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

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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 ofbiopharmaceutical 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)[3,4,5,6,7,8]. 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[3,4,5]. 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[11,12,13,14,15,16]. 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[17,18,19].

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[1,25,26,27,28,29,30], 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. Human ARPE-19 cell line
    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).
2. Change the medium three times per week.
3. Once the cells are grown to approximately 90% confluence (evaluated qualitatively), aspirate the medium and wash the cells with sterile 1x PBS.
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.
5. Stop trypsinization by adding complete medium containing 10% FBS (for volumes see **Table 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).

				Medium (mL)		
	Area (cm²)	**Seeding density for ARPE-19 cells (cells/well)**	**Application**	**For cell culture**	**To stop trypsin**	**Volume of trypsin (mL)**
Flask T75	75	5,00,000	ARPE-19 cell growth	10	7	3
6 Well plate	9.6	1,00,000	Seeding of transfected ARPE-19 cells	3	1	0.5
24 Well plate	2	50,000	Seeding of transfected hRPE cells	1	0.8	0.2

96 Well plate	0.32	5,000 for oxidative stress experiments with transfected cells (Fig. 1)	Oxidative stress experiments	0.2		
		3,000 for oxidative stress experiments with non-transfected cells plus proteins (Fig. 1)				

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.

2. Primary human RPE cells

    1. Isolate primary human RPE cells as described by Thumann et al.[17], and culture cells in complete medium supplemented with 20% FBS.
    2. Change the medium twice per week. Once the cells reach confluency (monitored visually), reduce FBS to 1% to avoid overgrowth.
    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 was collected 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.

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

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

1. Trypsinize ARPE-19 cells or primary human RPE cells as described in steps 1.1.3-1.1.5 of the protocol.
2. Perform electroporation with the commercially available transfection kit (see **Table of Materials**).
    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 p*SB100X* 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 p*SB100X*, 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.

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.

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.

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)

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

   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.

   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. 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**.

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

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

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

      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.

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. 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.

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.

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

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

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.

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. 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.

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

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.

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. 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.

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

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

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

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].

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.

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.

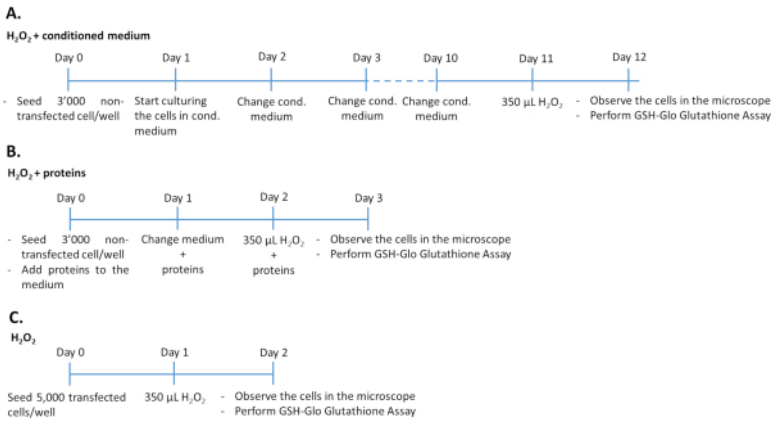

Figure 1: Timelines of the H_2O_2 assay in the three different experimental approaches. 3,000 non-transfected cells treated with the conditioned medium/recombinant proteins or 5,000 transfected cells were seeded in 96-well plates for treatment with H_2O_2. To determine the effect of conditioned medium, 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. Please click here to view a larger version of this figure.

4. Analysis of oxidative stress level and antioxidant capacity

1. **Glutathione assay**

    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.

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

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

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.

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.

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

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

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.

   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**.

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

2. **Cytotoxicity assay and microscopic analysis**

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.

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.

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

   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.

   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. 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.

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.

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

1. Trypsinize transfected cells as described above (steps 1.1.3-1.1.5 of the protocol).

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

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

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

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.

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**).

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.

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

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

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].

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

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

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

6. Pipette up and down.

7. Collect the lysate in 1.5 mL tubes.

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

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

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.

