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Isolation, Culture, and Genetic Engineering of Mammalian Primary Pigment Epithelial Cells for Non-Viral Gene Therapy

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Abstract

Age-related macular degeneration (AMD) is the most frequent cause of blindness in patients >60 years, affecting ~30 million people worldwide. AMD is a multifactorial disease influenced by environmental and genetic factors, which lead to functional impairment of the retina due to retinal pigment epithelial (RPE) cell degeneration followed by photoreceptor degradation. An ideal treatment would include the transplantation of healthy RPE cells secreting neuroprotective factors to prevent RPE cell death and photoreceptor degeneration. Due to the functional and genetic similarities and the possibility of a less invasive biopsy, the transplantation of iris pigment epithelial (IPE) cells was proposed as a substitute for the degenerated RPE. Secretion of neuroprotective factors by a low number of subretinally-transplanted cells can be achieved by *Sleeping Beauty* (*SB100X*) transposon-mediated transfection with genes coding for the pigment epithelium-derived factor (PEDF) and/or the granulocyte macrophage-colony stimulating factor (GM-CSF). We established the isolation, culture, and *SB100X*-mediated transfection of RPE and IPE cells from various species including rodents, pigs, and cattle. Globes are explanted and the cornea and lens are removed to access the iris and the retina. Using a custom-made spatula, IPE cells are removed from the isolated iris. To harvest RPE cells, a trypsin incubation may be required, depending on the species. Then, using RPE-customized spatula, cells are suspended in medium. After seeding, cells are monitored twice per week and, after reaching confluence, transfected by electroporation. Gene integration, expression, protein secretion, and function were confirmed by qPCR, WB, ELISA, immunofluorescence, and functional assays. Depending on the species, 30,000-5 million (RPE) and 10,000-1.5 million (IPE) cells can be isolated per eye. Genetically modified cells show significant PEDF/GM-CSF overexpression with the capacity to

reduce oxidative stress and offers a flexible system for *ex vivo* analyses and *in vivo* studies transferable to humans to develop ocular gene therapy approaches.

Introduction

Our group is focusing on the development of regenerative approaches to treat neuroretinal degeneration, i.e., AMD, by RPE- and IPE-based non-viral gene therapy. The pre-clinical establishment of such therapies necessitates *in vitro* models transferable to human beings. Thus, the goal of the study presented here is to deliver protocols for the isolation, culture, and genetic engineering of primary RPE and IPE cells. The rationale to establish the isolation of PE cells from multiple species is to robustly confirm safety and efficiency of the approach and increase its reproducibility and transferability[5]. The available human RPE cell line ARPE-19 differs from primary cells (e.g., they are less pigmented) and is, therefore, only of limited value for pre-clinical analyses[1]. Additionally, non-human mammalian cells can be purchased for less cost and in bigger quantities; human donor tissue can be obtained from various Eye Banks, but the availability is limited and expensive. Finally, new Advanced Therapy Medicinal Products (ATMP, i.e., cell, tissue, or Gene Therapy Medicinal Product) need to be applied in at least two different species before being tested in patients and these *in vivo* studies request the preparation of allogenic cell transplants.

Retinal neurodegenerative diseases are the leading cause of blindness in industrialized countries, comprising common diseases like AMD, as well as rare diseases like retinitis pigmentosa, in which the retinal cell death eventually leads to blindness. RPE cells, photoreceptor, and retinal ganglion cells (RGC) damage can in some cases be slowed, but there are currently no curative therapies available. ATMPs offer the potential to correct gene defects, integrate therapeutic

genes or replace degenerated cells, thereby enabling the development of regenerative and curative therapies for diseases such as AMD; 13 gene therapies already got marketing approval including a therapy to treat RPE65 mutation-associated retinal degeneration[2,3]. Among older adults (>60 years), ~30 million people worldwide are affected by either neovascular (nvAMD) or avascular (aAMD) AMD[4]. Both forms are induced by age-associated triggers including oxidative damage, function impairment and loss of RPE cells followed by photoreceptor degradation, among others (e.g. genetic risk alleles, smoking, hypertension)[5,6]. In nvAMD, pathogenesis is aggravated by an imbalance of angiogenic and anti-angiogenic factors in favor of the angiogenic Vascular Endothelial Growth Factor (VEGF) that induces choroidal neovascularization (CNV). To date, only nvAMD is treatable by monthly intravitreal injections of inhibitors of the VEGF protein to suppress the CNV; no effective treatment is yet available for aAMD[6,7].

Several studies evaluated cell-based therapies to replace the anti-VEGF therapy: studies made by Binder et al., in which freshly-harvested autologous RPE cells were transplanted into patients with nAMD[8,9,10], showed moderate visual improvement, but only a small group of patients reached a final visual acuity high enough to enable reading. Recently, a phase I clinical study used an embryonic stem cell-derived RPE patch to treat AMD with promising results; i.e., efficacy, stability, and safety of the RPE patch for up to 12 months in 2 of the 10 patients treated[11]. In addition, several groups have published studies in which autologous RPE-

Bruch's membrane-choroid patches were harvested from the peripheral retina and transplanted to the macula[12,13,14]; and induced pluripotent stem cell (iPSC)-derived RPE patches were generated for transplantation[15]. For aAMD, antibodies targeting the complement pathway have been tested in clinical trials[6,16] and a phase I study using a single intravitreal injection of an adeno-associated viral (AAV) vector coding the gene for the factor CD59 (AAVCAGsCD59) in patients with geographic atrophy (GA) was completed[17]; the phase II study recently started and aims to recruit 132 patients with advanced aAMD and to evaluate the outcome at 2 years post-intervention[18]. Finally, the FocuS study group has started a phase I/II multicenter clinical trial evaluating the safety, dose response and efficacy of a recombinant non-replicating AAV vector encoding a human complement factor[19].

