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How to cite

BASCUAS CASTILLO, Thaïs et al. Human retinal pigment epithelial cells overexpressing the neuroprotective proteins PEDF and GM-CSF to treat degeneration of the neural retina. In: Current Gene Therapy, 2022, vol. 22, n° 2, p. 168–183. doi: 10.2174/1566523221666210707123809

This publication URL: https://archive-ouverte.unige.ch//unige:154850
Publication DOI: 10.2174/1566523221666210707123809

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168 *Current Gene Therapy*, 2022, *22*, 168-183

RESEARCH ARTICLE

Human Retinal Pigment Epithelial Cells Overexpressing the Neuroprotective Proteins PEDF and GM-CSF to Treat Degeneration of the Neural Retina

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Abstract: ***Background*:** Non-viral transposon-mediated gene delivery can overcome viral vectors' limitations. Transposon gene delivery offers the safe and life-long expression of genes such as Pigment Epithelium-Derived Factor (PEDF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to counteract retinal degeneration by reducing oxidative stress damage.

***Objective*:** The study aimed at using *Sleeping Beauty* transposon to transfect human Retinal Pigment Epithelial (RPE) cells with the neuroprotective factors PEDF and GM-CSF to investigate the effect of these factors on oxidative stress damage.

***Methods*:** Human RPE cells were transfected with *PEDF* and *GM-CSF* by electroporation, using the hyperactive *Sleeping Beauty* transposon gene delivery system (*SB100X*). Gene expression was determined by RT-qPCR, and protein level by Western Blot as well as ELISA. The cellular stress level and the neuroprotective effect of the proteins were determined by measuring the concentrations of the antioxidant glutathione in human RPE cells, and conducting immunohistochemical examination of retinal integrity, inflammation, and apoptosis of rat Retina-Organotypic Cultures (ROC) exposed to H_2O_2.

***Results*:** Human RPE cells were efficiently transfected showing a significantly augmented gene expression and protein secretion. Human RPE cells overexpressing PEDF and/or GM-CSF or pretreated with recombinant proteins presented significantly increased glutathione levels post-H_2O_2 incubation than non-transfected/untreated controls. rPEDF and/or rGM-CSF-treated ROC exhibited decreased inflammatory reactions and cell degeneration.

***Conclusion*:** GM-CSF and/or PEDF could be delivered successfully to RPE cells with combined use of *SB100X* and electroporation. PEDF and/or GM-CSF reduced H_2O_2-mediated oxidative stress damage in RPE cells and ROC offering an encouraging technique to re-establish a cell protective environment to halt age-related retinal degeneration.

ARTICLE HISTORY

Received: December 09, 2020
Revised: April 23, 2021
Accepted: May 02, 2021

DOI:
10.2174/1566523221666210707123809

Keywords: Sleeping beauty transposon, PEDF, GM-CSF, age-related macular degeneration, RPE cells, non-viral gene delivery, oxidative stress damage, ocular gene therapy.

1. INTRODUCTION

Among ocular degenerative diseases, Age-Macular Degeneration (AMD) is the major cause of blindness in elderly people (>60 years) in industrialized countries with rising incidence due to the augmented lifetime and a growing senescent population [1]. There are two types of AMD, a neovascular (nvAMD) and an avascular (aAMD) form. Treatment for nvAMD consists of costly monthly intravitreal injections of anti-vascular endothelial cell growth factor (VEGF) molecules (*e.g.*, Avastin®, Lucentis®, and Eylea®); no treatment is available for aAMD [2]. The pathogenesis of both forms is caused by a multiple interworking metabolic, functional, genetic, and environmental elements [3]. Particularly, the development of aAMD has been associated with the accumulation of Reactive Oxygen Species (ROS) and immune cells, and the activation of the complement system, which lead to a decrease of neuroprotective proteins generated by the Retinal Pigment Epithelium (RPE), specifically Pigment Epithelium-Derived Factor (PEDF), Insulin-like Growth Factors (IGFs) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and finally the degeneration of RPE

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1566-5232/22 $65.00+.00

cells that leads to photoreceptor and Retinal Ganglion Cell (RGC) death [3-6].

A potential therapy approach to reconstitute the neuronal cell protective retinal milieu and thus stop retinal degeneration is the transplantation of transfected RPE cells overexpressing PEDF and GM-CSF to the RPE layer, which diminishes the effects of oxidative stress, reduces inflammation, and promotes cell survival [7-12]. The protective effect mediated by PEDF is because this factor inhibits VEGF-mediated proliferation and migration of vascular endothelial cells [13, 14], protects RPE cells and retinal neurons from glutamate and oxidative stress as well as ischemic conditions [15, 16]. In the central nervous system, in primary cortical neurons and cells of human neuroblastoma, GM-CSF inhibits programmed cell death by instigating B-cell lymphoma-2 (B-CL-2) and B-cell Lymphoma-extra Large (BCL-XL) gene transcription. In RGC cultures, GM-CSF decreased dose and time-dependently the expression of BCL-2 Associated Death promoter (BAD) [11]. A single injection of GM-CSF in the vitreous of RCS (Royal College of Surgeons) rats, a photoreceptor degeneration model, protected photoreceptors from death such that at 84 days post-natal, when photoreceptor degeneration should be completed, there was a 4-fold increase of photoreceptors compared to untreated animals [12].

Most gene delivery systems used in the manufacture of Gene Therapy Medicinal Products (GTMP) on the market and for ongoing clinical trials testing gene therapies have used viral vectors [17]. Adenoviral and Adeno-Associated Viral (AAV) vectors are efficient and do not appear to trigger an immediate severe immune response; however, since these vectors deliver the gene episomally, often treatment requires repeated administration with the risk of off-target dissemination and acute immune responses, especially when neutralizing antibodies are present [18-20]. Retroviral and lentiviral vectors integrate the transgene preferentially into active genes and oncogenes of the host cells' genome risking cancerogenesis and insertional mutagenesis [21-24].

During the last decade, transposons have been examined extensively as gene delivery vectors [25]. Transposons are DNA sequences that can move to different locations within a genome, being useful tools for non-viral gene delivery into mammalian cells [26]. We propose to use the hyperactive *Sleeping Beauty* (*SB100X*) transposon system, which has been shown to have very low immunogenicity [27], improved safety/toxicity profiles [28, 29], and the ability for large DNA cargo [30, 31], combined with electroporation, which has proved to have high transfection rate and is being used in several clinical trials [32, 33]; safety and tolerability were demonstrated, and some reports are showing both local and systemic responses [33]. In our laboratory, we have demonstrated that cultured primary RPE cells can be efficiently transfected with the *PEDF* gene using electroporation and the *SB100X* transposon system. We have shown that PEDF is secreted in the media of transfected RPE cells over 2 years and is expressed *in vivo* following transplantation as evidenced by a reduction of choroidal neovascularization (CNV) and corneal neovascularization in rats and rabbits [34-38].

Here, we report the successful and efficient transfection of human RPE cells with the genes coding for *PEDF* and *GM-CSF*, and show that in culture, the transfected cells are less affected by H_2O_2 treatment compared with non-transfected cells and that conditioned medium from transfected cells as well as rPEDF and rGM-CSF protects rat retina-organotypic cultures from H_2O_2 damage.

2. MATERIALS AND METHODS

2.1. Plasmid Constructs

The h*GM-CSF* cloning cassette sequence was obtained from the GeneArt Synthesis Service (ThermoFisher Scientific) and delivered in the commercial pMK-RQ-h*GMCSF* plasmid. It was designed to have a consensus Kozak sequence (5'-ACCATGT-3') at the N-terminal and a hinge sequence appended to a 6X Histidine (His) tag at the C-terminal, followed by the stop codon. Restriction enzyme sites for *NheI* and *BclI* were added on 5'- and 3'-ends, respectively. The h*GM-CSF* cloning cassette was isolated on an agarose gel after enzymatic restriction with *NheI* (NEB: #R3131) and *BclI* (NEB: R3160S). The pT2-CMV-*PEDF*-His transposon-plasmid (5,837 bp), encoding the recombinant His-tagged human PEDF protein [34] was used to clone the h*GMCSF* cloning cassette. The *PEDF* cloning cassette was removed from the pT2-CMV-*PEDF*-His construct by cutting at 1,150 bp and 2,463 bp with *NheI* and *BclI* restriction enzymes, respectively. The linearized backbone was then isolated and purified from agarose gel. For the pT2-CMV-*GMCSF*-His construct (5,015 pb), the h*GMCSF* cloning cassette and linearized backbone, both isolated from an agarose gel, were ligated and DH5α competent bacteria (Thermo Fisher Scientific: 18265017) were transformed with the ligation mix. Ampicillin resistant colonies carrying the pT2-CMV-*GMCSF*-His plasmid were analyzed by PCR and verified by Sanger sequencing. The plasmid isolation was done with PureLink HiPure Plasmid Filter Maxiprep Kit (ThermoFisher Scientific: K210016).

