Journal of Visualized Experiments

Electroporation-Based Genetic Modification of Primary Human Pigment Epithelial Cells Using the Sleeping Beauty Transposon System

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61987R2
Full Title:	Electroporation-Based Genetic Modification of Primary Human Pigment Epithelial Cells Using the Sleeping Beauty Transposon System
Corresponding Author:	Sandra Johnen Department of Ophthalmology, University Hospital RWTH Aachen Aachen, North Rhine-Westphalia GERMANY
Corresponding Author's Institution:	Department of Ophthalmology, University Hospital RWTH Aachen
Corresponding Author E-Mail:	sjohnen@ukaachen.de
Order of Authors:	Sandra Johnen
	Nina Harmening
	Zsuzsanna Izsvák
	Zoltán Ivics
	Peter Walter
	Gabriele Thumann
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Aachen, North Rhine-Westphalia, Germany
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	

TITLE:

Electroporation-Based Genetic Modification of Primary Human Pigment Epithelial Cells Using the Sleeping Beauty Transposon System

AUTHORS AND AFFILIATIONS:

Sandra Johnen[1], Nina Harmening[2,3], Zsuzsanna Izsvák[4], Zoltán Ivics[5], Peter Walter[1], Gabriele Thumann[2,3]

[1]Department of Ophthalmology, University Hospital RWTH Aachen, Aachen, Germany
[2]Experimental Ophthalmology, University of Geneva, Geneva, Switzerland
[3]Department of Ophthalmology, University Hospitals of Geneva, Geneva, Switzerland
[4]Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany
[5]Division of Medical Biotechnology, Paul-Ehrlich-Institute, Langen, Germany

CORRESPONDING AUTHOR:

Sandra Johnen (sjohnen@ukaachen.de)

EMAIL ADDRESSES OF CO-AUTHORS:

Nina Harmening (Nina.Harmening@unige.ch)
Zsuzsanna Izsvák (zizsvak@mdc-berlin.de)
Zoltán Ivics (Zoltan.Ivics@pei.de)
Peter Walter (pwalter@ukaachen.de)
Gabriele Thumann (Gabriele.Thumann@unige.ch)
Sandra Johnen (sjohnen@ukaachen.de)

KEYWORDS:

age-related macular degeneration, AMD, capillary electroporation system, non-viral transfection, pigment epithelium-derived factor, PEDF, primary pigment epithelial cells, *Sleeping Beauty* transposon system, SB

SUMMARY:

We have developed a protocol to transfect primary human pigment epithelial cells by electroporation with the gene encoding pigment epithelium-derived factor (PEDF) using the *Sleeping Beauty* (*SB*) transposon system. Successful transfection was demonstrated by quantitative polymerase chain reaction (qPCR), immunoblotting, and enzyme-linked immunosorbent assay (ELISA).

ABSTRACT:

Our increasingly aging society leads to a growing incidence of neurodegenerative diseases. So far, the pathological mechanisms are inadequately understood, thus impeding the establishment of defined treatments. Cell-based additive gene therapies for the increased expression of a protective factor are considered as a promising option to medicate neurodegenerative diseases, such as age-related macular degeneration (AMD). We have developed a method for the stable expression of the gene encoding pigment epithelium-derived factor (PEDF), which is characterized as a neuroprotective and anti-angiogenic protein in the nervous system, into the genome of primary human pigment epithelial (PE) cells using the *Sleeping Beauty* (*SB*) transposon system. Primary PE cells were isolated from human

donor eyes and maintained in culture. After reaching confluence, 1 x 10^4 cells were suspended in 11 µL of resuspension buffer and combined with 2 µL of a purified solution containing 30 ng of hyperactive *SB* (*SB100X*) transposase plasmid and 470 ng of *PEDF* transposon plasmid. Genetic modification was carried out with a capillary electroporation system using the following parameters: two pulses with a voltage of 1,100 V and a width of 20 ms. Transfected cells were transferred into culture plates containing medium supplemented with fetal bovine serum; antibiotics and antimycotics were added with the first medium exchange. Successful transfection was demonstrated in independently performed experiments. Quantitative polymerase chain reaction (qPCR) showed the increased expression of the *PEDF* transgene. PEDF secretion was significantly elevated and remained stable, as evaluated by immunoblotting, and quantified by enzyme-linked immunosorbent assay (ELISA). *SB100X*-mediated transfer allowed for a stable *PEDF* gene integration into the genome of PE cells and ensured the continuous secretion of PEDF, which is critical for the development of a cell-based gene addition therapy to treat AMD or other retinal degenerative diseases. Moreover, analysis of the integration profile of the *PEDF* transposon into human PE cells indicated an almost random genomic distribution.