Primarily, the goal of a regenerative AMD therapy is the transplantation of functional RPE cells, which were damaged or lost. However, IPE and RPE cells share many functional and genetic similarities (e.g., phagocytosis and retinol metabolism), and because IPE cells are more feasibly harvested, they have been proposed as an RPE substitute[20]. Even though it has been previously demonstrated that the IPE cell transplantation delays photoreceptor degeneration in animal models[21,22] and stabilizes visual function in patients with end-stage nvAMD, no significant improvement was observed in these patients[23]. The lack of efficacy may be due to the low number of transplanted cells, and/or the imbalance of neuroprotective retinal factors. An alternative approach would be to transplant transfected pigment epithelial cells that overexpress neuroprotective factors to restore retinal homeostasis, maintain remaining RPE cells, and protect photoreceptors and RGCs from degeneration. Consequently, we propose a new therapy that comprises the transplantation of functional RPE or IPE cells that have undergone genetic

engineering to secrete neuroprotective and anti-angiogenic proteins, such as PEDF, GM-CSF or insulin-like growth factors (IGFs). The advantage of developing and analyzing this approach in several species instead of using a cell line, only one species, or human tissue is: 1) increased reproducibility and transferability of the results as shown by numerous studies realized in independent laboratories and different species[1,24,25]; 2) pig or bovine cells are feasibly disposable without the sacrifice of additional animals; 3) the availability of especially pig and bovine cells allows large test series to produce robust results; 4) the knowledge to isolate, culture and genetically modify cells from the mostly used models enables *in vivo* analyses in multiple species[24,25,26] and thus offers an improved risk-benefit ratio for the first treated patients; 5) the flexibility of the protocol presented allows its use in various models and experimental set ups and for all ocular cell based therapies with and without genetic engineering. In contrast, alternative techniques as cell lines or human tissue are only of limited transferability and/or limited disposability. Cell lines such as the ARPE-19 are ideal for preliminary experiments; however, low pigmentation and high proliferation differ significantly from primary cells[1]. RPE and IPE cells, which are isolated from human donor tissue offer a precious source for transferable *in vitro* experiments; however, we obtain human tissue from an US-American Eye Bank meaning that the tissue is at least two days old (after enucleation) and requires a long and expensive transport, but local donor tissue is not available in sufficient amounts for a productive research. The advantage of the use of primary cells is confirmed by multiple studies from other groups[27,28].

For the development of a cell-based non-viral gene therapy using the *SB100X* transposon system for transfecting primary RPE and IPE cells with the genes coding for PEDF and/or GM-CSF to treat nvAMD and aAMD, respectively[29,30,31,32],

we first established the transfection of ARPE-19 cells[1]. Next, the isolation and transfection protocols were established in readily accessible bovine and porcine primary cells. Now, the isolation and transfection of primary RPE and IPE cells from five different species has been established, from small (as mouse) to large mammals (as cattle). It was confirmed in primary RPE and IPE cells derived from human donor eyes[30]. The Good Manufacturing Practices (GMP)-compliant production of the ATMP was validated using human donor tissue as well[33]. Finally, both safety and efficiency of the approach were assessed *in vivo* in three different species for which the protocol has been adapted: mouse, rat, and rabbit. In the clinical set-up, an iris biopsy will be harvested from the patient and IPE cells will be isolated and transfected in the clean room, before the cells will be transplanted subretinally back into the same patient. The entire process will take place during a single surgical session that lasts approximately 60 minutes. The development of the treatment approach and the evaluation of its efficiency requested excellent *in vitro* and *ex vivo* models to implement robust and efficient gene delivery methods, to analyze efficiency of gene delivery, therapeutic protein production and neuroprotective effects, and to produce cell transplants to test the approach *in vivo*[1,24,25,29,30]. It is worth mentioning that the therapy has the ethical approval for a clinical phase Ib/IIa trial from the ethical commission for research of the Canton of Geneva (no. 2019-00250) and currently last pre-clinical data requested for authorization by Swiss regulatory authorities are collected using the presented protocol. In this regard, pre-clinical *in vivo* data demonstrated a significant reduction in CNV and excellent safety[24,25,31].

Here, the isolation and culture of RPE/IPE cells from bovine, pig, rabbit, rat and mouse, and the use of the integrative *SB100X* transposon system combined with electroporation as an efficient gene-delivery method is described. Particularly, primary PE cells were transfected to overexpress PEDF and GM-CSF. The collection of these protocols enables the *in vitro* and *in vivo* studies to be performed in all pre-clinical phases of ATMP development. Moreover, the set-up has the potential to be adapted to other genes of interest and diseases.

Protocol

The protocols in which animals were involved were carried out by certified personnel and after authorization by the cantonal Département de la sécurité, de l'emploi et de la santé (DSES), Domaine de l'expérimentation animale of Geneva, Switzerland, and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (approval no. GE/94/17). Adult healthy Brown Norway rats, C57BL/6 mice, and New Zealand white rabbits were euthanized by an overdose of Pentobarbital (150 mg/kg) diluted in 0.9% NaCl injected intraperitoneal and the eyes were enucleated immediately after sacrifice. Porcine and bovine eyes were obtained from a local slaughterhouse within 6 hours of sacrifice and were transported to the laboratory on ice.

1. Before preparation

1. Prepare complete medium (DMEM/Ham's F12 supplemented with 10% fetal bovine serum (FBS), 80 U/mL penicillin / 80 μg/mL streptomycin, and 2.5 μg/mL amphotericin B). Heat the medium, 1x PBS, and 0.25% trypsin (if necessary) in a 37 °C water bath.

2. Put a sterile drape into the hood to prepare an aseptic working place. Introduce all needed sterile instruments and materials inside the hood.
   NOTE: Only the enucleation of the eyes and cleaning of remaining muscle tissue and skin are the procedures

carried out outside the hood, the rest of the steps must be performed inside the hood.

2. Isolation of rat/mouse PE cells

1. Use curved scissors and Colibri forceps to enucleate the eyes after euthanizing the animal. Clean the remaining muscle tissue and skin from the eyes using scissors and forceps (non-sterile).

   NOTE: The size of the scissors and forceps used for the enucleation and cleaning of the eyes depends on the species (e.g., for rat and mouse the instruments are going to be smaller than the ones used for pig and cattle) (see **Figure S1**).

   1. Collect the eyes in a 50 mL tube filled with non-sterile PBS and transfer the tube to the laminar flow hood. Disinfect the eyes by submerging for 2 min in iodine-based solution, then transfer them to a Petri dish filled with sterile PBS.

2. Opening of the bulb

   1. After transferring the eyes to a sterile Petri dish, hold one firmly close to the optic nerve with Colibri or pointed forceps. Punch a hole near the limit of the iris (between pars plana and ora serrata) with an 18 G needle. Insert small scissors in the hole and cut around the iris. Remove the anterior segment (cornea, lens, and iris) and put it in a Petri dish. Leave the bulb with the vitreous until RPE cells are isolated.

3. Isolation of IPE cells

   1. Remove the lens and with fine forceps delicately pull out the iris containing the IPE cells. Place the iris in a Petri dish, wash with sterile PBS and leave it in PBS until more iris are prepared.