The plasmid pT2-CAGGS- *Venus* [39] coding for the Venus yellow fluorescent protein was used to determine the transfection efficiency by fluorescence microscopy and image-based cytometry. The commercial plasmid pCMV (CAT)T7-SB100X (p*SB100X*) carrying the hyperactive *Sleeping Beauty* transposase (*SB100X*) was included in all transfections [39]; p*SB100X* and pT2-CAGGS-*Venus* were kindly provided by Prof. Zsuzsanna Izsvak from Max-Delbrück Center in Berlin.

2.2. Cells and Culture Conditions

The human cell line ARPE-19 (ATCC CRL-2302) was cultured as described in the study by Johnen *et al.* [40].

Human eyes from 8 donors (age 81±10 years; 1 male and 7 females) were obtained from the Lions Gift of Sight

Eye Bank (Saint Paul, MN). Informed consent was obtained in accordance with the Declaration of Helsinki before the eyes were collected 11.2±4.5 hours post-mortem. Procedures for the use of human eye globes was authorized by the Cantonal Ethical Commission for Research (no. 2016-01726). Primary human RPE cells were isolated as described before [37]. Cell survival and proliferation after transfection were optimized by supplementing the DMEM/Ham's F-12 medium with 20% FBS, changed two times per week. As soon as the cells reached confluence, the FBS concentration was reduced to 1% to decrease cell proliferation.

2.3. Electroporation of ARPE-19 and Primary Human RPE Cells

Electroporation was carried out using the 10 µL Kit of the Neon Transfection System (ThermoFisher Scientific) according to the manufacturer's instruction. For ARPE-19 cells, the electroporation parameters consisted of two pulses of 1,350 V for 20 ms (pulse width); primary cells were electroporated with two pulses of 1,100 V for 20 ms [40]. $1x10^5$ ARPE-19 or $5x10^4$ primary human RPE cells were transfected with p*SB100X* transposase and pT2-CMV-*PEDF*-His and/or pT2-CMV-*GMCSF*-His transposon, using the ratios 1:16 for single (p*SB100X* + pT2-CMV-*PEDF*-His or pT2-CMV-*GMCSF*-His) or 1:16:16 for double (pSB100X + pT2-CMV-*PEDF*-His + pT2-CMV-*GMCSF*-His) transfected cells as described before [6, 37, 40]. Transfected ARPE-19 and primary human RPE cells were seeded into 6-well and 24-well plates, respectively, without antibiotics or antimycotics. Penicillin (80 U/mL), streptomycin (80 µg/mL), and amphotericin B (2.5 µg/mL) were added to the medium three days after electroporation with the first medium exchange. To determine cell growth and transfection efficiency, cells were monitored weekly microscopically. In addition, ARPE-19 cells were analyzed for gene expression and protein secretion at 7, 14, 21, 28, 42, 57, and 70 days post-transfection, and human RPE cells at the end of the cell culture (76±45 days post-transfection). Because in primary cells there could be differences in cell growth due to donor variation, for the quantification of secreted PEDF and GM-CSF, before the cultures were terminated, the medium was changed to 0.5 mL of complete medium, and after 24 hours, the medium was collected, the cells were trypsinized using 0.05% trypsin-0.02% EDTA and counted in a Neubauer chamber; the protein concentration was expressed as ng/d (day)/$5x10^3$ cells.

2.4. Transfection Efficiency

At 7, 14, 21, 28, 42, 57, and 70 days post-transfection, ARPE-19 cells were monitored and photographed with bright field and fluorescence microscopy (EVOS FL Color Imaging System, ThermoFisher Scientific). To quantify the transfection efficiency, the percentage of yellow fluorescent cells was determined for each time point using Tali image-based cytometry (ThermoFisher Scientific) following the manufacturer's instruction. Briefly, transfected ARPE-19 cells were trypsinized and 25 µL of the cell suspension was used for the analysis. The result was considered valid if at least 100 cells were counted by the device. A similar analysis was carried out in transfected human RPE cells performing Tali image-based cytometry at the termination of cell cultures (76±45 days post-transfection); in this case, the Leica DMI4000B microscope (Leica Microsystems) was used.

2.5. Reverse Transcriptase Quantitative PCR

For the quantification of *PEDF* and *GM-CSF* gene expression, Reverse Transcriptase quantitative PCR (RT-qPCR) was performed as follows. Total RNA was isolated from pellets of transfected ARPE-19 at 7, 14, 21, 28, 42, 57, and 70 days after transfection, and at 76±45 days for transfected human RPE cells using the RNeasy Mini Kit (QIAGEN: 74204) in combination with the RNase-free DNase Set (QIAGEN: 79254) according to the manufacturer's instructions. Reverse transcription was carried out on 0.5 µg total RNA using the SuperMix qScript (Quantabio: 95048-025) following the manufacturer's protocol. The cDNA diluted 1:4 and 5 µL (approx. 30 ng of cDNA/reaction) were used for qPCR in duplicates using the LightCycler 480 Instrument II (Roche Molecular Systems) employing the PerfeCTa SYBR Green FastMix (Quantabio: 95072-012) in a final volume of 20 µL. The primers (ThermoFisher Scientific) listed in Table **1** were used at a final concentration of 0.3 µM. The used conditions were the following: initial denaturation at 95°C for 5 min, 40 cycles with denaturation at 95°C for 10 sec, annealing (Table **1**), and elongation at 72°C for 15 sec followed by a melting curve to confirm the specific amplification from each primer pair. Data were analyzed with the LightCycler 480 SW1.5.1 software and evaluated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), which calculates relative gene expression [41]; *GAPDH* was used as housekeeping gene and the expression was described relative to the non-transfected cells.

The transposon copy number was quantified as developed by Kolacsek *et al.* [42]. Briefly, genomic DNA from transfected ARPE-19 cells was isolated 35 days post-transfection using the QIAamp DNA Mini Kit (QIAGEN: 51304) according to the manufacturer's protocol for cultured cells. DNA with known transposon insertions (kindly provided by Dr. Kolacsek) was used as a standard to calculate the copy number by interpolation of the $2^{-\Delta\Delta Ct}$. As internal control gene, the Ribonuclease P RNA component H1 (*RPPH1*) was used since it is present in a single copy in the human genome, and inverted repeat/direct repeat left (*IRDRL*) to detect the transposon sequence. The final volume of PCR reactions was 20 µL with 30 ng of genomic DNA and a final concentration of 0.3 µM for the primers (Table **1**) using the same conditions as described above.

Table 1. Primer pair sequences and annealing time/temperature used for RT-qPCR.

Gene	Sequence (5' - 3')	Annealing Time/Temperature
GADPH	F: ATC CCA TCA CCA TCT TCC AG	15 sec/60°C
-	R: ATG AGT CCT TCC ACG ATA CC	
GM-CSF	F: GAC ACT GCT GCT GAG ATG AA	30 sec/62°C
-	R: GGG GAT GAC AAG CAG AAA GT	
IRDRL	F: CTC GTT TTT CAA CTA CTC CAC AAA TTT CT	15 sec/60°C
-	R: GTG TCA TGC ACA AAG TAG ATG TCC TA	
PEDF (endogenous)	F: GCT GGC TTT GAG TGG AAC GA	15 sec/60°C
-	R: GTG TCC TGT GGA ATC TGC TG	
PEDF (recombinant)	F: CCT GCA GGA GAT GAA GCT GCA	15 sec/60°C
-	R: TCC ACC TGA GTC AGC TTG ATG	
RPPH1	F: AGC TGA GTG CGT CCT GTC ACT	15 sec/60°C
-	R: TCT GGC CCT AGT CTC AGA CCT T	

2.6. ELISA

ELISAquant kit (BioProducts MD: PED613-10-Human) and DuoSet ELISA kit (R&D Systems: DY215-05) were used to quantify total PEDF and GM-CSF, respectively, in culture media of transfected ARPE-19 and primary human RPE according to the manufacturers' protocols. Absorbance was measured with the FLUOstar Omega plate reader (B-MG Labtech). For primary cells, the protein concentration was expressed in ng/d/5x10^3cells.

Phosphorylated Akt (pAkt) in cell lysates from transfected ARPE-19 cells treated with 350 µM H_2O_2 was quantified using the DuoSet IC ELISA kit (R&D System: DY-C887B-2) according to the manufacturer's instructions.

2.7. Retina-organotypic Culture (ROC)

Adult healthy Brown Norway rats (8 to 10-week-old, Charles River Laboratories) were euthanized by an overdose of Pentobarbital (150 mg/kg, Thiopental Inresa, Ospedalia AG) diluted in 0.9% NaCl (Laboratorium Dr. Bichsel AG) injected intraperitoneally. Based on former work [43, 44], retinas were isolated immediately after euthanasia and cultured at standard conditions (37°C, 5% CO_2, 95% air) for up to 13 days in Ames' medium (Sigma-Aldrich: A1420-10X1L) supplemented with 2% B-27 (50X) (ThermoFisher Scientific: 17504044), 1% N-2 (100X) (ThermoFisher Scientific: 17502048), 80 U/mL penicillin, 80 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, placed on stainless steel grid supports and protective nitrocellulose membranes at the medium-air interface. The medium was changed daily.

