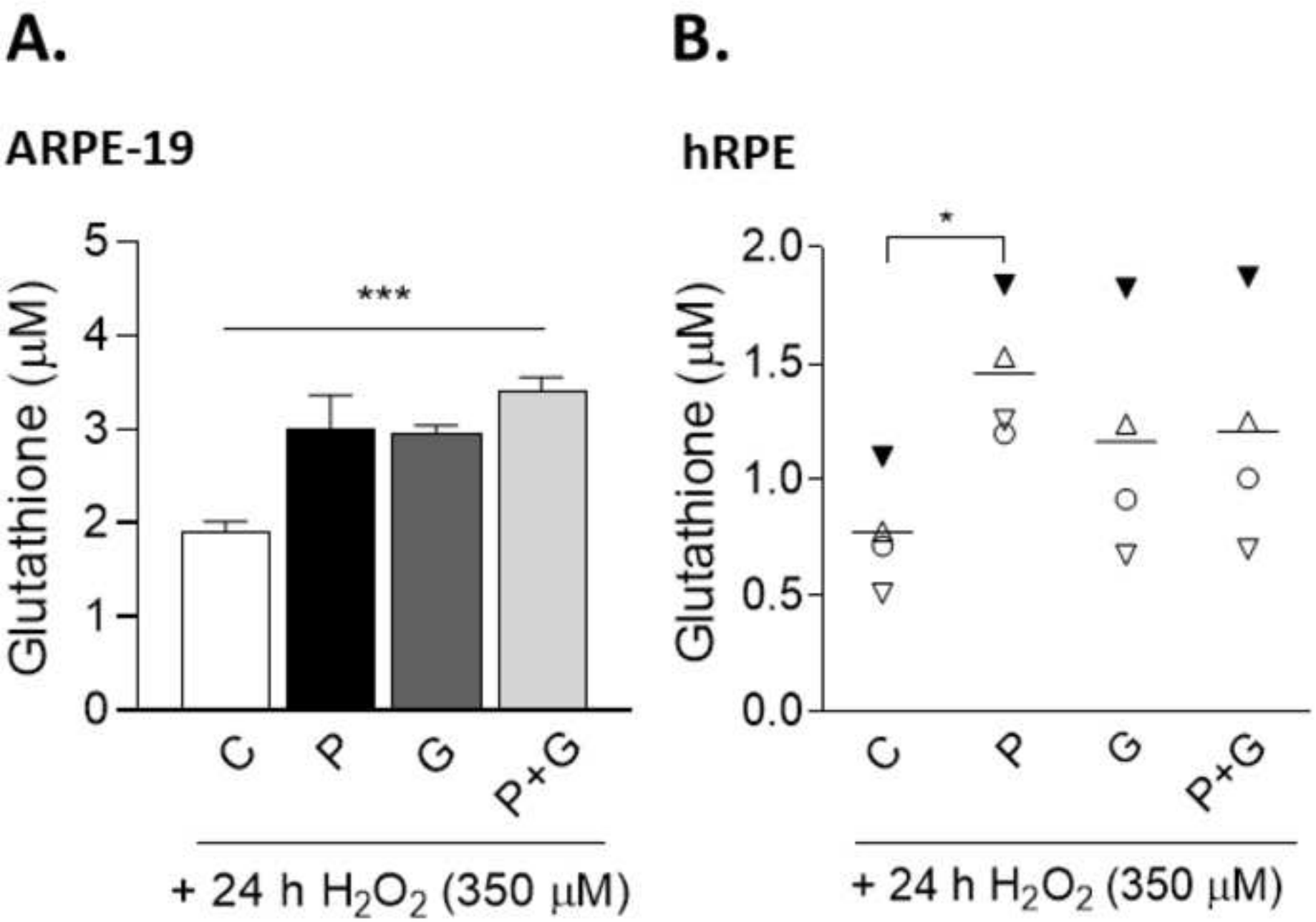