2. Repeat steps 2.2.1 to 2.3.1 for all eyes to be prepared that day.

3. Add 50 µL of 0.25% trypsin per iris and incubate for 10 min at 37 °C. Remove trypsin, add 150 µL of complete medium per iris and scrape the IPE delicately with a flat fire-polished Pasteur pipette; use fine forceps to immobilize the tissue. Collect the cell suspension and put in a 1.5 mL tube. Use 10 µL of the cell suspension diluted 1:3 with trypan blue to count the cells in the Neubauer chamber[34,35].

4. If not transfected immediately, seed 200,000 cells/ well in a 24-well plate (100,000 cells/cm^2) in 1 mL of complete medium (10% FBS) (for seeding see **Table 1**). Place the plate in an incubator and culture it at 37 °C, 5% CO_2.

   NOTE: It might be necessary to pool several eyes together to have enough cells for seeding.

4. Isolation of RPE cells

   1. Remove vitreous humor and retina from the posterior segment with thin forceps. Avoid damaging the retinal pigment epithelium.

   2. Cut the segment in half with a #10 scalpel to have the globe completely open and wash with sterile PBS.

   3. Add 50 µL of 0.25% trypsin per eye and incubate for 10 min at 37 °C. Remove trypsin and add 150 µL of complete medium per globe and scrape the RPE cells delicately with a round scalpel; use fine forceps to immobilize the tissue. Collect the cell suspension and put it in a 1.5 mL tube. Take 10 µL of the cell suspension; dilute 1:4 with trypan blue to count the cells in the Neubauer chamber.

   4. See step 2.3.4.

NOTE: It might be necessary to pool several eyes together to have enough cells for seeding.

3. Isolation of rabbit PE cells

1. Perform cleaning and disinfection as described in steps 2.1 to 2.1.1.

2. Put one eye on a sterile gauze compress and hold it firmly close to the optic nerve. Open the eye with the scalpel #11 and scissors about 2 mm under the limbus. Remove the anterior segment (cornea, lens, and iris) and put it in a Petri dish. Leave the bulb with the vitreous until RPE cells are isolated.

3. Isolation of IPE cells

   1. Perform step 2.3.1. Remove the ciliary body from the iris by cutting with a scalpel #10.

   2. After the preparation of 2 iris, incubate with 1 mL of 0.25 % trypsin at 37 °C for 10 min; during this time, the RPE cells can be isolated (see step 3.4). Remove the trypsin and add 1 mL complete medium to the iris and isolate the cells by carefully scratching with a flat fire-polished Pasteur pipette. Resuspend the cells carefully by pipetting and transfer the cell suspension into a 1.5 mL tube. Take 10 µL of the cell suspension and dilute 1:3 with trypan blue to count the cells in the Neubauer chamber.

   3. See step 2.3.4.

4. Isolation of RPE cells

   1. Perform step 2.4.1.

   2. Put a sterile gauze into a 12 well plate and put the bulb over the gauze.

   3. Wash with PBS and perform step 2.4.3.

   4. Centrifuge the cells 10 min at 120 x *g*.

5. See step 2.3.4.

4. Isolation of pig PE cells

1. Perform cleaning as described in step 2.1. Wash with PBS and disinfect the eyes by submerging for 2 min in iodine-based solution, rinse with PBS. Continue with step 3.2.

2. Isolation of IPE cells

   1. Perform step 3.3.1. After the preparation of 2 iris, add 1 mL of complete medium and isolate the cells by carefully scratching with a flat fire-polished Pasteur pipette. Transfer the cell suspension into a 1.5 mL tube. Take 10 µL of the cell suspension and dilute 1:4 with trypan blue to count the cells in the Neubauer chamber.

   2. See step 2.3.4.

3. Isolation of RPE cells

   1. Perform step 2.4.1. Place the bulb in a Petri dish and wash with PBS. Fill the bulb with 1 mL complete medium.

   2. Using a curved fire-polished Pasteur pipette, carefully remove RPE cells. Make sure to scrape from the bottom to the top to avoid slipping down of the choroid-Bruch's membrane complex. Collect the cell suspension within the bulb using a 1,000 µL pipette and transfer into a 1.5 mL tube for resuspension. Take 10 µL of the cell suspension and dilute 1:8 with trypan blue to count the cells in the Neubauer chamber.

   3. See step 2.3.4.

5. Isolation of bovine PE cells

1. Perform cleaning as described in step 2.1. Wash with PBS and disinfect the eyes by submerging for 2 min in iodine-based solution, rinse with PBS. Continue with step 3.2.

2. Isolation of IPE cells

   1. Perform step 3.3.1. After the preparation of two iris, incubate with 2 mL of 0.25% trypsin at 37 °C for 10 min. During this time, the RPE cells can be isolated (see step 5.3).

   2. Remove the trypsin and add 2 mL complete medium to the iris and isolate the cells by carefully scratching with a flat fire-polished Pasteur pipette. Transfer the cell suspension into a 15 mL tube. Centrifuge the cells 10 min at 120 x *g*. Take 10 µL of the cell suspension and dilute 1:4 with trypan blue to count the cells in the Neubauer chamber.

   3. If not transfected immediately, seed 320,000 cells/ well in a 6-well plate in 3 mL of complete medium (10% FBS) (for seeding see **Table 1**). Place the plate in an incubator and culture it at 37 °C, 5% CO_2.

3. Isolation of RPE cells

   1. Follow step 2.4.1. Place the bulb in a Petri dish and wash with PBS. After preparation of 2 eyes fill the bulb about ¾ with trypsin and incubate for 25 min at 37 °C with the lid of the Petri dish on top of the bulbi.

   2. Remove the trypsin and add 1 mL complete medium. Perform step 4.3.2. Centrifuge the cells 10 min at 120 x *g*.

   3. See step 5.2.3.

6. Cultivation - medium change

1. Culture the cells in DMEM/Ham's F12, supplemented with 10% FBS, 80 U/mL penicillin / 80 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, at 37°C and 5% CO_2 in a humidified incubator. After 3-4 days pipette up and down to collect non-adherent cells and put half of the volume in another well. Fill up to 1 mL with complete medium.
   NOTE: This allows having a surface large enough for all cells isolated to attach and maximize the output.

2. After another 3-4 days repeat the cell collection but this time by pooling the non-adherent cells from two wells into one well (e.g., A1+B1 in C1). Add medium to all wells. Observe the cells and change the medium 2 times per week (for 6 and 24-well plates use 3 and 1 mL/ well, respectively). When cells reach confluence, switch to complete medium with 1% FBS or use the cells for experiments (e.g., transfection).
   NOTE: RPE and IPE cells are confluent after 3-4, and 4-5 weeks after isolation, respectively. The cell culture purity was corroborated checking the cell morphology (pigmented cells) and specific markers as described by Johnen and colleagues[36].