For the studies of the neuroprotective function of PEDF and GM-CSF, after isolation, retina explants were treated with 500 ng/mL recombinant PEDF (BioProducts MD: PED-F-005) and/or GM-CSF (PeproTech: 300-03) for three consecutive days. On the third day of treatment, retinas were exposed to 350 µM Hydrogen Peroxide (H_2O_2) (Merck: 107209) for 3 hours [45]. Retinas were fixed and stained with hemalum and eosin (H&E), and immunohistological staining was freshly done after isolation (untreated control) at 3-4 days and 1-2 weeks.

2.8. Neuroprotection of PEDF and GM-CSF in Retinal Cells

Five thousand ARPE-19 cells, seeded in a 96-well plate in 200 µL/well of complete medium, were the day after, exposed to 50, 100, 350, 500, and 700 µM H_2O_2 for 24 hours. The oxidative stress damage was determined by measuring the levels of the antioxidant glutathione using the GSH-Glo Glutathione Assay for adherent mammalian cells (Promega: V6912) according to the manufacturer's instructions.

To determine the effect of the proteins PEDF and GM-CSF on oxidative stress, different studies were carried out in 96-well plates following protocols described before [6]. Briefly, 1) 3,000 non-transfected ARPE-19 cells were seeded in complete medium containing commercially recombinant PEDF (BioProducts, MD: PEDF-005) and GM-CSF (Pepro-Tech: 300-03) proteins (5, 50 and 500 ng/mL); the dose of the proteins selected was based on previous work [7, 11]. 2) 3,000 non-transfected ARPE-19 or primary human RPE cells were seeded in 200 µL/well of medium containing 500 ng/mL (PEDF) and/or 50 ng/mL (GM-CSF) of protein purified from transfected ARPE-19 cell culture medium (see Supplementary Material_**S1**). 3) 3,000 primary human RPE cells non-transfected were seeded and cultured for 10 days with 200 µL/well of conditioned medium (100%) from transfected ARPE-19 cells. 4) 5,000 PEDF- and/or GM-CSF--transfected ARPE-19 or primary human RPE cells were seeded in 200 µL/well of medium, and the day after, the cells were exposed to the H_2O_2 treatment for 24 hours. In each case after H_2O_2 treatment, as an index of oxidative stress, the level of glutathione was determined using the kit GSH-Glo Glutathione Assay for adherent mammalian cells according to the manufacturer's instructions.

To define the protective effect of GM-CSF on the human RPE against damage through induction of the Akt pathway [11], 300,000 GM-CSF-transfected ARPE-19 cells (21 days post-transfection) were seeded in 6-well plates, and the day after, the cells were treated with 350 µM H_2O_2 for 24 hours. The Ser473-phosphorylated Akt (pAkt) was determined in cell lysates by WB (Supplementary Material **S1**) and ELISA as detailed in the study by Bascuas *et al.* [6].

The neuroprotective effect of PEDF and GM-CSF was examined in rat ROC treated with the proteins (500 ng/mL PEDF and/or GM-CSF) for three consecutive days after isolation, and on the third day, exposed to 350 µM H_2O_2 for 3 hours, by studying apoptosis with TUNEL assay using the *in situ* Cell Death Detection Kit Fluorescein (Roche: 11684795910) following the manufacturer's instructions. In addition, the expression of the inflammatory markers GFAP and Iba-1, and rhodopsin for photoreceptor degeneration was examined by immunohistochemistry as detailed in Supplementary Material **S1**. The manipulation of animals was carried out by certified personnel using protocols in accordance with the Animal Welfare Department of the Canton de Genève and European regulations for the care and use of laboratory animals and was approved by the Commission Cantonale pour les Expériences sur les Animaux (CCEA), Switzerland (approval no. GE/116/19).

2.9. Statistics

Statistical analysis was realized using GraphPad Prism software for Windows (Version 8.0, GraphPad). Student's t-test and analysis of variance (ANOVA) were performed for experiments carried out with the human cell line ARPE-19. For primary human RPE cell experiments, the Mann-Whitney and Kruskal-Wallis tests were performed to account for the high variability of cells isolated from 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. Data are expressed as mean ± SD.

3. RESULTS

3.1. Transfection Efficiency

For ARPE-19 cells, the percentage of Venus$^+$ cells studied by Tali image-based cytometry was 98±6% (mean fluorescence intensity (MFI) of 5,785±1,255) and stable for the 138 days that the cells were monitored (Fig. **1A**); this result was corroborated microscopically as essentially all cells were fluorescent (Fig. **1C**). For primary human RPE cells (n=8 donors), transfection efficiency was 21±12% (MFI of 1,845±279) and highly variable ranging from 40±12% for cells isolated from donor 5 to 5.7±1.5% for cells isolated from donor 8 (Fig. **1B**). This is also shown microscopically in Fig. (**1D**), left and right panel for donor 5 (104 days post-transfection) and donor 8 (27 days post-transfection), respectively. Table **2** shows age (81±9 years), gender, time from death to preservation (11.2±4.5 hours), days of cultivation before (72±50) and after (76±45) transfection, and symbol reference used for each donor in the dot plots.

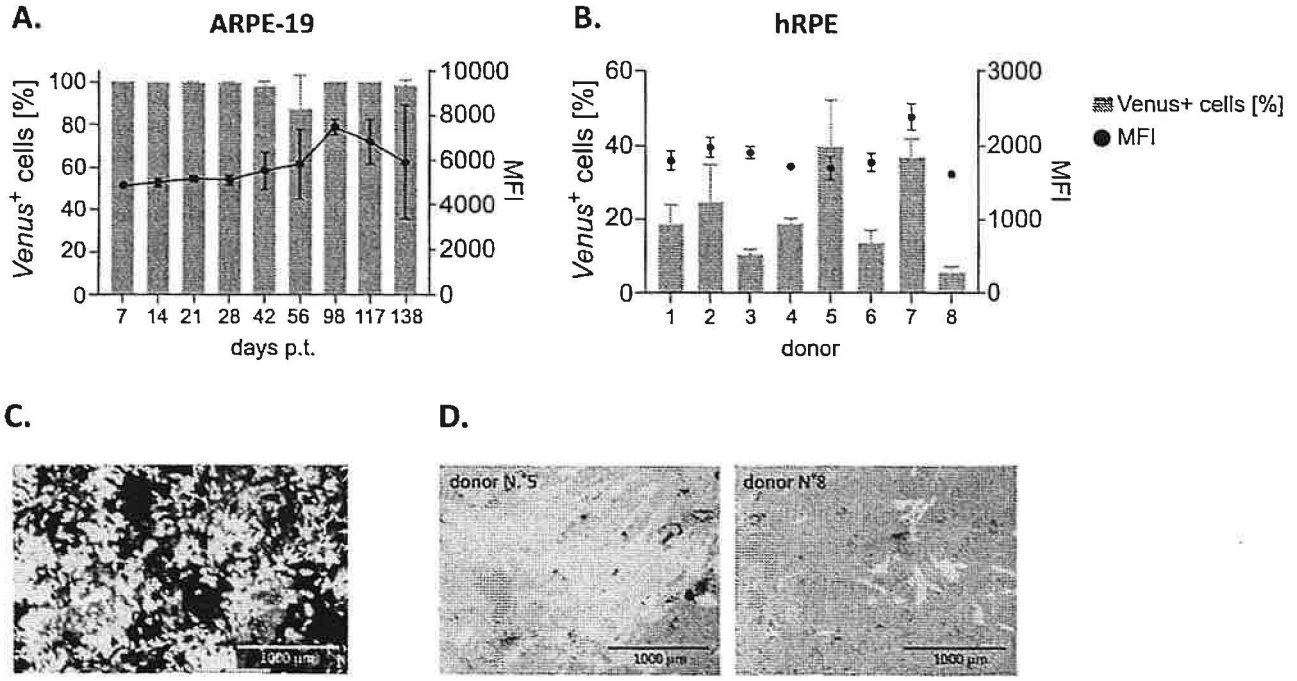

Fig. (1). Study of the transfection efficiency using the reporter gene *Venus*. (**A**). Percentage of Venus$^+$ ARPE-19 cells followed for 138 days; the graph shows n=3 replicates for each day. (**B**). Transfection efficiency for primary human RPE cells (n=8 donors) (the day of termination of the culture for each donor is shown in Table **2**). The graph shows n=3 replicates for each donor. (**C**). Fluorescent micrograph of transfected ARPE-19 cells at 21 days post-transfection. (**D**). Fluorescent micrograph of human RPE cells (donor 5 [left panel], donor 8 [right panel]) (magnification: 40x). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Characteristics of human retinal pigment epithelial cell donor.