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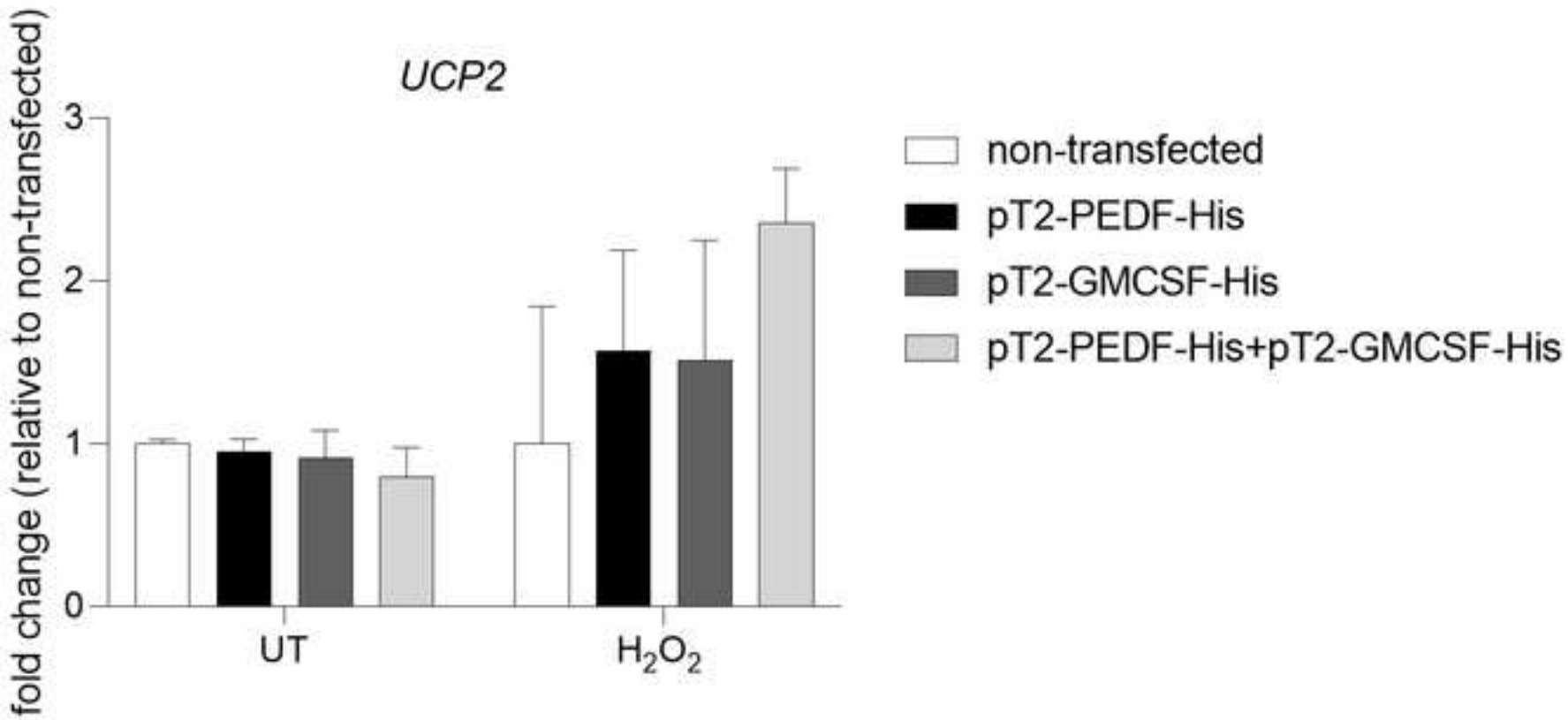