7. Electroporation of primary PE cells

1. Perform electroporation as described before[1,37].

2. Depending on the number of cells transfected use 6-, 24- or 48-well plates (see **Table 2**) to seed the cells in medium without antibiotics or antimycotics. For the following 2 weeks add drops with medium containing penicillin (80 U/mL), streptomycin (80 µg/mL), and

amphotericin B (2.5 μg/mL) twice a week. Exchange the medium completely 2 weeks after transfection.

3. To determine cell growth, transfection efficiency, and protein secretion, monitor the cells weekly by microscopy and analyze the cell culture supernatant by western blot. Before termination of the culture, take a 24 h cell culture supernatant to quantify protein secretion by ELISA, count the cells, measure fluorescence by image-based cytometry (in case of *Venus*-transfected cells) following manufactures' instructions (see **Table of Materials**), and collect the cell pellet for RT-qPCR-based gene expression analysis.

NOTE: These methods are not included in the present paper since it is not the purpose to explain in detail the analysis of the cells but rather their isolation. The cell seeding density is the same for all species (100,000 cells/cm^2) but because the number of isolated cells varies, different plates were used. In addition, for mice, rat, and rabbit it might be necessary to pool 2-3 eyes to have enough cells for seeding.

Species	Trypsin treatment	N° IPE cells	N° RPE cells	Plate for seeding (100,000 cells/cm^2)
Mouse/rat	Yes	~50,000	~150,000	24-well plates
Rabbit	Yes	~350,000	~2,500,000	24-well plates
Pig	No	~1,000,000	~3,000,000	24-well plates
Bovine	Yes	~1,700,000	~5,000,000	6-well plates

Table 1: Number of primary PE cells isolated from eyes from different species.

| Name	Area	Volume medium	Trypsin	Volume medium to stop action of trypsin	Seeding density
6-well plate	9.6 cm²	3.0 mL	0.5 mL	1.0 mL	$3x10^5$
24-well plate	2.0 cm²	1.0 mL	0.2 mL	0.8 mL	$5x10^4$
48-well plate	1.1 cm²	0.5 mL	0.1 mL	0.4 mL	$0.5-1x10^4$

Table 2: Cell culture volumes and seeding densities.

Representative Results

**PE isolation from different mammal species**

Using the aforementioned protocols, IPE and RPE cells were successfully isolated and cultured from five different species. The number of cells obtained from each procedure depends on the species and size of the eye (**Table 1**). As

shown in **Figure 1**, cells show typical PE cell morphology and pigmentation (except for rabbit cells shown, derived from albino New Zealand White (NZW) rabbits). At 21 days post-isolation, the cells are confluent, ready to be used for further experiments (e.g., transfections). It must be noted that cultures from all species are monitored and controlled further up to 2 years confirming normal morphology and stable transgene expression (data not shown).