INTRODUCTION:

Advanced age is described to be the main risk for neurodegenerative diseases. Age-related macular degeneration (AMD), a polygenic disease leading to severe vision loss in patients older than 60 years of age, belongs to the four most common causes of blindness and vision impairment[1] and is expected to increase to 288 million people in 2040[2]. Dysfunctions of the retinal pigment epithelium (RPE), a single layer of tightly packed cells located between the choriocapillaris and the retinal photoreceptors, contribute to the pathogenesis of AMD. The RPE fulfills multiple tasks that are essential for a normal retinal function[3] and secretes a variety of growth factors and factors essential to maintain the structural integrity of the retina and the choriocapillaris, thereby supporting photoreceptor survival and providing a basis for the circulation and the supply of nutrients.

In healthy eyes, pigment epithelium-derived factor (PEDF) is responsible for balancing the effects of vascular endothelial growth factor (VEGF) and protects neurons against apoptosis, prevents endothelial cell proliferation, and stabilizes the capillary endothelium. A shifted VEGF-to-PEDF ratio is related to ocular neovascularization, which was observed in animal models[4,5] as well as in samples of patients with choroidal neovascularization (CNV) due to AMD and proliferative diabetic retinopathy[6–10]. The enhanced VEGF concentration is the target for the current standard treatment. The anti-VEGF pharmaceuticals bevacizumab, ranibizumab, aflibercept and, most recently, brolucizumab improve visual acuity in about one third of CNV patients or rather stabilize vision in 90% of cases[11–13]. However, the frequent, often monthly, intravitreal injections bear the risk of adverse events[14], impair patient compliance, and represent a significant economic burden to healthcare systems[15]. Moreover, a certain percentage of patients (2%–20%) do not respond or only poorly react to the anti-VEGF therapy[16–19]. These negative concomitants necessitate the development of alternative treatments, e.g., intraocular implants, cell and/or gene therapeutic approaches.

Gene therapy has evolved as promising treatment for hereditary and non-hereditary diseases and intends to restore non-functional gene sequences or suppress malfunctioned ones. For polygenic diseases, where identification and replacement of the causative factors is hardly

possible, strategies aim for the continuous delivery of a protective factor. In the case of AMD, various additive therapies have been developed, such as the stable expression of endostatin and angiostatin[20], the VEGF antagonist soluble fms-like tyrosine kinase-1 (sFLT-1)[21,22], the complement regulatory protein cluster of differentiation 59 (CD59)[23] or PEDF[24,25]. The eye, and especially the retina, is an excellent target for a gene-based medication due to the enclosed structure, good accessibility, small size, and immune privilege, thus allowing for a localized delivery of low therapeutic doses and making transplants less susceptible to rejection. Moreover, the eye enables non-invasive monitoring, and the retina can be examined by different imaging techniques.

Viral vectors are, because of their high transduction efficiency, the main vehicle to deliver therapeutic genes into target cells. However, depending on the viral vector used, different adverse reactions have been described, such as immune and inflammatory responses[26], mutagenic and oncogenic effects[27,28], or dissemination in other tissues[29]. Practical limitations include a restricted packaging size[30] as well as difficulties and costs associated with the production of clinical grade lots[31,32]. These drawbacks have promoted the further development of non-viral, plasmid-based vectors that are transferred via lipo-/polyplexes, ultrasound or electroporation. However, genomic integration of the transgene into the host genome is usually not promoted with plasmid vectors, thus resulting in a transient expression.