-	No	Age	Gender	Death to Preservation (Hours)	Death to Isolation (Days)	Cultivation Before Transfection (days)	Cultivation After Transfection (Days)	Symbol in Graph
-	1	60	F	13.7	7	131	114	●
-	2	80	M	20.7	8	140	36	○
-	3	86	F	12.8	8	85	45	▼
-	4	86	F	8.5	5	26	133	▽
-	5	78	F	10.2	6	28	104	■
-	6	95	F	7.8	5	104	31	□
-	7	83	F	7.2	7	40	116	▲
-	8	83	F	8.9	6	18	27	△
mean	-	**81**	-	**11.2**	**6.5**	**72**	**76**	-
SD	-	10	-	4.5	1.2	50	45	-

3.2. Gene Expression and Integration Analysis

PCR analysis of the genomic DNA of the transfected cells revealed the integration of 1.00±0.04 and 5.00±1.00 copies of the genes for *PEDF* and *GM-CSF*, respectively (Fig. **2A**). For ARPE-19 cells, gene expression was determined by RT-qPCR weekly for 10 weeks; for primary human RPE cells (n = 8 donors), gene expression was determined at the end of the cell culture (76±45 days post-transfection). Transfected ARPE-19 cells revealed a significant increased gene expression of both *PEDF* and *GM-CSF* compared with non-transfected cells. For *PEDF*, a 401±596-fold increase (min 70±30 [56 days post-transfection], max 1747±446 [7 days post-transfection]) for single ($p < 0.0001$) and 244±286-fold increase (min 50±34 [56 days post-transfection], max 880±142 [7 days post-transfection]) for double transfected cells ($p < 0.0001$) was observed compared with non-transfected cells from day 7-70 post-transfection (Fig.**2B**). For *GM-CSF*, the increase was 2752±246-fold (min 593±39 [7 days post-transfection], max 7012±1004 [70 days post-transfection]) for single ($p = 0.0003$) and 1278±1650-fold (min 92±11 [7 days post-transfection], max 4215±2415 [42 days post-transfection]) for double ($p<0.0001$) transfected cells (Fig. **2C**). A significantly augmented gene expression was also observed in transfected primary human RPE cells for both genes compared with non-transfected human RPE cells. For *PEDF*, an increase of 1.9±0.5-fold was observed for single ($p = 0.0094$) and 4.2±4.3-fold for double ($p = 0.0019$) transfected cells (Fig. **3A**). For *GM-CSF*, the increase was 1217±2091-fold for single ($p = 0.0006$) and 697±1460-fold for double ($p = 0.0067$) transfected cells (Fig. **3B**). Gene expression data for human RPE cells is the average of the measurements at the end of the cell culture (76±45 days post-transfection; the day of cell culture termination for each donor is shown in Table **2**).

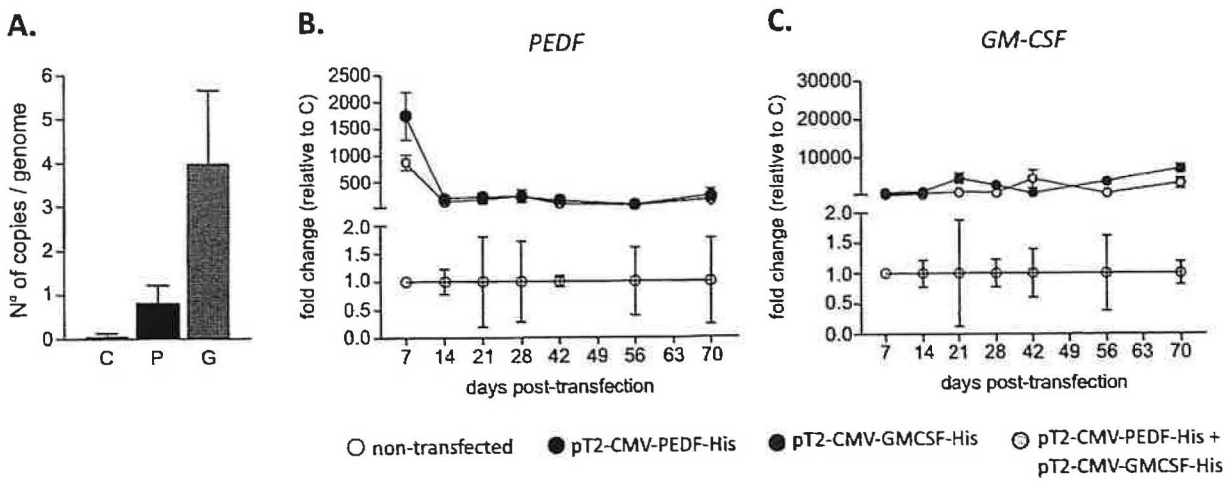

Fig. (2). Genomic analysis of *PEDF* and *GM-CSF* in transfected ARPE-19 cells. (**A**). Quantification of transposon insertions by RT-qPCR into the host cell (ARPE-19) genome (n=3 replicates). (**B-C**). Gene expression for both, single and double transfected *PEDF* and *GM-CSF* ARPE-19 cells compared with non-transfected cells until termination of the culture (n=3 replicates). mRNA values were normalized to the *GADPH* gene and expressed relative to mRNA levels of the non-transfected cells.

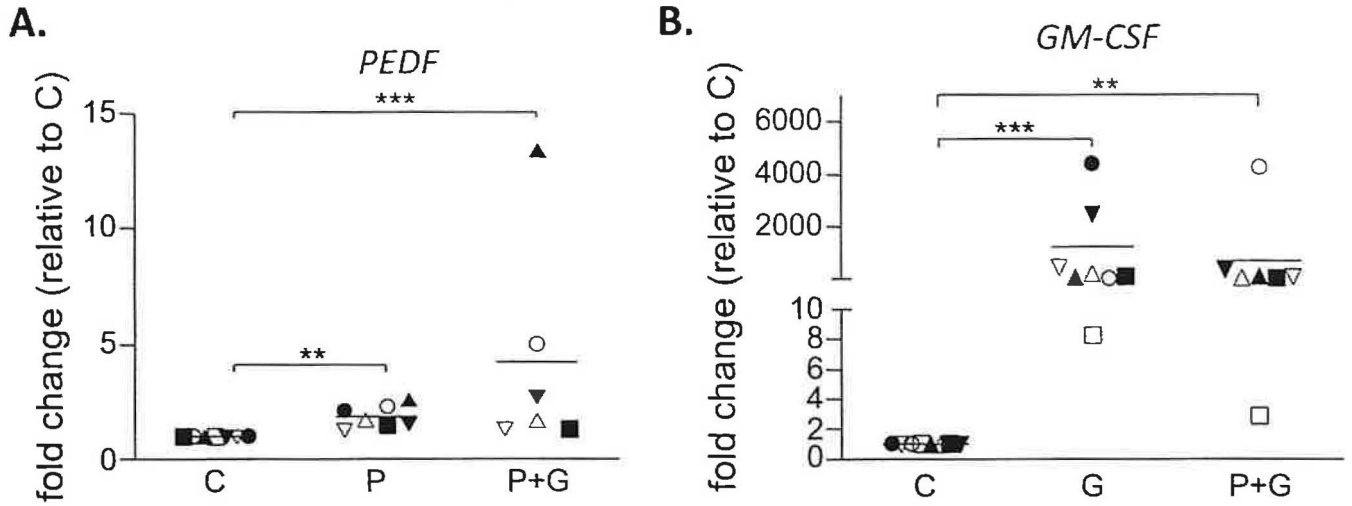

Fig. (3). *PEDF* and *GM-CSF* gene expression after transfection of primary human RPE cells. Gene expression of *PEDF* (**A**) and *GM-CSF* (**B**) in single and double transfected primary human RPE cells at 76±45 days post-transfection (2-3 replicates from each donor). mRNA values were normalized to the *GADPH* gene and expressed relative to mRNA levels of the non-transfected cells. The symbol donor references are presented in Table **2**.