Dedifferentiation, cellular stress, and changes in gene expression were excluded by RT-qPCR and immunohistochemistry. A panel of genes (VEGF, CRALBP, CATD, ZO-1, KRT8) analyzed in transfected human RPE cells (ARPE-19 cells) confirmed normal RPE expression pattern[1]; which could be confirmed in primary bovine IPE cells by immunofluorescence for RPE65 (**Figure 2**). In addition, Johnen and colleagues corroborated the ZO-1 immunostaining in primary porcine IPE and RPE cells[36].
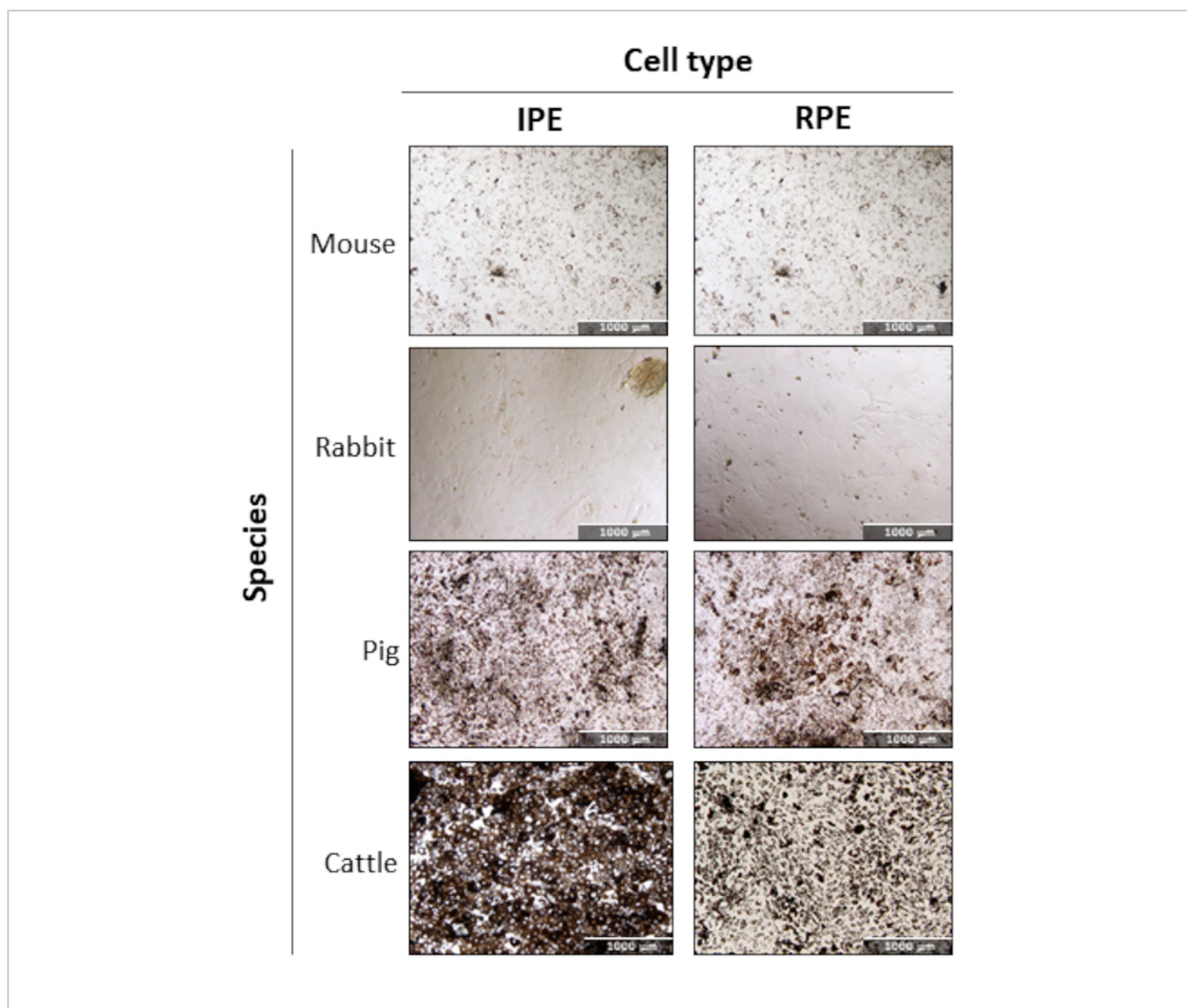
Figure 1: Micrographs of PE cells from various mammals at 21 days post-isolation. IPE and RPE cells from mouse, rabbit (albino NZW rabbit), pig, and bovine are shown at day 21 post-isolation. For all species, the cultures shown are confluent. Note that the rabbit PE cells are not pigmented (original magnification, 50x). Please click here to view a larger version of this figure.
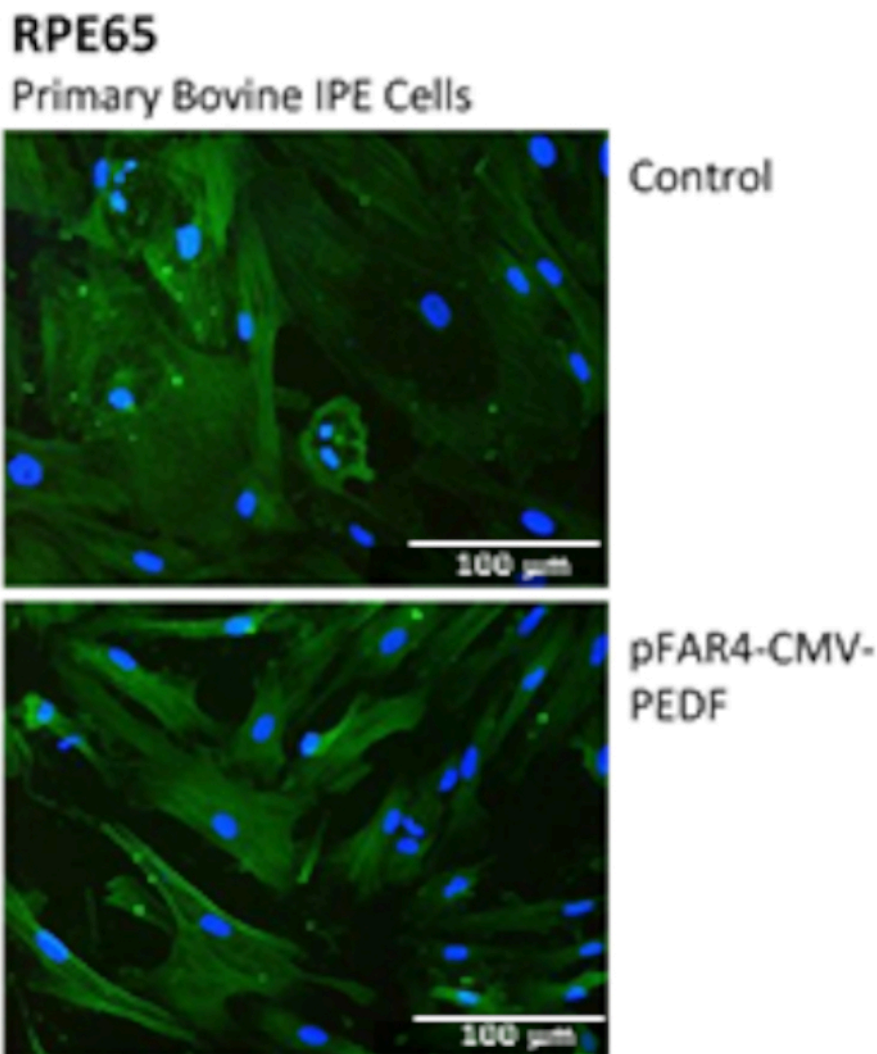
Figure 2: RPE65 immunostaining of primary IPE cells. RPE65 (green) staining of bovine IPE cells transfected with the pFAR4-CMV-PEDF transposon plasmid using an initial cell number of 1×10^4 cells compared to non-transfected control cells (original magnification, 200x). Nuclei was stained with DAPI (blue). Please click here to view a larger version of this figure.

Viability of transfected primary RPE

Cell viability is shown in **Figure 3**. To exclude a potential toxicity of the buffer used for the electroporation process, pre-cultured primary RPE cells were suspended in electroporation buffer from a commercially available kit, or in a nutrient buffer developed by a pharmaceutical company (confidential composition) and electroporated (E) (without addition of plasmid) as described in step 7 of the protocol. The cell viability was studied 3 ± 1 days post-transfection using a commercially available cytotoxicity assay kit (see **Table of Materials**) following the manufacturer's instructions. The assays were always performed with a control without power