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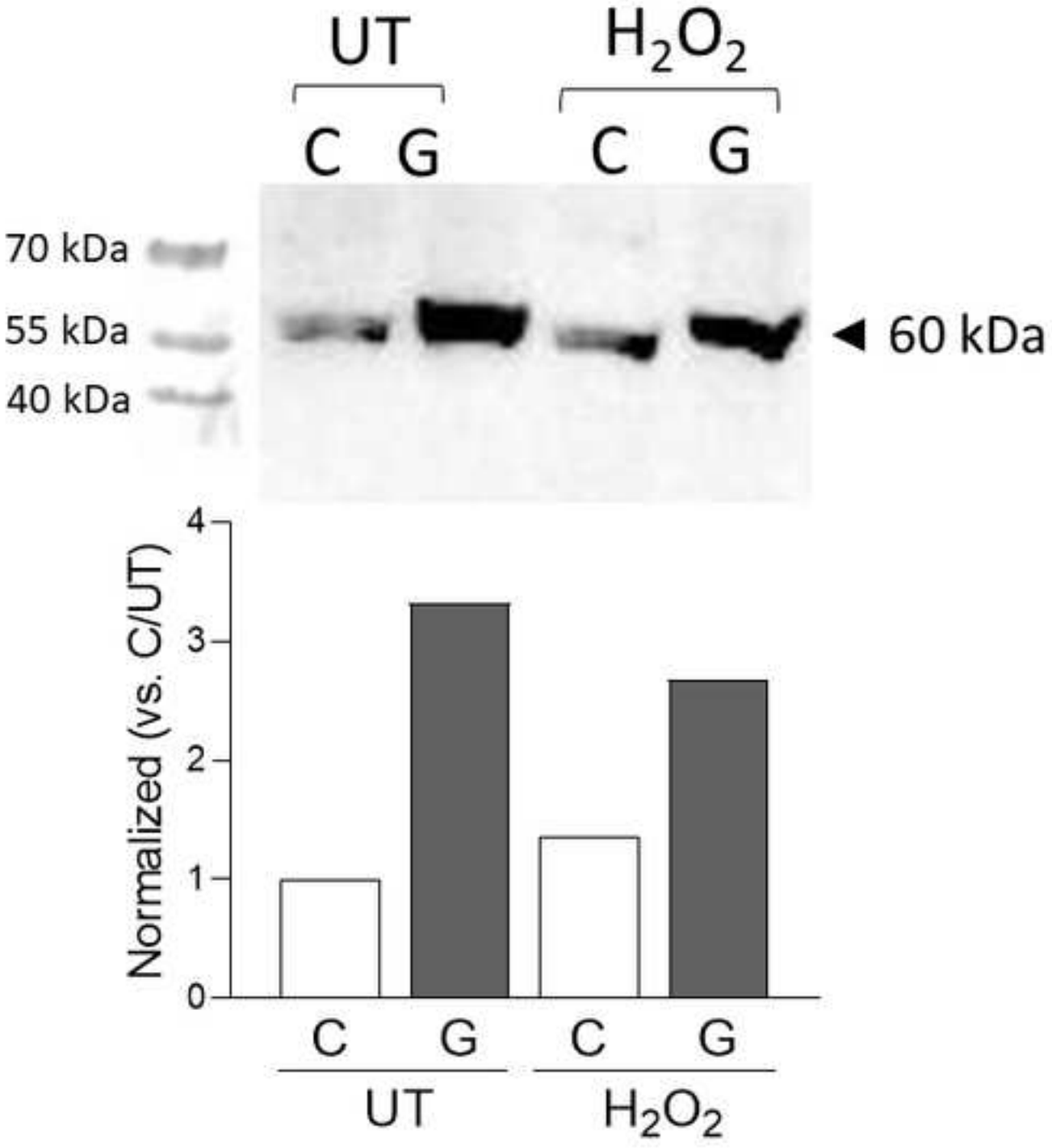

Table 1

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	Area (cm^2)
Flask T75	75
6 Well plate	9.6
24 Well plate	2
96 Well plate	0.32

Seeding density for ARPE-19 cells (cells/well)
500,000
100,000
50,000
5,000 for oxidative stress experiments with transfected cells (Fig. 1)
3,000 for oxidative stress experiments with non-transfected cells plus proteins (Fig. 1)

	Medium (mL)		
Application	**For cell culture**	**To stop trypsin**	**Volume of trypsin (mL)**
ARPE-19 cell growth	10	7	3
Seeding of transfected ARPE-19 cells	3	1	0.5
Seeding of transfected hRPE cells	1	0.8	0.2
Oxidative stress experiments	0.2		

Table 2

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	No	age	gender	death to preservation (hours)	death to isolation (days)	cultivation before transfection (days)	cultivation after transfection (days)	Symbol in graph
	2	80	M	20.7	8	140	36	○
	3	86	F	12.8	8	85	45	▼
	4	86	F	8.5	5	26	133	▽
	8	83	F	8.9	6	18	27	△
mean		**83.8**		**12.7**	**6.8**	**67.3**	**60.3**	
SD		2.9		5.7	1.5	57.0	49.1	

Table of Materials

Click here to access/download;Table of Materials;Table of Materials_20201019.xlsx