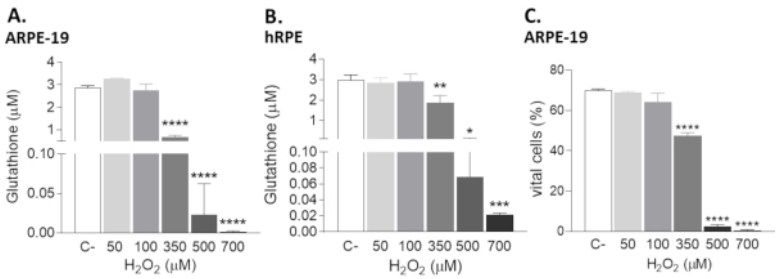

Figure 2: Oxidative stress level evidenced as glutathione level and cell viability, in human RPE cells treated with H_2O_2. (**A**) ARPE-19 cells exposed to several concentrations of H_2O_2 showed significantly decreased glutathione levels (in brackets) at 350 µM (0.66 µM), 500 µM (0.022 µM), and 700 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]. Please click here to view a larger version of this figure.

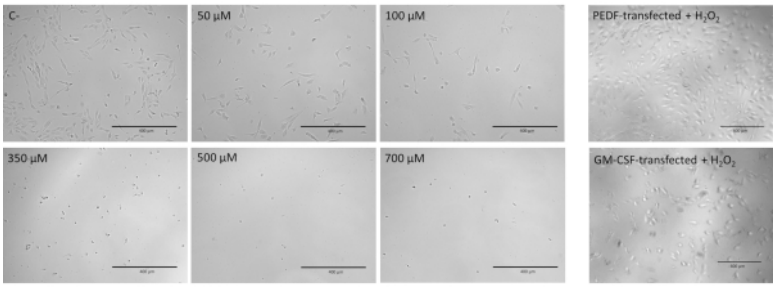

Figure 3: Morphology of non-transfected and PEDF- or GM-CSF-transfected ARPE-19 cells treated with H_2O_2. Cells treated with increasing concentrations of H_2O_2 show fewer cells in the culture wells and display a more rounded, less spread morphology, a known sign of cellular stress. Note that for PEDF-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