(Co-P) (not electroporated). No impact on cell viability was observed for any of the buffers tested (**Figure 3**).
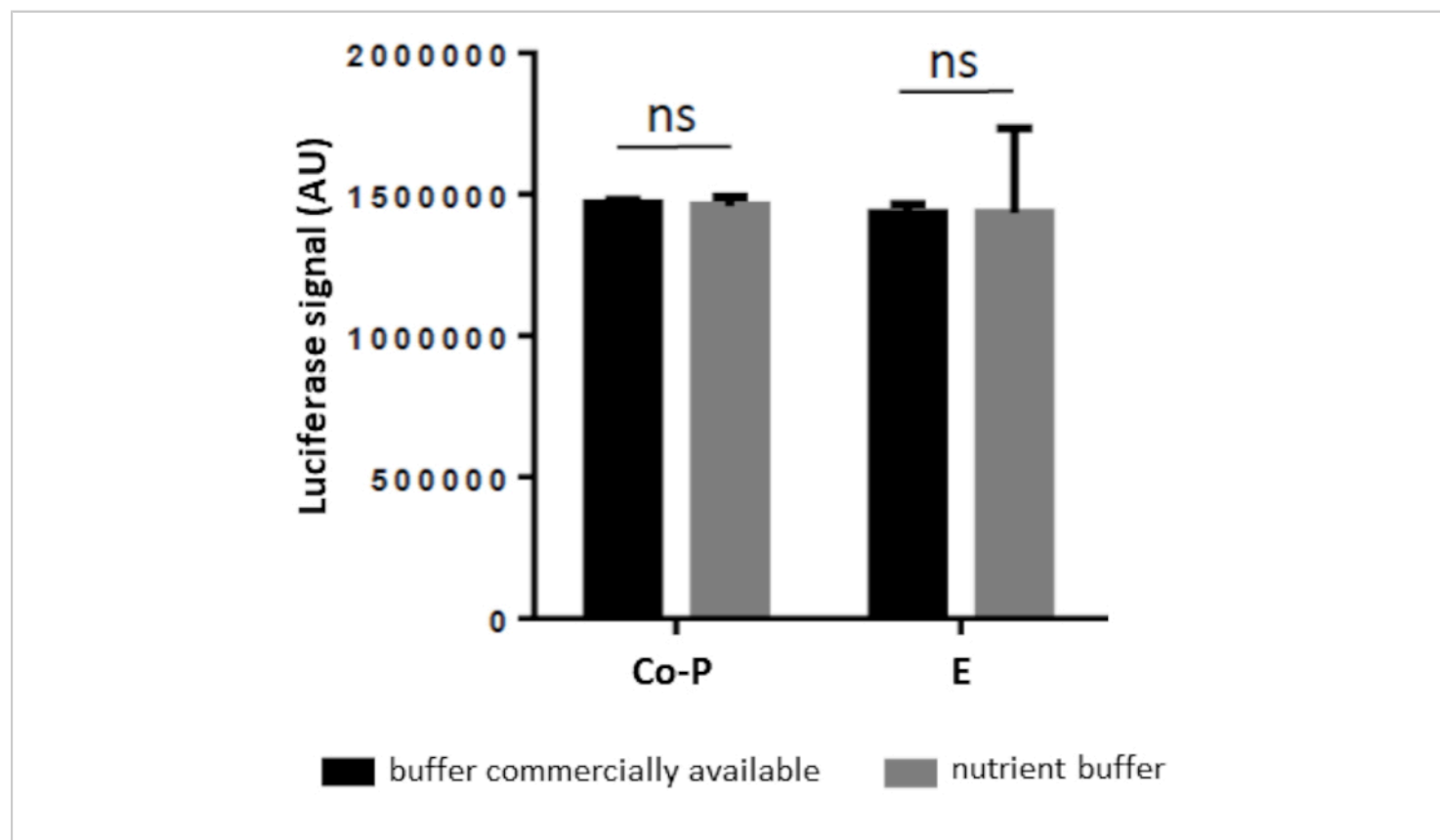
Figure 3: Viability of primary RPE cells suspended in different buffers. 1×10^4 cells were suspended in electroporation buffer from a commercially available kit or in a nutrient buffer. Cell viability was measured 3 ± 1 days post-transfection using a commercially available cytotoxicity assay kit. No differences in viability were observed between the different buffers; data are represented as mean $\pm$ SD (n=2 donors, 3 replicates/donor). E: electroporated cells, Co-P: control without power. AU: arbitrary units. Please click here to view a larger version of this figure.
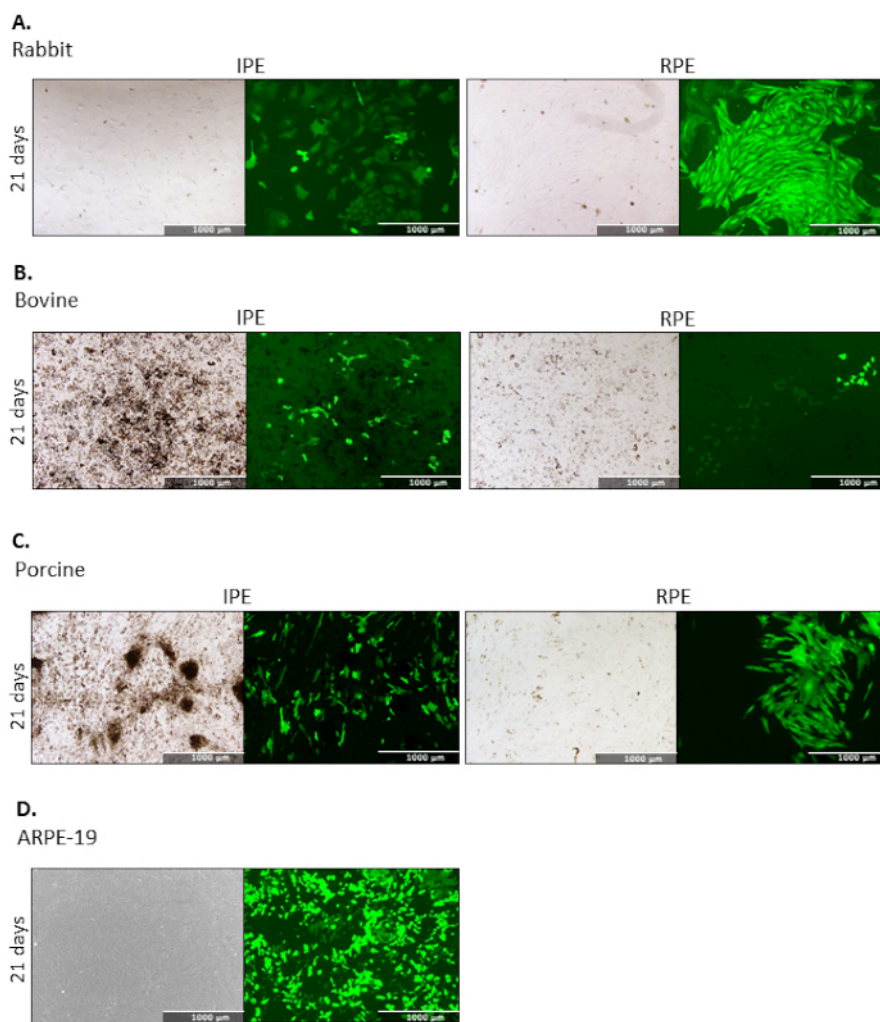
Transfections of pre-cultured PE cells with the *Venus* reporter gene

50,000-100,000 pre-cultured PE cells from different species were transfected with the yellow fluorescent Venus protein (pT2-CAGGS-Venus) using the hyperactive *SB100X* transposon gene delivery system. Micrographs shown in **Figure 4** corroborate that the cells were successfully transfected (fluorescent cells at 21 days post-transfection).