Transposons are naturally occurring DNA fragments that change their position within the genome, a characteristic that has been adopted for gene therapy. Due to an active integration mechanism, transposon-based vector systems allow for a continuous and constant expression of the inserted transgene. The *Sleeping Beauty* (*SB*) transposon, reconstituted from an ancient Tc1/*mariner*-type transposon found in fish[33] and further improved by molecular evolution resulting in the hyperactive variant *SB100X*[34], enabled efficient transposition in various primary cells and was used for the phenotypic correction in different disease models[35]. At present, 13 clinical trials have been initiated using the *SB* transposon system. The *SB100X* transposon system consists of two components: the transposon, which comprises the gene of interest flanked by terminal inverted repeats (TIRs), and the transposase, which mobilizes the transposon. Following plasmid DNA delivery to the cells, the transposase binds the TIRs and catalyzes the excision and integration of the transposon into the cell's genome.

We have developed a non-viral cell-based additive therapy for the treatment of neovascular AMD. The approach comprises the electroporation-based insertion of the *PEDF* gene into primary pigment epithelial (PE) cells by means of the *SB100X* transposon system[36–38]. The genetic information of the transposase and PEDF are provided on separate plasmids, thereby enabling the adjustment of the ideal *SB100X*-to-*PEDF* transposon ratio. Electroporation is performed using a pipette-based capillary transfection system that is characterized by a maximized gap size between the electrodes while minimizing their surface area. The device was shown to achieve excellent transfection rates in a wide range of mammalian cells[39–41]. The small electrode surface area provides a uniform electric field and reduces the various side effects of electrolysis[42].

The anti-angiogenic functionality of PEDF secreted by transfected pigment epithelial cells was shown in various in vitro experiments analyzing the sprouting, migration, and apoptosis of human umbilical vein endothelial cells[43]. In addition, transplantation of PEDF-transfected cells

in a rabbit model of corneal neovascularization[44] as well as a rat model of CNV[43,45,46] showed decline of neovascularization.

Here, we describe a detailed protocol for the stable insertion of the *PEDF* gene into primary human RPE cells via the *SB100X* transposon system using a capillary transfection system. The transfected cells were kept in culture for 21 days and subsequently analyzed in terms of *PEDF* gene expression by quantitative polymerase chain reaction (qPCR) and in terms of PEDF protein secretion by immunoblotting and enzyme-linked immunosorbent assay (ELISA, **Figure 1**).

PROTOCOL:

Human donor eyes were obtained from the Aachen Cornea Bank of the Department of Ophthalmology (University Hospital RWTH Aachen) after obtaining informed consent in accordance with the Declaration of Helsinki protocols. Procedures for the collection and use of human samples have been approved by the institutional ethics committee.

1. Isolation of primary human RPE cells

1.1. Lay out sterile protective clothing and gloves. Place a sterile drape under a laminar flow.

1.2. Place sterile preparation instruments and other necessary sterile equipment under the laminar flow.

1.3. Record the receipt of the eye globes, the beginning of the preparation as well as possible abnormalities. Register donor age, gender, cause of death, and the period between time of death and eye removal.

1.4. Place the eye globe in a sterile gauze compress and hold it in one hand.

1.5. Separate the anterior segment from the posterior segment by a circumferential cut approximately 3.5 mm posterior to the limbus using a scalpel and an extra fine pointed eye scissor.

1.6. After tilting the posterior segment downwards, carefully remove the vitreous and the retina using colibri forceps.

1.7. Discreetly rearrange the collapsed RPE/choroidea complex using straight iris forceps and subsequently hold it in position with curved iris forceps.

1.8. Fill the posterior eye cup with 1 mL of Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 80 U/mL penicillin and 80 µg/mL streptomycin (Pen/Strep), and 2.5 µg/mL amphotericin B (AmphoB).

NOTE: Contrary to the isolation of primary RPE cells from pig or bovine eyes[36,37], the posterior eye cup doesn't have to be filled and incubated with trypsin.

1.9. Harvest the RPE cells by gently brushing the retinal pigment epithelium from the optic nerve toward the limbus with a fire-polished curved glass Pasteur pipette, while fixating the RPE/choroidea complex at the limbus using colibri forceps. Transfer the cell suspension into a Petri dish.

1.10. Repeat the steps 1.8 and 1.9 and collect all cells in the Petri dish. Resuspend the cell suspension carefully by pipetting up and down using a single channel pipette (100–1,000 µL).