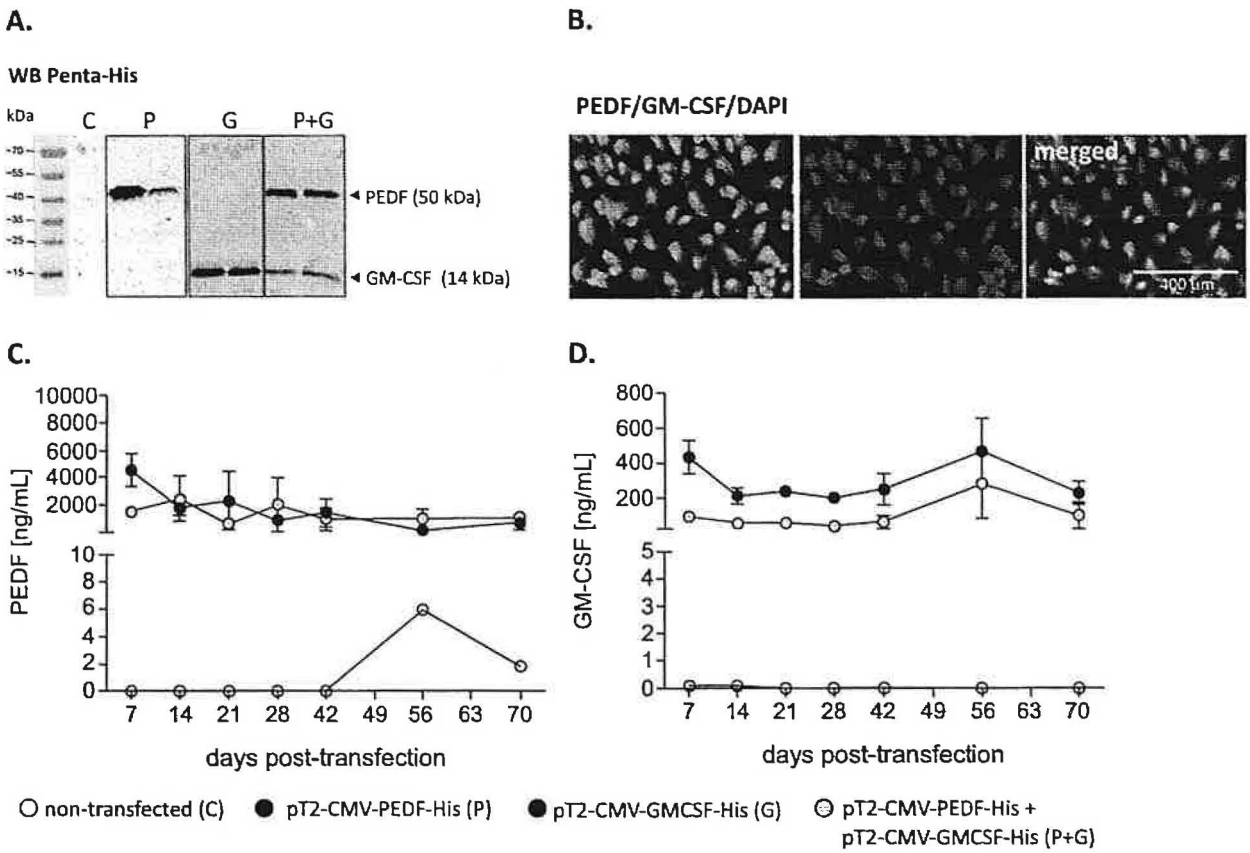

Fig. (4). GM-CSF and PEDF secretion after transfection of ARPE-19 cells. (**A**). PEDF (50 kDa) and GM-CSF (14 kDa) were detected by WB in ARPE-19 culture media collected at day 28 post-transfection using anti-penta-His antibodies. (**B**). Immunohistochemical analysis of co-transfected ARPE-19 cells using specific monoclonal antibodies. (**C-D**). Protein quantification of PEDF (left panel) and GM-CSF (right panel) in transfected ARPE-19 cells *via* ELISA (n=3 replicates). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3. Secretion of PEDF and GM-CSF in Cells after Transfection

The secretion of the proteins was confirmed by WB specific for penta-His in supernatant of single and double transfected ARPE-19 cells; since the blots were developed with anti-His antibodies, no signal was detected in control non-transfected cells (C) (Fig.**4A**). The secretion of both PEDF and GM-CSF in double-transfected cells studied by immunofluorescence (described in Supplementary Material **S1**) was illustrated by double staining of GM-CSF and PEDF (Fig.**4B**).

For the 10 weeks that cells were followed in culture, APRE-19 cells secreted significantly more PEDF, 1694±1450-fold (min 136±63 [56 days post-transfection], max 4550±1211 [7 days post-transfection]) for single (p=0.0022) and 1390±667-fold (min 607±396 [21 days post-transfection], max 2494±1662 [14 days post-transfection]) for double (p=0.0005) transfected cells compared with non-transfected from day 7-70 post-transfection (Fig. **4C**). GM-CSF protein secretion was also augmented in transfected cells; 289±111-fold (min 200±24 [28 days post-transfection], max 467±186 [56 days post-transfection]) for single (p=0.0012) and 99±82-fold (min 39±6 [28 days post-transfection], max 281±199 [56 days post-transfection]) for double (p=0.0431) transfected cells compared with non-transfected cells from day 7-70 post-transfection (Fig. **4D**).

Significantly higher protein secretion was also observed in PEDF-transfected primary human RPE cells; non-transfected human RPE cells secreted 0.6±0.4 ng PEDF/d/5x10^3 cells whereas PEDF transfected cells secreted 22.2±14.5 ng/d/5x10^3 cells for single (p = 0.0016) and 21±13.6 ng PEDF/d/5x10^3 cells for double (p = 0.0042) transfected cells (Fig. **5A**). GM-CSF protein section was also significantly higher in GM-CSF-transfected cells; the protein secretion from non-transfected human RPE was not detectable by ELISA whereas GM-CSF transfected cells secreted 4.2±4.2 ng/d/5x10^3 cells for single (p = 0.0003) and 2.8±4.2 ng PEDF/d/5x10^3 cells for double (p = 0.011) transfected cells (Fig. **5B**). Protein secretion data for human RPE cells is the average of the measurements at the end of the cell culture (76±45 days post-transfection; the day of cell culture termination for each donor is shown in Table **2**).

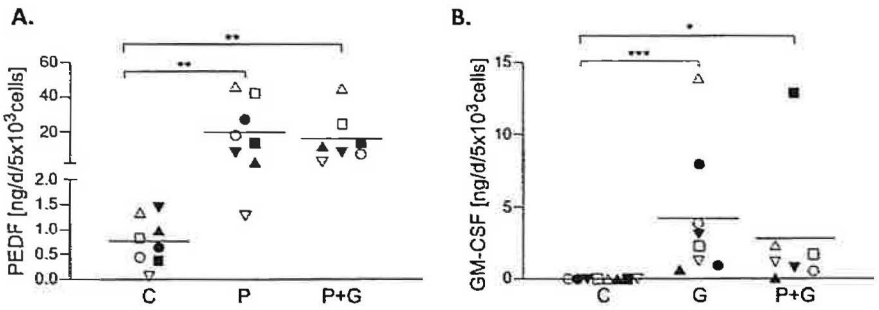

Fig. (5). Protein secretion in transfected primary human RPE cells. PEDF (**A**) and GM-CSF (**B**) protein quantification *via* ELISA in supernatants of single and double transfected primary human RPE cells (n=8 donors, 2-3 replicates/donor). C: non-transfected cells, P: PEDF- transfected cells, G: GM-CSF-transfected cells, P+G: PEDF, and GM-CSF double transfected cells. The symbol donor reference and day of cell culture termination are given in Table **2**.

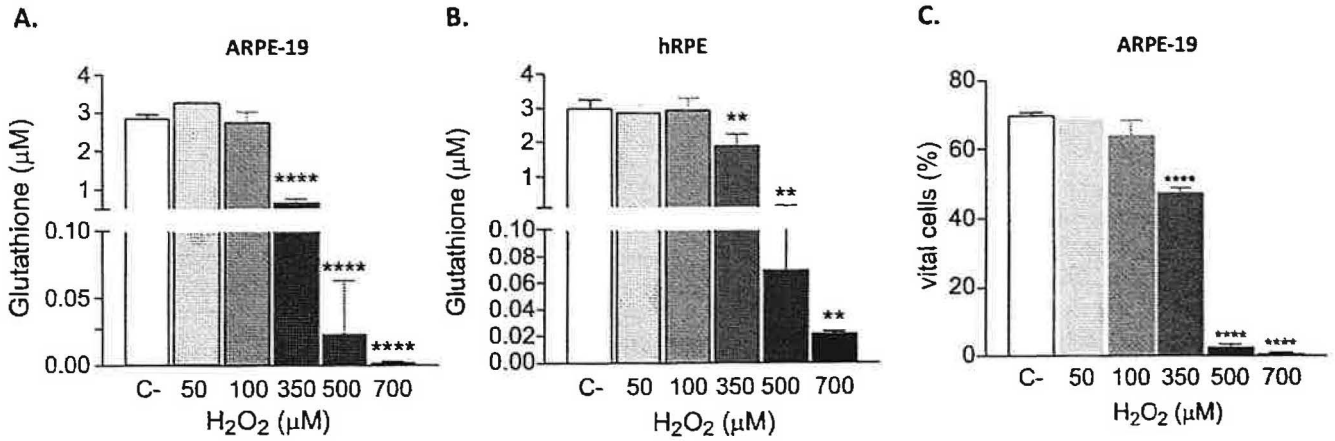

Fig. (6). (**A**). Oxidative stress model. Glutathione levels shown for ARPE-19 cells (**A**) and primary human RPE cells (**B**) after incubation with varying concentrations of H_2O_2. 350 μM was the minimal H_2O_2 concentration that generated significant oxidative harm compared with the negative control. (**C**). Effect of varying concentrations of H_2O_2 on cell viability. Data are represented from n=3 replicates.

3.4. Neuroprotection of PEDF and GM-CSF in Retinal Pigment Epithelium Cells

The effect of 24 hours treatment with varying concentrations of H_2O_2 on glutathione production is shown in Fig. **6A** for ARPE-19 and Fig. **6B** for primary human RPE. H_2O_2 at 50 μM and 100 μM does not influence glutathione generation; at 350 μM, there is a significant decrease of glutathione, 77% in ARPE-19 and 37% in primary human RPE. For ARPE-19 cells, a significant decrease in cell viability was observed from 350 μM onwards (Fig **6C**). Morphologically, cells treated with H_2O_2 treatment are rounder and spread less with increasing H_2O_2 concentration (Supplementary Material **S2**, Fig. **S2.1**).