Name of Material/ Equipment/Sofware	Company	Catalog Number
24-well plates	Corning	353047
6-well plates	Greiner	7657160
96-well culture plate white with clear flat bottom	Costar	3610
96-well plates	Corning	353072
Acrylamid 40%	Biorad	161-0144
Amphotericin B	AMIMED	4-05F00-H
Antibody anti-GMCSF	ThermoFisher Scientific	PA5-24184
Antibody anti-mouse IgG/IgA/IgM	Agilent	P0260
Antibody anti-PEDF	Santa Cruz Biotechnology Inc	sc-390172
Antibody anti-penta-His	Qiagen	34660
Antibody anti-phospho-Akt	Cell Signaling Technology	9271
Antibody anti-rabbit IgG H&L-HRP	Abcam	ab6721
Antibody donkey anti-rabbit Alexa Fluor 594	ThermoFisher Scientific	A11034
Antibody goat anti-mouse Alexa 488	ThermoFisher Scientific	A-11029
ARPE-19 cell line	ATCC	CRL-2302
BSA	Sigma-Aldrich	A9418-500G
chamber culture glass slides	Corning	354118
CytoTox-Glo Cytotoxicity Assay	Promega	G9291
DAPI	Sigma-Aldrich	D9542-5MG
DMEM/Ham`s F12	Sigma-Aldrich	D8062
Duo Set ELISA kit	R&D Systems	DY215-05
EDTA	ThermoFisher Scientific	78440
ELISAquant kit	BioProducts MD	PED613-10-Human
Eyes (human)	Lions Gift of Sight Eye Bank (Saint Paul, MN)	
FBS	Brunschwig	P40-37500
Fluoromount Aqueous Mounting Medium	Sigma-Aldrich	F4680-25ML
FLUOstar Omega plate reader	BMG Labtech	
GraphPad Prism software (version 8.0)	GraphPad Software, Inc.	
GSH-Glo Glutathione Assay	Promega	V6912
hydrogen peroxide (H_2O_2)	Merck	107209
ImageJ software (image processing program)	W.S. Rasband, NIH, Bethesda, MD, USA; https://imagej.nih.gov/ij/; 1997–2014	
Imidazol	Axonlab	A1378.0010
Leica DMI4000B microscope	Leica Microsystems	
LightCycler 480 Instrument II	Roche Molecular Systems	
LightCycler 480 SW1.5.1 software	Roche Molecular Systems	
NaCl	Sigma-Aldrich	71376-1000
NaH_2PO_4	Axonlab	3468.1000
Neon Transfection System	ThermoFisher Scientific	MPK5000
Neon Transfection System 10 µL Kit	ThermoFisher Scientific	MPK1096
Neubauer chamber	Marienfeld-superior	640010
Ni-NTA superflow	Qiagen	30410
Nitrocellulose	VWR	732-3197

Omega Lum G Gel Imaging System	Aplegen Life Science	
PBS 1X	Sigma-Aldrich	D8537
Penicillin/Streptomycin	Sigma-Aldrich	P0781-100
PerfeCTa SYBR Green FastMix	Quantabio	95072-012
PFA	Sigma-Aldrich	158127-100G
Pierce BCA Protein Assay Kit	ThermoFisher Scientific	23227
Primers	Invitrogen	
pSB100X (250 ng/µL)		
pT2-CMV-GMCSF-His plasmid DNA (250 ng/µL)		
pT2-CMV-PEDF-His plasmid DNA (250 ng/µL)		
QIAamp DNA Mini Kit	QIAGEN	51304
recombinant hGM-CSF	Peprotech	100-11
recombinant hPEDF	BioProductsMD	004-096
ReliaPrep RNA Cell Miniprep System	Promega	Z6011
RIPA buffer	ThermoFisher Scientific	89901
RNase-free DNase Set	QIAGEN	79254
RNeasy Mini Kit	QIAGEN	74204
SDS	Applichem	A2572
Semi-dry transfer system for WB	Bio-Rad	
SuperMix qScript	Quantabio	95048-025
Tris-buffered saline (TBS)	ThermoFisher Scientific	15504020
Triton X-100	AppliChem	A4975
Trypsin/EDTA	Sigma-Aldrich	T4174
Tween	AppliChem	A1390
Urea	ThermoFisher Scientific	29700
WesternBright ECL HRP substrate	Advansta	K-12045-D50
Whatman nitrocellulose membrane	Chemie Brunschwig	MNSC04530301