Figure 5 shows the quantification of the transfection efficiency in pre-cultured pig RPE cells (n=6 donors, 50,000 cells/transfection) transfected with *Venus* (pT2-CAGGS-Venus). The percentage of fluorescent cells and MFI was measured using image-based cytometry following the manufacturer's instructions at the day of termination of the cell culture (30 ± 5 days post-transfection). Mean percentage of fluorescent cells was $50 \pm 30\%$ but variable ranging

from 95 ± 6% (donor 2) to 28 ± 1% (donor 4) (**Figure 5**). In all experiments, transfected ARPE-19 cells were used as positive control. Additionally, transfection efficiency was always compared to the negative controls: Co-P (control without power [not electroporated]) and Co+P (control with power [electroporated but without addition of plasmids]). The experiment has been also performed with IPE cells (data not shown).

Figure 4: Micrographs of pre-cultured PE cells transfected with pT2-CAGGS-Venus. *Venus*-transfected rabbit (**A**), bovine (**B**), and porcine (**C**) PE cells are shown at 21 days post-transfection. As a positive control, 50,000 ARPE-19 cells were transfected with *Venus* (**D**). Left micrograph: bright field, right micrograph: GFP (480 nm) filter (original magnification, 50x). Transfections were done in triplicate and negative controls were included: Co-P (control without power [not electroporated]) and Co+P (control with power [electroporated but without addition of plasmids]) (not shown). Please click here to view a larger version of this figure.
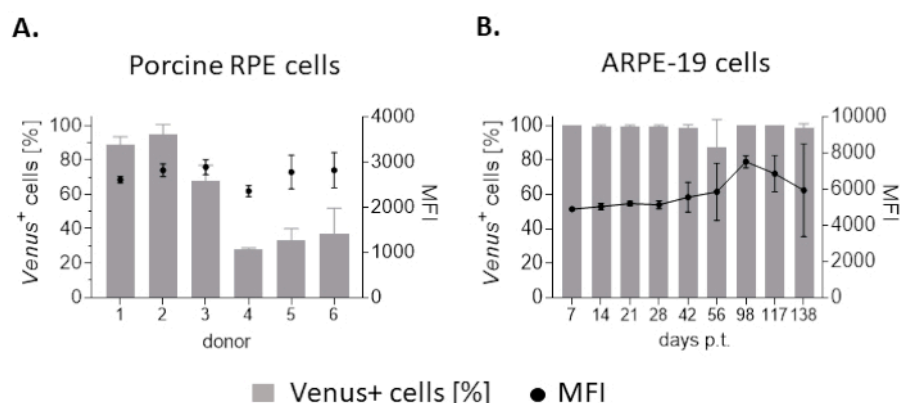
Figure 5: Transfection efficiency in porcine RPE cells transfected with the *Venus* reporter gene. (**A**) 50,000 primary porcine RPE cells were transfected with pT2-CAGGS-Venus, the overall mean transfection efficiency was 50 ± 30% and the mean MFI was 2712 ± 197 at the day of termination of the cell cultures (30±5 days post-transfection). The graph shows the mean ± SD (n=3 replicates) of 6 different animals. (**B**) The percentage of Venus[+] ARPE-19 cells used as a positive control, was 98±6% and was stable for the 138 days the cells were followed. The mean fluorescence intensity (MFI) was 5,785 ± 1,255. The graph shows the mean ± SD (n=3 replicates) for each day. Co-P (control without power [not electroporated]) and Co+P (control with power [electroporated but without addition of plasmids]) were included in all transfection experiments (not shown). Please click here to view a larger version of this figure.

Transfections of freshly PE cells with *Venus* reporter gene

10,000-50,000 PE cells freshly isolated from rabbits were transfected with the yellow fluorescent protein Venus (pFAR4-CMV-Venus), and cell cultures were monitored by microscopy. In **Figure 6** fluorescent cells can be observed at day 21 post-transfection. The percentage of fluorescent cells measured by image-based cytometry was 53 ± 29% for IPE cells and 28 ± 23% for RPE cells (data not shown).
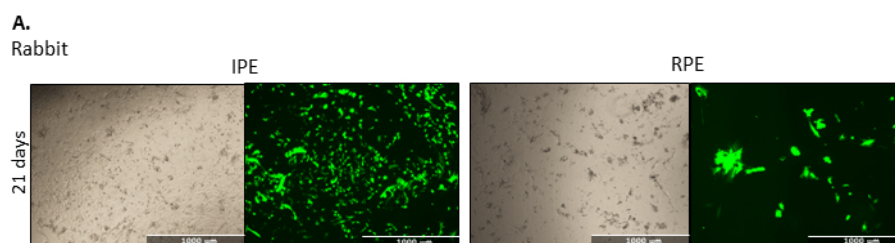
Figure 6: Micrographs of freshly transfected PE cells isolated from rabbit. 50,000 IPE and RPE cells from rabbit were transfected with pFAR4-CMV-Venus. The fluorescence is shown at day 21 post-transfection. Left micrograph: bright field, right micrograph: GFP (480 nm) filter. In all cases, transfections were done in triplicate (original magnification, 50x). Please click here to view a larger version of this figure.