Next, ARPE-19 and human RPE cell medium was supplemented with 5, 50, and 500 ng/mL GM-CSF and/or PEDF for three days, starting two days before the 24-hours H_2O_2 treatment. After incubation with 500 ng/mL PEDF, ARPE-19 cells produced 1.83 μM of glutathione, *versus* 0.83 μM of glutathione when cell medium was not supplemented with protective proteins (C) ($p = 0.0261$); treatment with 50 ng/mL showed a similar behavior but no significant differences were observed (Fig. **7A**). Comparable results were obtained with GM-CSF treatment (Fig. **7A**), but in this case, glutathione levels differed significantly already with a concentration of 50 ng/mL (1.3 μM) ($p = 0.037$). Thus, the concentrations chosen for further experiments were 500 ng/mL for PEDF and 50 ng/mL for GM-CSF. Regarding proteins extracted from culture media of transfected ARPE-19 cells, similar results were observed using 500 ng/mL PEDF and 50 ng/mL GM-CSF, with glutathione levels of 0.9 μM for PEDF ($p=0.0037$), and 0.8 μM for GM-CSF ($p=0.293$), *versus* 0.4 μM observed for the control (C) (Fig.**7B**). Treated primary human RPE cells produced higher glutathione levels (PEDF: 2.6 μM [purchased], 2.5 μM [purified]), (GM-CSF: 2.9 μM [purchased], 3.3 μM [purified]), (PEDF plus GM-CSF: 3.0 μM [purchased], 2.9 μM [purified]) *versus* 1.9 μM observed for the control (C) ($p=0.0059$ [purchased], and $p=0.0037$ [purified]) (Fig. **7C**). In addition, primary human RPE cells cultured in conditioned medium from transfected ARPE-19 cells for ten days presented increased glutathione level, 0.21 μM for the three protein-treated group, compared with 0.19 μM observed for the control (C) ($p = 0.0001$) (Fig. **7D**).

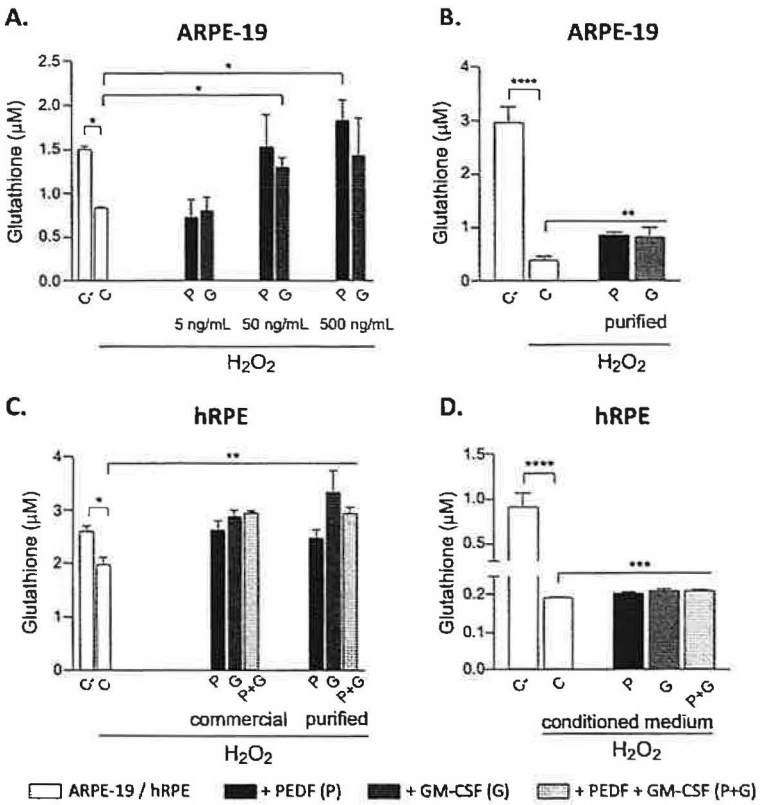

Fig. (7). (**A**). Neuroprotective effects of GM-CSF and PEDF. (**A**). Glutathione levels in ARPE-19 cells treated with 5, 50 or 500 ng/mL PEDF or GM-CSF commercial proteins for three days. (**B**). Glutathione levels in ARPE-19 cells treated with 500 ng/mL PEDF or 50 ng/mL GM-CSF proteins purified from culture media of ARPE-19 cells transfected with the genes coding for the respective proteins. (**C**). Glutathione levels in primary human RPE cells treated with 500 ng/mL PEDF and/or 50 ng/mL GM-CSF commercial and purified proteins (data shown for one donor). (**D**). Glutathione levels in primary human RPE cells treated with conditioned medium during 10 days before starting the H_2O_2 treatment (data shown for one donor). Data are represented from n=3 replicates. C-: non-treated cells (either H_2O_2 or proteins), 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.

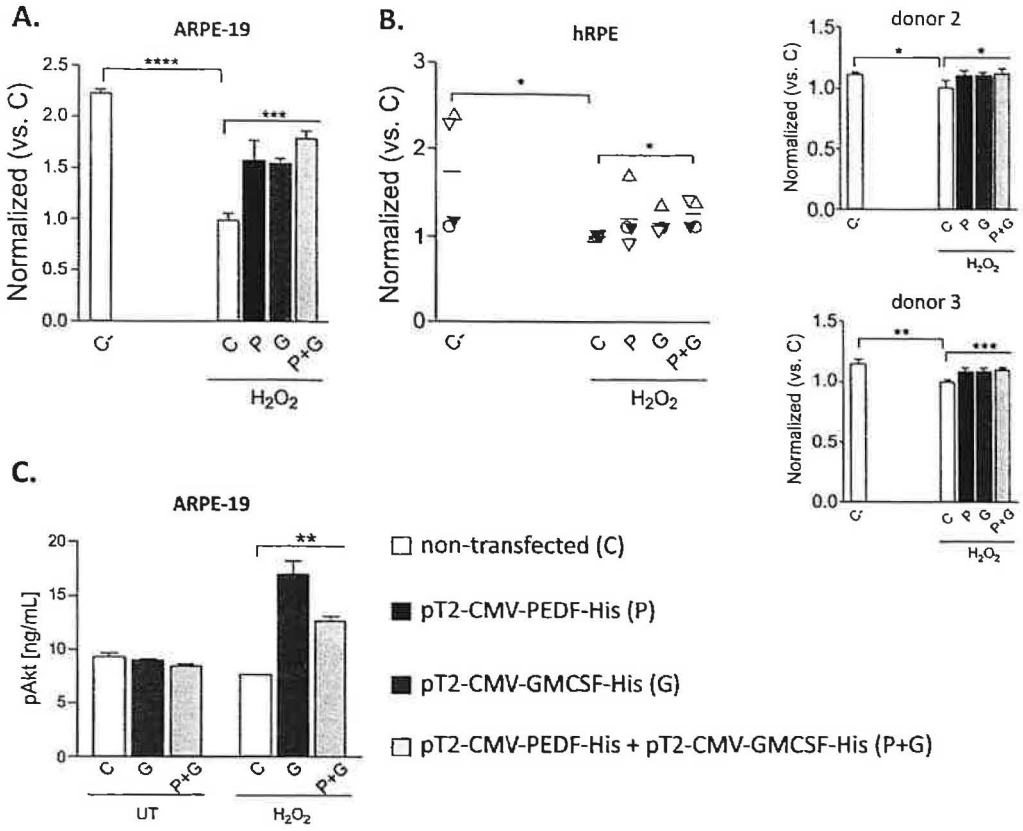

Fig. (8). Effect of H_2O_2 on glutathione and Ser473-phosphorylated AKT levels in ARPE-19 and human RPE cells transfected by the genes coding for GM-CSF and PEDF. (A). Levels of glutathione normalized to control (C) are shown for transfected ARPE-19 cells incubated with 350 μM H_2O_2 for 24 hours (56 days post-transfection). For normalization, the glutathione levels in transfected cells were divided by the control value (H_2O_2 treated cells, not transfected) (n=3 replicates). **(B).** The graphs visualize the normalized values of glutathione for transfected human RPE cells (n=4 donors) after the H_2O_2 treatment. **(C).** Quantification of pAkt performed by ELISA in GM-CSF-transfected ARPE-19 cells exposed to 350 μM H_2O_2 for 24 hours. C-: non-transfected cells without H_2O_2 treatment, C: non-transfected cells treated with H_2O_2, UT: cells non-treated with H_2O_2

All groups of transfected ARPE-19 cells treated with 350 μM H_2O_2 for 24 hours revealed significantly increased glutathione level (1.5-fold increase for GM-CSF- and PEDF-single transfected cells, and 1.7-fold increase for double transfected cells) in relation to non-transfected cells exposed to the oxidant agent (C) ($p < 0.0001$) (Fig. **8A**). For transfected primary human RPE cells, comparing the mean values of all donors (n = 4 donors), significant differences were observed only for double transfected cells (1.3-fold increase) *versus* the control (C) ($p = 0.0246$); however, analyzing the donors individually, significant differences were observed for all transfected groups compared with the control (C) for donor N°2 ($p = 0.0222$) and N°3 ($p = 0.0010$), with an approx. 1.1-fold increase for all transfected groups for both donors (Fig. **8B**).