Comments/Description

Allows to check the cells before measuring the luminescence (GSH-Glo Assay)

See Table 1 in Supplementary Materials	
Mátés et al., 2009. Provide by Prof. Zsuzsanna Izsvak	
Constructed using the existing pT2-CMV-PEDF-EGFP plasmid reported in Johnen, S. *et al.* (2012) IOVS, 53 (8), 4787-4796.	
Constructed using the existing pT2-CMV-PEDF-EGFP plasmid reported in Johnen, S. *et al.* (2012) IOVS, 53 (8), 4787-4796.	

letter to editor and reviewers_JoVE_Bascuas_20201020 Click here to access/download;Rebuttal Letter;letter to editor and reviewers_JoVE_Bascuas_20201020.pdf

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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.

Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.tif

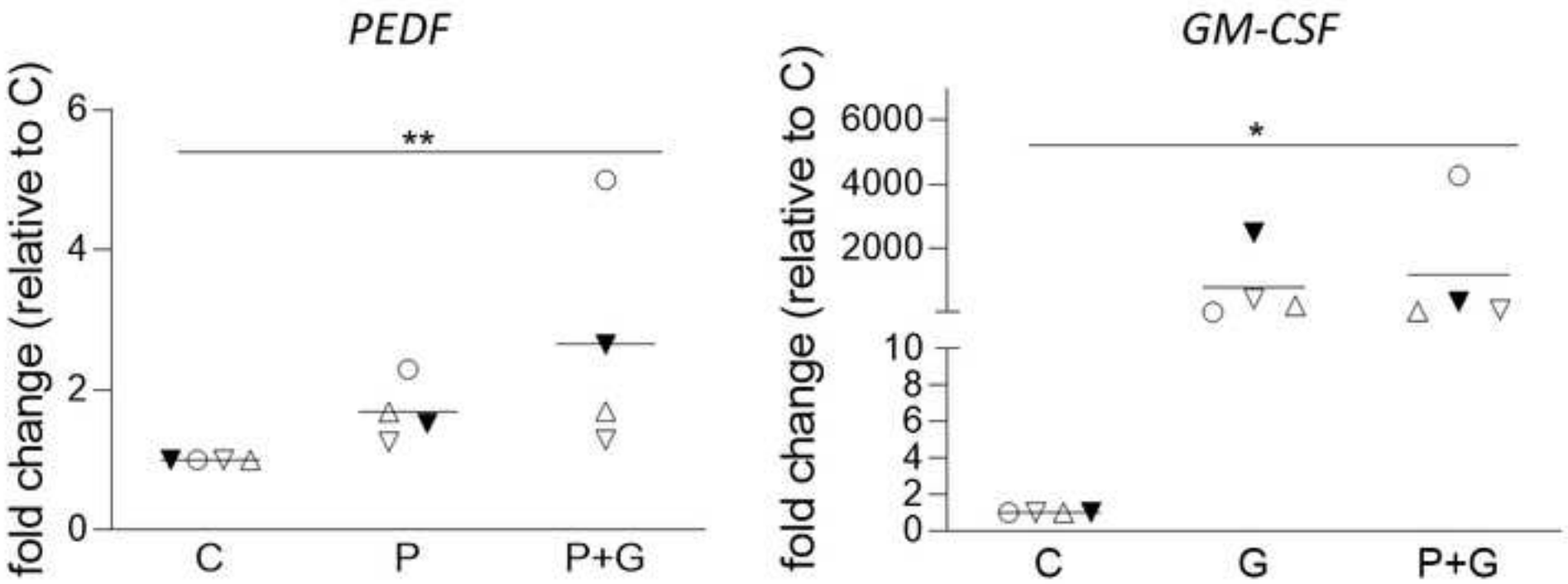
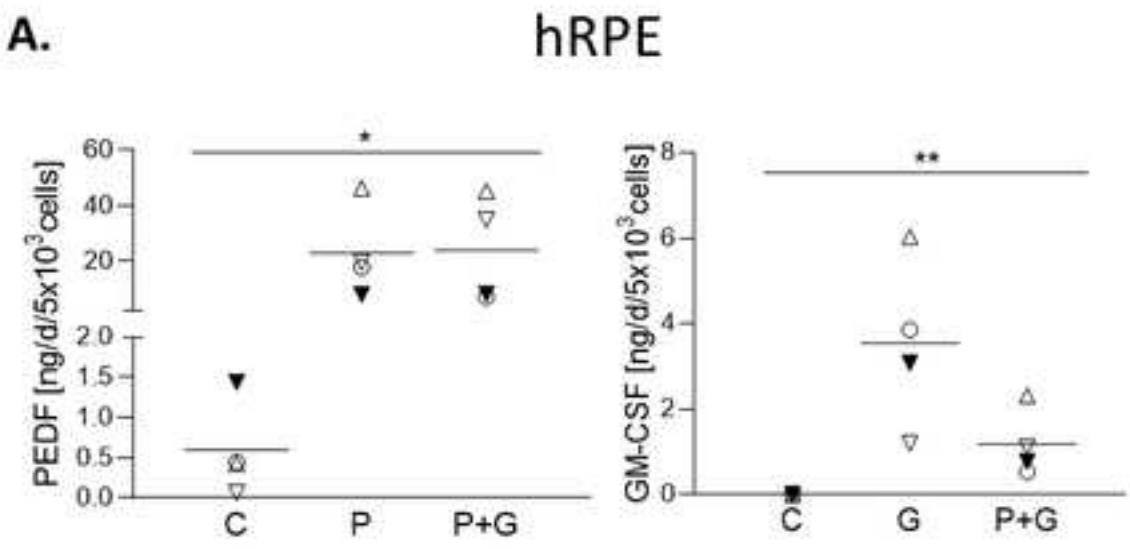