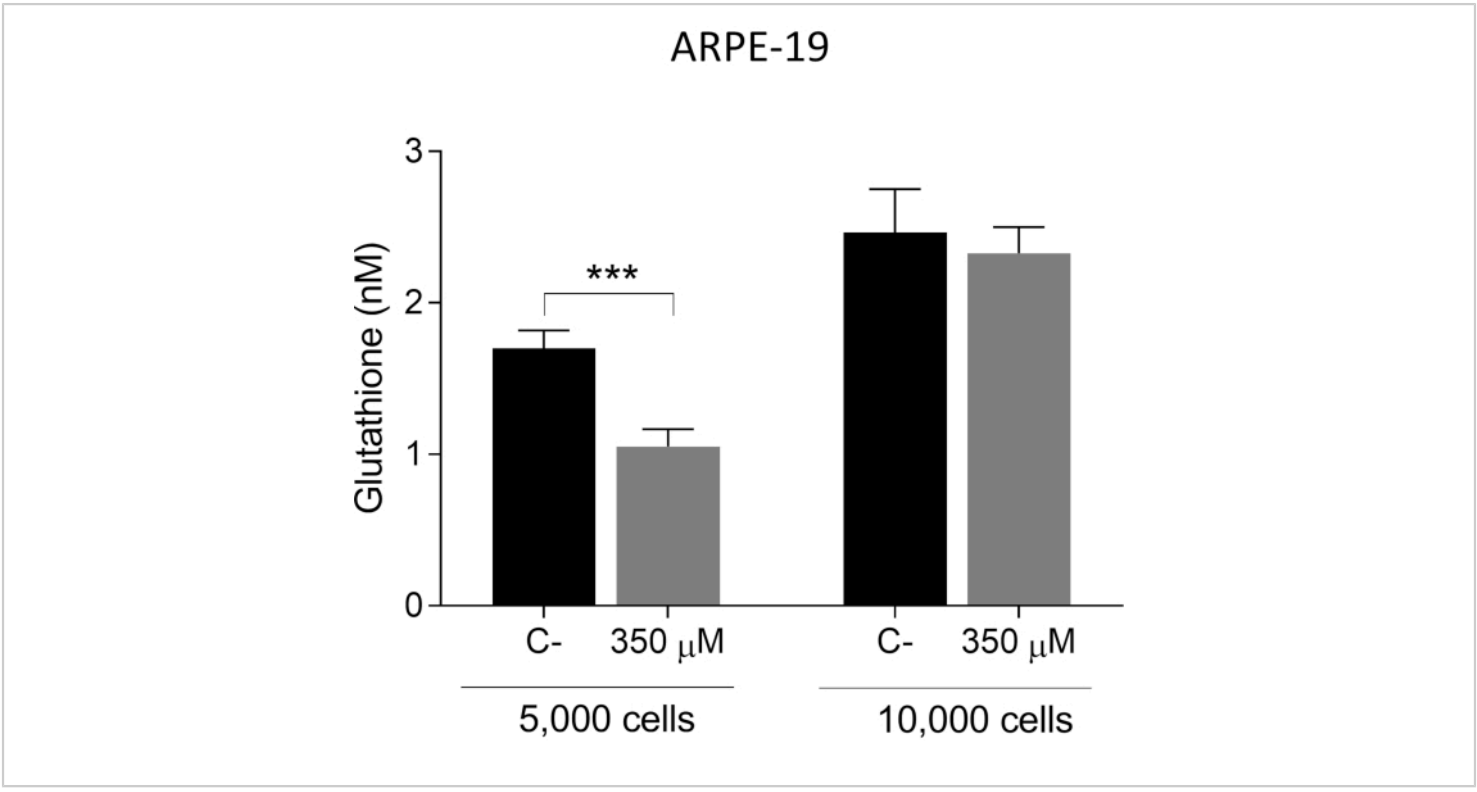

Figure 4: Influence of cell number on the effect of H_2O_2-induced oxidative stress. 5,000 and 10,000 ARPE-19 cells/well were seeded in 96-well plates. After 24 h, cells were treated with 350 µM H_2O_2 for 24 h. Significant differences in glutathione levels were observed in the wells seeded with 5,000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

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.

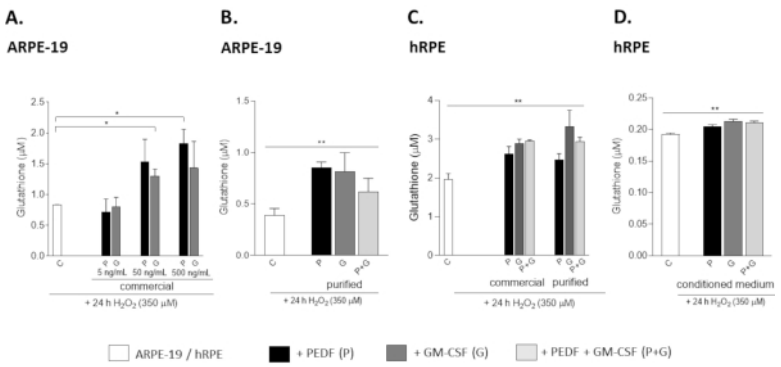

Figure 5: Glutathione level as a marker of the antioxidant capacity of PEDF and GM-CSF. (**A**) Treatment of ARPE-19 cells with 500 ng/mL PEDF or 50 ng/mL GM-CSF for 3 days before and during 24 h H_2O_2 exposure increased the level of glutathione from 0.83 µM (C) to 1.83 µM (PEDF) and 1.3 µM (GM-CSF), 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]. Please click here to view a larger version of this figure.

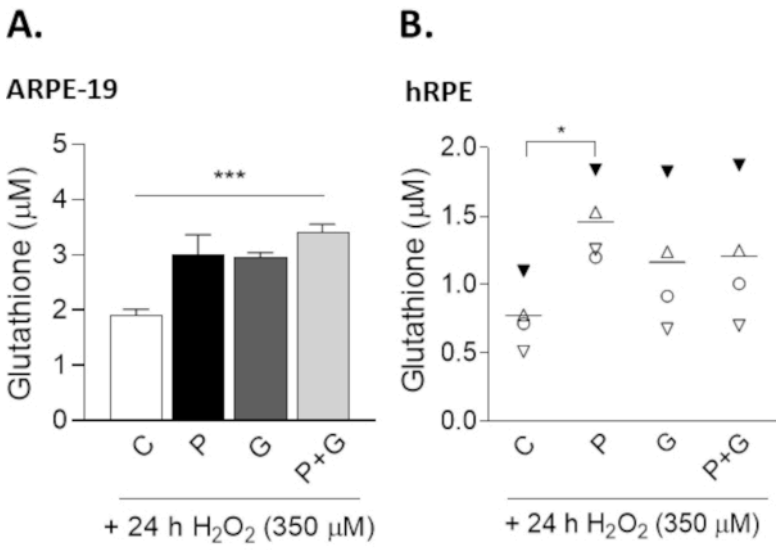

Figure 6: Glutathione level as a marker of the antioxidant capacity of PEDF- and GM-CSF-transfected human RPE cells. (**A**) The levels of glutathione of transfected ARPE-19 cells exposed to 350 µM H_2O_2 for 24 h (56 days post-transfection) were significantly higher compared to non-transfected cells (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]. Please click here to view a larger version of this figure.