Transfections of PE cells with the therapeutic genes *PEDF* and *GM-CSF*

50,000 PE cells were transfected with *PEDF* and protein secretion was monitored by WB (**Figure 7**). The WB signal for transfected cells was higher compared with the non-transfected cells for all species and days studied; no decrease in protein secretion was observed within this time.
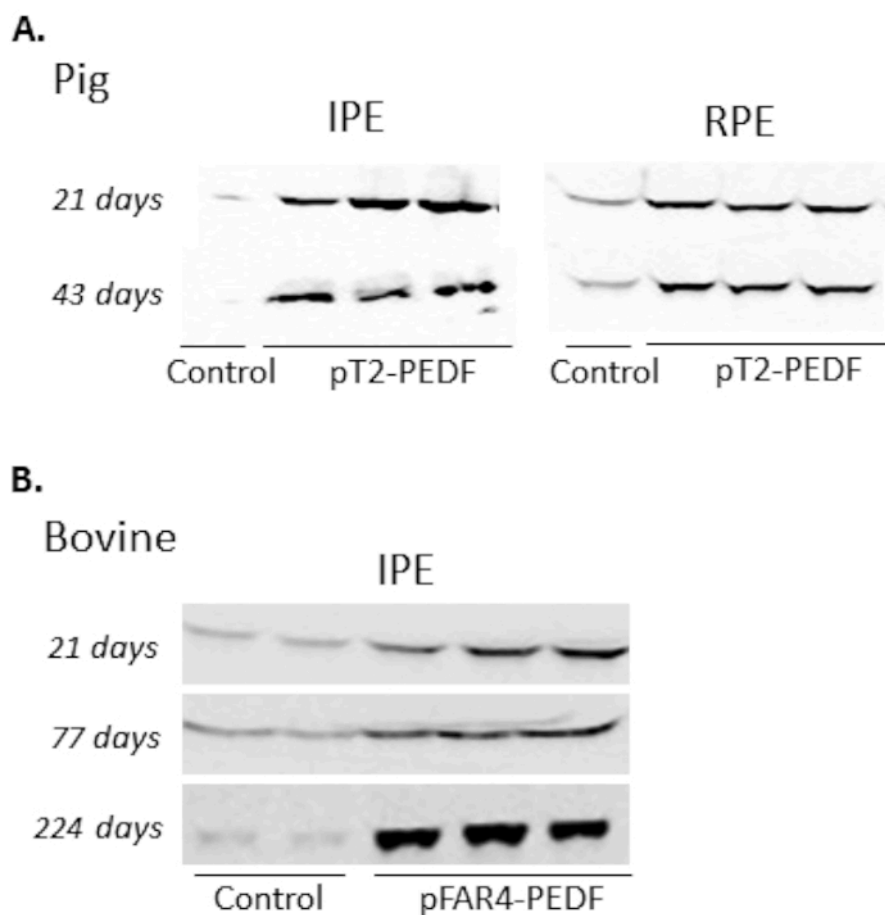
Figure 7: WB analysis of PEDF secretion of transfected PE cells. WB analysis of supernatants from pig (pre-cultured) (**A**), and cattle (freshly) (**B**) *PEDF*-transfected PE cells show a higher PEDF secretion compared with the control (non-transfected cells) and stable over time. Please click here to view a larger version of this figure.

Figure S1: Instruments used for PE isolation depending on the species. (**A**) Non-sterile instruments used for the enucleation of the eyes and cleaning of remaining muscle tissue and skin. Set 1 is used for rat, mouse, and rabbit, and set 2 is used for pig and cattle eyes. (**B**) Sterile instruments used for PE isolation. Note the different size of scissors and forceps used depending on the eye size. Round and flat fire-polished Pasteur pipettes are used for the scrape of RPE and IPE, respectively. Please click here to download this File.

Discussion

Having standardized methods to isolate and culture PE cells is fundamental in developing new therapy approaches for retinal degenerative diseases. With the protocols presented here, PE cells can be successfully isolated from different species and cultured for long periods (up to now, the longest culture was maintained for 2 years[1,38]); typical PE cell morphology, pigmentation and function was observed (**Figure 1**, **Figure 2**). Notice that particularly for pure RPE cultures, it is important to extract completely the retina to

avoid contamination with neural retinal cells; for IPE cells, the ciliary body should be removed from the iris as explained in the protocol. Confluence of the cells is achieved in a relatively short time (~21 days), after which the cells are ready for transfection with therapeutic genes or for use in other experiments (e.g., exposure to toxic agents, proteins, etc.). Moreover, the protocols are not only crucial for pre-clinical *in vitro* and *in vivo* testing, but also important for human application, i.e., the validation of transfected PE cell transplants; particularly for the treatment of AMD as in development by our group, where IPE cells will be isolated from an iris biopsy, immediately transfected, and transplanted subretinally to the same patient within one surgical session. The isolation and subretinal transplantation of autologous IPE cells was established in rabbits[39] and patients[23]. Since the transplantation of autologous IPE cells was successful but not sufficient to restore vision, transfection of the cells to overexpress neuroprotective factors, such as PEDF and GM-CSF, had been added to the approach and established in three more species (mouse, rat, and cattle). With the methods described here for the isolation, culture and genetic modification of PE cells, respective cell transplants can now be prepared from various species to test toxicity and efficiency of the approach *in vivo*.

It was shown that, using the hyperactive *SB100X* transposon system, PE cells from various origin can be efficiently modified to overexpress PEDF (**Figure 7**); similar results using rat IPE and RPE[24] and human RPE cells[29,30] have been published. The transfection with PEDF has been proposed to treat nvAMD by inhibition of VEGF-mediated neovascularization and protection of RPE cells and retinal neurons from glutamate and oxidative stress as well as ischemia[40,41]. Corroboration of stable long-term protein secretion (**Figure 7**) confirmed PE cells as a reliable biological

"drug delivery system". In this regard, transfection efficiency studied with the reporter gene *Venus* (**Figure 4**, **Figure 5**, **Figure 6**) confirmed high transfection efficiencies of ~20-100% (species- and donor-dependent) as reported in Johnen *et al.*[1].

It should be noted that though the size of the plasmids used was variable, from pFAR miniplasmids (~3,000 bp) to plasmids with a pT2-backbone (~5,000 bp)[30], transfection efficiencies were comparably high. Differences seen in transfection efficiency are probably due to variability in the time from enucleation to cell isolation and in tissue preservation but did not alter cell morphology neither the time that the cells reach confluence (~21 days after isolation), or time that the cells were followed after transfection. In general, an important consideration to achieve a successful transfection is that the source of PE cells should be as fresh as possible; this is particularly important for transfections with freshly isolated PE cells.

Comparing with cell sheet transplants, the therapy our group is developing (transfected cells transplanted subretinally as a cell suspension) is less invasive since the first requires large retinotomies with the associated risks of proliferative vitreoretinopathy and subretinal fibrosis[42]. In addition, in sheet grafts for wet AMD the area of CNV is removed together with the adjacent choroid and therefore, graft survival could be compromised[43]. Finally, the idea of using a cell patch is not compatible with the proposed procedure, where the isolation, modification and transplantation of the cells is done during a single surgical session. On the other hand, inherent issues of PE cells transplanted as cell suspensions are potential cell death during delivery, lack of adhesion, and variance in cellular distribution; but these drawbacks could be overcome by tissue engineering approaches[42]; in

addition, cell survival could be improved by the use of pro-survival agents[44,45,46,47]. We believe the transplantation of primary PE cells overexpressing neuroprotective factors such PEDF and GM-CSF as a cell suspension is a promising approach to treat AMD, and to develop this therapy the protocols presented here are crucial since they allow the standardization of the isolation, culture, and analysis of the primary PE cells.

In summary, the protocol delivers an advanced model system for the development and pre-clinical *in vitro* and *in vivo* testing of ocular advanced medicinal therapies in five different species, robust enough to compensate for individual variances in cell quality from the different sources.

Disclosures

The authors have nothing to disclose.

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