Figure S2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S2.tif



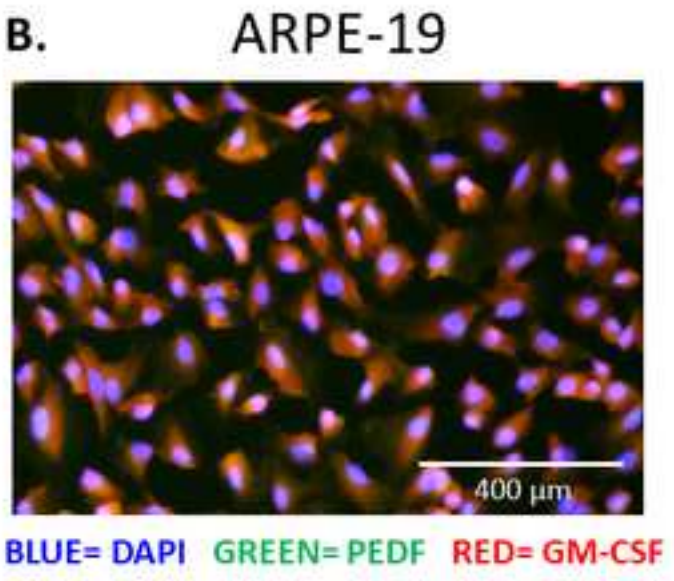


Table S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Table S1_20201019.xlsx

Gene	Sequence (5' - 3')	Annealing time/temperature
GADPH	F: ATC CCA TCA CCA TCT TCC AG R: ATG AGT CCT TCC ACG ATA CC	15sec/60°C
GM-CSF	F: GAC ACT GCT GCT GAG ATG AA R: GGG GAT GAC AAG CAG AAA GT	30sec/62°C
PEDF (endogenous)	F: GCT GGC TTT GAG TGG AAC GA R: GTG TCC TGT GGA ATC TGC TG	15sec/60°C
PEDF (recombinant)	F: CCT GCA GGA GAT GAA GCT GCA R: TCC ACC TGA GTC AGC TTG ATG	15sec/60°C
UCP2	F: CTACAAGACCATTGCACGAGAGG R: AGCTGCTCATAGGTGACAAACAT	30sec/60°C

Permission Letter_Hum Gene Ther_Bascuas

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September 29, 2020

Dear Thais:

Copyright permission is granted to use figures from the abstract below for your methodological paper to be submitted to JoVE.