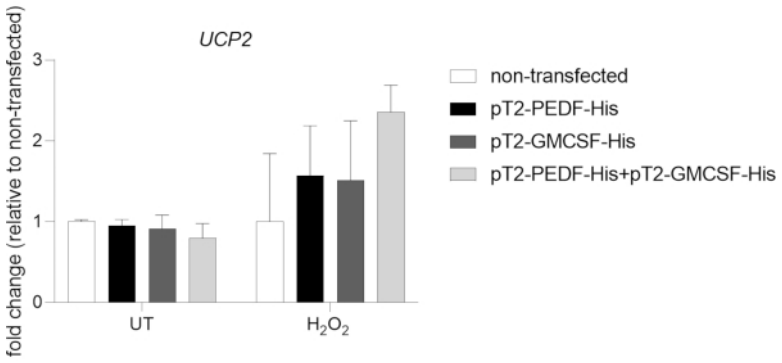

Figure 7: *UCP2* gene expression in transfected ARPE-19 cells treated with H_2O_2. Since *UCP2* gene expression can be used to examine mitochondrial oxidative damage, we examined the effect of the overexpression of PEDF and GM-CSF by transfected ARPE-19 cells. Transfected ARPE-19 cells treated with H_2O_2, 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. Please click here to view a larger version of this figure.

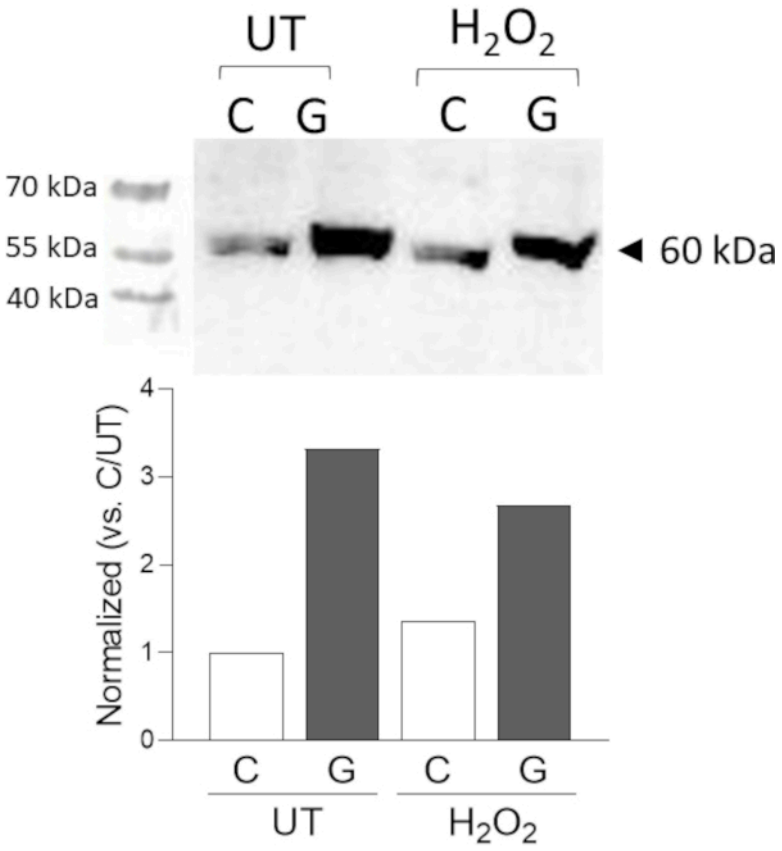

Figure 8: Western Blot of phosphorylated Akt (Ser473) from a cell lysate of GM-CSF-transfected ARPE-19 cells. The WB demonstrated that GM-CSF enhances the phosphorylation of Akt in both, untreated and H_2O_2-treated cultures (UT: 3.32; H_2O_2: 2.69). The values are normalized to non-transfected non-H_2O_2-treated 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. Please click here to view a larger version of this figure.

Table S1: Primer pair sequences and annealing time/ temperature used for RT-qPCR. Please click here to download this table.

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]. Please click here to download this file.

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]. Please click here to download this file.

Supplementary material. Please click here to download this file.

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.

Disclosures

The authors have nothing to disclose.

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