The quantification of Ser473-phosphorylated Akt showed higher amounts in lysates from GM-CSF-transfected cells in relation to non-transfected cells exposed to H_2O_2 in both the ELISA analysis (Fig. **8C**) and the WB (Supplementary Material **S2**, Fig. **S2.2**).

3.5. Neuroprotection Effect of PEDF and GM-CSF in Retinal Organotypic Culture

Cultured rat retinas (n = 6) maintained tissue integrity for up to 13 days (Fig. **9A**). At 3-4 days of culture, the retinal layers were still organized, however, some vacuolization has been present (arrow). At 6-8 days of culture, retinal morphology was largely normal with only a few signs of degeneration (arrow). At 12-13 days of culture, photoreceptors were mostly lost (yellow arrow); however, the neuronal layers were still distinguishable (white arrows), with only a minor loss of cells. No microglial activation was observed (Iba-1) in retinas treated with 350 μM H_2O_2 for 3 hours, whether the retinas were treated or not with proteins for 3 days before H_2O_2 treatment (Fig. **9B**). Increased protein expression of GFAP and loss of rhodopsin in photoreceptors illustrate inflammation and cell damage detected by fluorescent green GFAP and red Rhodopsin staining after oxidative stress induction by H_2O_2 incubation at 3 days of culture (Fig. **9C** [H]). Treatment of retinas with 500 ng/mL recombinant PEDF (+P) and 500 ng/mL GM-CSF (+G) for 3 days before treatment with H_2O_2 for 3 hours reduced GFAP and rho-

dopsin loss (Fig. **9C** [H+P, H+G]). By TUNEL assay, we observed that retinas treated with 350 μM H_2O_2 for 3 hours had a large number of apoptotic cells, comparable to the number of apoptotic cells observed when retinas were treated with DNase I (Fig. **10** [H]). Three days of treatment with 500 ng/mL recombinant PEDF or GM-CSF before 3-hours treatment with H_2O_2 reduced the number of apoptotic cells (Fig. **10** [H+P], [H+G]).

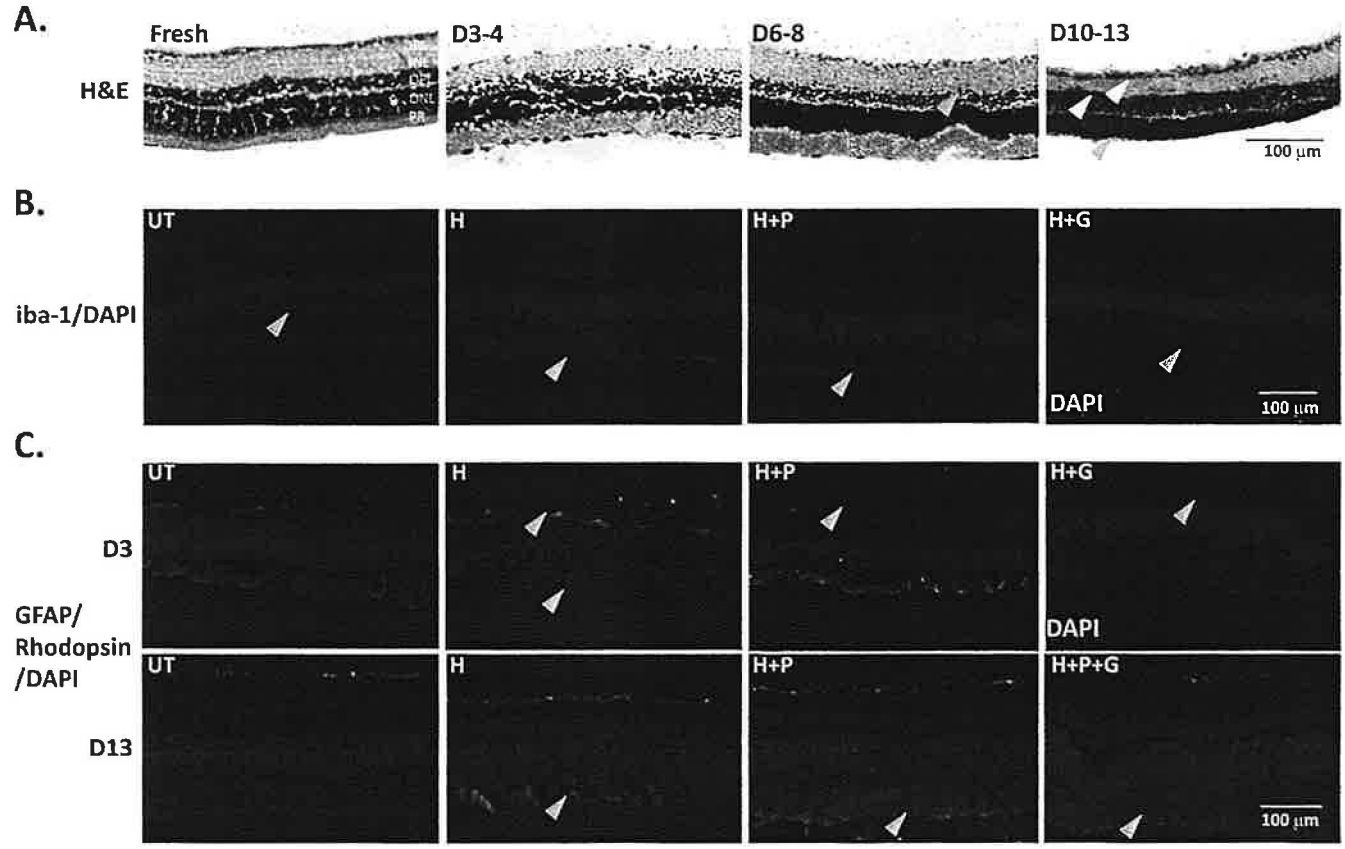

Fig. (9). Impact of GM-CSF and PEDF on morphology of retinas treated with H_2O_2. (**A**). Histological study of rat retinas cultured for 13 days. (**B**). Immunostaining with anti-Iba-1 (green) of retinas treated with 350 μM H_2O_2 for 3 hours, exposed or not to 3 days of protein treatment before H_2O_2 treatment. (**C**). Immunostaining with anti-GFAP (green) and anti-Rhodopsin (red) in H_2O_2-treated retinas, exposed or not to 3 days of protein treatment before H_2O_2 treatment. Notice that due to limited availability of retinal tissue, not all treatment combinations could be tested with all antibodies at all time points. UT, untreated; RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR, photoreceptor layer. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Fig. (10). Impact of GM-CSF and PEDF on viability of retinal cell treated with H_2O_2 studied by TUNEL assay. H_2O_2 treatment (H) increased the number of apoptotic cells (green). Pre-treatment with PEDF (P) or GM-CSF (G) was able to compensate the effect and reduce the number of retinal apoptotic cells. PoCo: positive control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

Even though gene therapy has entered clinical routine by 13 GTMP currently on the market, used gene delivery systems still suffer from limitations. A possibility to overcome these limitations regarding the safety and efficiency of several gene delivery systems may be the use of transposon-mediated gene delivery in combination with electroporation. In particular, the hyperactive *SB* transposon system has the advantage of high transfection efficiency, the ability to integrate large inserts, stable and safe integration since it does not preferably integrate transcriptional active sequences, and enters the cells by plasmids, reducing the immunogenicity and facilitating its production [39, 46-48], features that led to the decision to develop a non-viral gene therapy against retinal degeneration based on the *SB* system. It is worth mentioning that 12 clinical trials are being carried out using the SB transposon system, most of them based on the genetic modification of T cells for the treatment of B cell malignancies demonstrating high benefit but none acute or late toxicities [17]. The use of electroporation for its part has advantages such as yielding high transfection efficiency and usability with most cell types; compared with other gene delivery systems, it is faster, inexpensive, and safe (non-mutagenic) [32]. There are several clinical trials using electroporation for both gene therapy and non-gene therapy approaches (*e.g.*, delivery of chemotherapeutic drugs) [33, 49, 50]. Regarding the potential negative impact that electroporation can have on cell viability because of the formation of pores in the cell membrane [51], we did not observe significant cell death after transfection. This is consistent with studies, which have reported that with the use of capillary electroporation systems, such as the Neon transfection system, cell viability and transfection rate, especially for primary cells, are higher than after applying conventional electroporation [52]. It is assumed that the capillary electroporation system provides a uniform electric field that diminishes the side effects of electrolysis due to the small surface area of the electrode [52]. Thus, we combined the advantages of both, the *SB100X* transposon system and electroporation, achieving a significant transfection efficiency with negligible cellular toxicity.