P318: Non-virally transfected primary human pigment epithelium cells overexpressing the oxidative stress reduction factors PEDF and GM-CSF to treat retinal neurodegeneration
By Thais Bascuas et al
Human Gene Therapy. Nov 2019.A1-A221
http://doi.org/10.1089/hum.2019.29095.abstracts
Published in Volume: 30 Issue 11: November 7, 2019

Kind regards,

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SUPPLEMENTARY MATERIAL

The following methods and results are included as supplementary material since they present data that illustrates the ability to transfect RPE cells with the *PEDF* and *GM-CSF* genes, the efficient gene expression, and the secretion of PEDF and GM-CSF by transfected cells before carrying out the oxidative stress experiments. Even though these data are essential to demonstrate that cells transfected with *PEDF* and *GM-CSF* genes protect cells against oxidative stress, they are not included in the manuscript since they are not relevant to the objectives of the manuscript, which is the analysis of the antioxidant capacity of neuroprotective factors (PEDF and GM-CSF) expressed by genetically modified cells using an *in vitro* cell model of oxidative stress damage. The details of the methods and procedures for the transfection and analysis of transfection efficiency of RPE cells are going to be submitted as a separate manuscript to Bioelectrochemistry (Bascuas *et al.*, 2020).

A. MATERIALS AND METHODS

1. Real-Time quantitative PCR

Relative *PEDF* and *GM-CSF* gene expression was analyzed by Real-Time quantitative PCR (RT-qPCR). Total RNA was isolated from cell pellets of transfected hRPE cells at 60±49 days post-transfection (for donor's details see **Table 2** of the manuscript) using a commercially available RNA isolation kit (see **Table of Materials**) following the manufacturer's instructions; DNase treatment was also included. Besides, the study of *UCP2* gene expression was carried out in pellets from transfected ARPE-19 cells treated with H_2O_2 (for cell seeding and H_2O_2 treatment see steps 4.3.1-4.3.4 of the manuscript); RNA isolation was done using an isolation kit from low number of cells (see **Table of Materials**) following the manufacturer's instruction. RNA was quantified by spectrophotometry using a plate reader and the corresponding LVisPlate. Reverse transcription was carried out with 0.5 µg total RNA using a commercially available mix containing an optimized M-MLV Reverse Transcriptase (see **Table of Materials**) following the manufacturer's instructions. The cDNA was diluted 1:4, for the *PEDF* and *GM-CSF* genes, and 1:3 for the *UCP2* gene (due to the lower cell number seeded in H_2O_2 experiments). 5 µL of the cDNA solution (approx. 30 ng of cDNA/reaction) was used for qPCR, performed in duplicates (final volume of 20 µL) in a plate-based real-time PCR amplification and detection instrument (see **Table of Materials**), employing a ready-to-use reaction cocktail containing all components (including SYBR Green) (see **Table of Materials**) except primers and DNA template. The primers listed in **Table S1** were used at a final concentration of 0.3 µM. For *PEDF* and *GM-CSF* genes the following conditions were used: initial denaturation at 95 °C for 5 min, 40 cycles with denaturation at 95 °C for 10 sec, annealing (see **Table S1**), and elongation at 72 °C for 15 sec followed by a melting curve to confirm the primer-specific amplification. For *UCP2* gene the initial denaturation was 95 °C for 10 min, 40 cycles with denaturation at 95°C for 15 sec, annealing at 60 °C for 30 sec, and elongation at 72 °C for 32 sec. Data were evaluated using the comparative Ct method ($2^{-\Delta\Delta Ct}$)[36]; *GAPDH* was used as housekeeping gene and the results were expressed relative to the non-transfected cells.

[Place **Table S1** here]

2. SDS-PAGE and Western Blot (WB) analysis

WBs were performed to verify PEDF and GM-CSF secretion (both proteins carried the His-tag), in cell culture medium from transfected ARPE-19 and primary hRPE cells; media was collected at 7, 14, 21, 28, 42, 57, and 70 days post-transfection. Endogenous levels of Ser473-phosphorylated Akt was analyzed in cell lysates of transfected ARPE-19 cells. 15 µL of medium or lysates were mixed with an equal volume of 2x SDS sample buffer and heated for 5 min at 95 °C. Proteins were separated on a 10% SDS-PAGE gel and transferred onto a 0.45 µm pore-size Whatman nitrocellulose membrane using the semi-dry transfer system. Blots were blocked with 3% BSA in Tris-buffered saline (TBS) for 1 h at RT. Nitrocellulose membranes were incubated on a shaker for 2 h at RT with anti-penta-His (mouse monoclonal; 1:500 in 3% BSA/TBS), or anti-phospho-Akt (Ser473) (rabbit monoclonal; 1:1000 in 5% BSA/TBS) antibodies. After storage overnight at 4 °C, the membranes were washed 3x with TBS-Tween/Triton X-100-buffer for 10 min, and then incubated with horseradish peroxidase-conjugated anti-mouse IgG/IgA/IgM (rabbit polyclonal; 1:1000 in 10% non-fat dry milk/TBS for penta-His) or anti-rabbit IgG H&L (goat polyclonal; 1:2000 diluted in 5% non-fat dry milk/TSB for pAkt) for 1 h at RT. Protein bands were visualized by chemiluminescence using an HRP substrate (see **Table of Materials**); 10 photographs using cumulative exposure, every 10 sec were taken and the signal was evaluated using an open-source image processing program (see **Table of Materials**).