An ideal treatment for retinal neurodegeneration associated with loss of RPE cells would be the implantation of functional RPE cells secreting neuroprotective factors necessary to maintain the RPE cell layer, and in consequence, the photoreceptors and RGCs. The study conducted by Binder & col., in which a cell suspension of freshly harvested autologous RPE was transplanted to patients with nAMD [53-55], showed moderate visual improvement in more than half of the cases, but with useful reading, acuity was observed only in a small group of patients. Recently, Da Cruz & col. have described a phase I clinical trial using a RPE-patch derived from embryonic stem cells to treat AMD with promising results [56]. In addition, different groups have published studies in which autologous RPE–Bruch's membrane–choroid patches were transplanted [57-59], and induced pluripotent stem cell (iPSC)-derived RPE patches have been generated [60]. The lack of improvement in several of these studies may be due to the low number of transplanted cells, and/or the low levels of neuroprotective factors secreted by the cells. Thus, the transplantation of pigment epithelial cells transfected to overexpress the neuroprotective factors would be a promising approach.

As expected from former work [40], the transfection efficiencies, as evidenced by fluorescence of the expressed *Venus* gene, were ~100% for ARPE-19 cells and ~20% for primary human RPE cells. We have shown that using the *SB100X* transposon system in combination with electroporation, ARPE-19 cell and primary human RPE cells can be successfully transfected with the *PEDF* gene and the *GM-CSF* gene as well as with both genes. Secretion of both, GM-CSF and PEDF, was stable for the time the cells were maintained in culture (10 weeks). The lower level of PEDF secretion compared to GM-CSF secretion might be explained by the number of gene copies integrated into the genome, specifically 1 copy for PEDF and 5 for GM-CSF; however, other unknown factors could be additionally involved. Secretion of PEDF and GM-CSF showed wide variation among donors, which can be expected considering differences of time from death to preservation of eyes (7.2 to 20.7 hours), time from death to isolation (5 to 8 days), donor's age, gender, and existing pathologies. It should be noted that in our final clinical approach, in which it is planned to subretinal transplant transfected freshly isolated autologous iris pigment epithelial (IPE) cells in patients with dry AMD, variances are expected to be smaller since the tissue is going to be fresher than in our *in vitro* system, without the impact of the time from death to enucleation and preservation conditions of the eye, *i.e.*, in the clinic, the tissue is going to be used within ~1 hour after isolation from an alive person *vs.* days (in the present work 6.5±1.2 days) after eye enucleation from a dead donor (Table **2**).

Since oxidative stress of RPE cells is considered to be a key factor in the pathogenesis of aAMD [61, 62], treatment of the disease should consider strategies to reduce oxidative stress in RPE cells. Since it has been reported that PEDF lowers oxidative stress in retinal neurons [7, 63-66] and that GM-CSF augments the activation of the PI3K/Akt and ERK1/2 pathways, which is implicated in cell survival of neuronal cells [11, 67, 68], we have investigated the effect of the proteins on H_2O_2-mediated RPE cell functionality and survival. H_2O_2 was chosen as oxidant agent since it is a well-established model of oxidative stress damage in retinal cells [69-72]. Using glutathione as an index of cell functionality, we have shown that both PEDF and GM-CSF whether purchased or isolated from culture medium conditioned by transfected cells increased the level of glutathione in ARPE-19 and primary human RPE cells; in addition, a higher amount of phosphorylated Akt was observed in GM-CSF transfected ARPE-19 cells. Therefore, PEDF and GM-CSF prevent damage and protect RPE from the toxic impact of H_2O_2. All transfected groups had significantly increased levels of glutathione related to non-transfected cells; however, there was no significant alteration observed between the different transfected groups for both ARPE-19 and primary

cells. The higher differences observed in ARPE-19 compared with primary cells are explained by a generally higher protein secretion level compared with primary cells (Fig. 4). In this regard, Pang & col. showed that treatment of RGCs with PEDF dose-dependently secured against glutamate-induced toxicity. Additionally, RGC viability was sensitive to trophic factors (bFGF, BDNF, CNTF) withdrawal, and PEDF successfully inhibited related damage in a concentration-dependent manner [64]. In addition, Schallenberg & col. observed that RGCs treated with pro-apoptotic drugs (staurosporine or glutamate) diminished the numbers of dying cells in a dose-dependent manner after being treated with increasing concentrations of GM-CSF [11]. In transfected primary human RPE cells, differences are partly masked by the high inter-donor variability as already discussed. Thus, when all donors were analyzed together, significant differences were observed only in double transfected cells (Fig. 7, dot plot), but when donors were analyzed individually, in 2 out of 4 donors, differences were seen for all transfected in comparison to non-transfected cells under oxidative stress conditions.

To define whether the beneficial effects of GM-CSF and PEDF can be translated from cell culture to tissues and eventually to whole organisms, we analyzed the effect of the proteins in rat retinas organotypic cultures. The establishment of a ROC model for oxidative stress is important to test therapeutic approaches in the whole organ taking into account the complex interplay between the different neuronal retinal cells [73-75]. The results from the experiments with rat retinas after oxidative stress induction showed that the supplementation of the medium with the proteins improved tissue integrity, *i.e.* lower inflammation and better preservation of photoreceptors. In addition, exposure of the retinas to PEDF/GM-CSF decreased apoptotic cell death and increased cell viability compared with the non-treated control, and this effect could be seen even after 6 days of culture. Supporting these results, it was recently described that PEDF can support photoreceptor survival in rd10 retina models [76] and the rescue of the photoreceptor cell degeneration in microphthalmia (Mitf) mice [77].

The monitoring of the glutathione redox state in biological samples is essential for evaluating the detoxification status of the cells and tissues against oxidative and free radicals mediated cell injury [6], therefore, we used a GSH assay to analyze the benefit of our therapy approach. Further, studies have described that oxidative stress is mostly generated in mitochondria [78], and particularly, PEDF attenuates the effects of oxidative stress by increasing the mitochondrial uncoupling protein 2 (UCP2) expression [7, 65]. Thus, the gene expression analysis of *UCP2*, which acts as a sensor of mitochondrial oxidative stress [65, 79] is currently analyzed [6].

Since the final goal is to transplant IPE cells overexpressing the neuroprotective factors, we are currently carrying out experiments transfecting primary human IPE cells with *PEDF* and *GM-CSF*. Here, we have confirmed the long-term overexpression of the proteins in transfected human RPE cells; similar experiments with human IPE cells are ongoing showing promising preliminary results. The protective effect of PEDF and GM-CSF in the context of oxidative stress could be determined. Furthermore, based on the results presented here, we recently optimized a murine disease model of aAMD [80], and the first subretinal transplantations with retinal pigment epithelial cells in ongoing experiments are promising.

CONCLUSION

The non-viral hyperactive *SB* transposon-mediated gene delivery system combined with electroporation has been shown to overcome crucial limitations of viral vectors, offering an efficient and safe method for genetic modification of human cells as it was reflected in our results in which we observed a sustained gene and protein secretion of chosen GOIs. PEDF and/or GM-CSF reduced oxidative stress damage in pigment epithelial cells. The results have been confirmed by studies in retinal organotypic cultures in which PEDF and GM-CSF improved tissue preservation and cell vitality. We believe our results support the hypothesis that PE cells overexpressing GM-CSF and PEDF, transplanted subretinally, would be a promising approach to treat aAMD patients.

AUTHORS' CONTRIBUTIONS

GT, MK, and NH conceived and supervised the project. TB drafted the manuscript, designed and performed most experiments, and analyzed the data. MK and NH participated in the experimental design and data analysis. HZ carried out experiments involving retinal organotypic cultures. MA, CPS, and ST participated in the production of plasmid constructs. TB, MK, NH, and GT reviewed and revised the paper. All authors contributed to the project and manuscript preparation with enriching discussions. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The cantonal ethical commission for research, Geneva, approved the use of the donor tissue (project no. 2016-01726).

The protocols in which animals were involved (retinal organotypic cultures) were carried out by certified personnel in accordance with the Animal Welfare Department of the Canton de Genève and European regulations for the care and use of laboratory animals and were approved by the Commission Cantonale pour les Expériences sur les Animaux (C-CEA), Switzerland (approval no. GE/116/19).

HUMAN AND ANIMAL RIGHTS

For the human eyes obtained from the Minnesota Lions Eye Bank (Saint Paul, MN), the eyes were enucleated after informed consent was obtained in accordance with the Declaration of Helsinki protocols. All the reported experiments on animals were conducted in accordance with the guidelines

established by the European regulations (Directive 2010/63/EU) for the care of animals used for scientific purposes.

CONSENT FOR PUBLICATION

The eyes were enucleated after informed consent was obtained.

AVAILABILITY OF DATA AND MATERIALS

All raw data supporting our findings are available on the repository Yareta (https://doi.org/10.26037/yareta:nknjbdlqwrc7perfksgofnskm4, excel file SNF_*in vitro* data_20201209), and in case of sensitive data upon request.

FUNDING

This work was funded by the Swiss National Science Foundation (SNSF) (project no. 31003A_160195/1).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors thank Gregg Sealy and Alain Conti for excellent technical assistance, and Prof. Nino Sorgente for his support in the editing of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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