3. Immunohistological anti-PEDF and anti-GM-CSF staining

PEDF and GM-CSF protein production by ARPE-19 cells transfected with the *PEDF* and/or the *GM-CSF* gene was additionally determined by immunofluorescence. Transfected cells grown to approximately 80% confluence (verified qualitatively) in 8 chamber culture glass slides were fixed with 4% PFA at RT for 15 min and washed 3x in PBS. The fixed cells were permeabilized and blocked with 0.25% Triton X-100/1% BSA/PBS for 30 min at RT. The fixed cells were incubated overnight at 4°C with the anti-PEDF (mouse polyclonal IgG2b 1:200), anti-GM-CSF (rabbit polyclonal IgG 1:30) antibodies, or both, diluted in 0.05% Triton X-100/1% BSA/PBS. After washing with 5x for 5 min with 0.05% Triton X-100/1% BSA/PBS at RT, the cells were incubated at RT for 1 h with the secondary antibodies goat anti-mouse Alexa 488 (1:600) and donkey anti-rabbit Alexa Fluor 594 (1:500) diluted in 0.05% Triton X-100/1% BSA/PBS. After washing 5x for 5 min with 0.05% Triton X-100/1% BSA/PBS the cells were incubated for 20 sec with DAPI (5 mg/mL) diluted 1:1000 in PBS, washed 5x for 5 min with PBS, and mounted with a mounting medium (see **Table of Materials)**; cells were stored at 4 °C in the dark until photographed using fluorescence microscopy.

4. ELISA

For PEDF and GM-CSF quantification in culture medium of transfected ARPE-19 and primary hRPE cells commercially available ELISA kits (see **Table of Materials**) were used according to the manufacturers' instructions. Absorbance was measured at 450 nm (650 nm was used as

reference wavelength) in a plate reader. The protein concentration was expressed in ng/24 h/5 x 10^3 cells.

5. Statistical analysis

Student's t-test and the analysis of variance (ANOVA) were used for experiments carried out with the human cell line ARPE-19. For primary hRPE cell experiments, the more robust Mann-Whitney and Kruskal-Wallis tests were used to account for the high variability of cells isolated from human donors of variable age, gender, pathology, and time of enucleation. A value of $p<0.05$ was considered statistically significant and is indicated with * (<0.05), ** (<0.01), *** (<0.001), and **** (<0.0001). When the ANOVA or the Kruskal-Wallis test detected statistical differences, a post-hoc test was done using Tukey's multiple comparison test or Dunn's multiple comparison test, respectively, to compare the individual groups; shown in the figures is only significance.

B. REPRESENTATIVE RESULTS

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

RT-qPCR analysis revealed increased *PEDF* and *GM-CSF* gene expression in transfected cells compared to the non-transfected control cells (Fig. S1). Gene expression data is the average of the measurements at the termination of the cell culture (60±49 days post-transfection; the day of cell culture termination for each donor is specified in **Table 2** of the manuscript).

[Place Figure S1 here]

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

PEDF and GM-CSF secretion into the media by transfected cells was quantified by ELISA (Fig. S2A). Note the significant increase of PEDF secretion in cells transfected with PEDF and PEDF plus GM-CSF. Also, the GM-CSF secretion was higher in both GM-CSF and PEDF plus GM-CSF transfected cells. Fig. 2A shows the average of measurements at the termination of the cultures (60±49 days post-transfection; the day of cell culture termination for each donor is specified in **Table 2** of the manuscript). The immunofluorescent staining of double transfected ARPE-19 cells confirmed the co-secretion of PEDF and GM-CSF (Fig. S2B).

[Place Figure S2 here]