1.11. Seed the RPE cell suspension of each eye globe into three wells of a 24-well cell culture plate and fill them up to 1 mL with DMEM/F12 supplemented with FBS, Pen/Strep, and AmphoB.

1.12. Maintain the RPE cell cultures at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 until confluence is reached. Change the cell culture medium twice a week.

2. Electroporation of primary human RPE cells

2.1. Preparation of the *SB100X* transposase/*PEDF* transposon plasmid mixture

2.1.1. Purify the *SB100X* transposase and *PEDF* transposon plasmid DNA using customary plasmid purification kits, which are identified to be suitable for the use in applications such as transfection, according to the protocol of the manufacturer.

2.1.2. Quantify the plasmid DNA contents using a microvolume spectrophotometer and adjust them to a concentration of 250 ng/µL with 10 mM Tris-HCl (pH 8.5).

2.1.3. Mix one portion of 250 ng/µL *SB100X* transposase plasmid DNA (e.g., 2.5 µL) with 16 portions of 250 ng/µL *PEDF* transposon plasmid DNA (e.g., 40 µL). Residual plasmid mixture can be stored at -20 °C.

NOTE: Multiple repeated freezing and thawing cycles of the plasmid mixture should be avoided.

2.1.4. Place 2 µL of the *SB100X* transposase/*PEDF* transposon plasmid mixture, containing 29.4 ng of *SB100X* transposase plasmid and 470.6 ng of PEDF transposon plasmid, in a sterile 1.5 mL safe-lock microcentrifuge tube. Keep on ice until mixed with cells.

2.2. Set-up of the capillary transfection system

NOTE: Electroporation of primary human RPE cells has been performed via a capillary transfection system using the 10 µL kit according to the manufacturer's protocol. The system comprises the transfection device, the transfection pipette, and the pipette station. The kit contains 10 µL transfection tips, buffer tubes, electrolytic buffer E (buffer E), and resuspension buffer R (buffer R).

2.2.1. Connect the pipette station to the transfection device.

2.2.2. Fill a buffer tube with 3 mL of buffer E and insert it into the pipette station until a click sound is heard.

NOTE: Ensure that the side electrode of the buffer tube is connected to the side ball plunger of the pipette station.

2.2.3. For the electroporation of primary human RPE cells, set the following pulse conditions on the transfection device: 1,100 V (pulse voltage), 20 ms (pulse width), two pulses (pulse number).

2.3. Preparation of the cultivated primary human RPE cells

2.3.1. Document shape and morphology of the primary RPE cell cultures via phase contrast microscopy. Take low magnification micrographs to demonstrate the growth of the RPE cells to a confluent and integrated monolayer as well as higher magnification micrographs to point out the typical cobblestone morphology of the RPE cells.

2.3.2. Aspirate the cell culture medium and wash the cells twice with 1 mL buffered phosphate saline (PBS, pH 7.4).

2.3.3. Trypsinize primary human RPE cells with 0.5 mL 0.05% trypsin-EDTA at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 7–15 min (maximum). Check the cell detachment microscopically.

2.3.4. Stop trypsinization with 1 mL of DMEM/F12 supplemented with FBS. Take a 20 µL aliquot for cell counting using a hemocytometer. Centrifuge the RPE cell suspension at 106 x *g* for 10 min.

2.3.5. Mix 20 µL aliquot with 20 µL trypan blue solution. Pipette 10 µL in each half of the hemocytometer and count the cells under a phase contrast microscope.

2.3.6. After centrifugation, resuspend the RPE cell pellet in 1 mL of PBS.

2.3.7. Take 10,000–100,000 RPE cells per transfection reaction and centrifuge them at 106 x *g* for 10 min.

NOTE: Beside the transfection reactions with electric field application and addition of plasmid DNA, each approach also includes two different control cultures: (1) without electric field application and without plasmid DNA; (2) with electric field application, but without plasmid DNA.

2.3.8. Resuspend the cell pellet in 11 µL of buffer R and combine it with 2 µL of the *SB100X* transposase/*PEDF* transposon plasmid mixture (see step 2.1.4).

NOTE: After resuspension in buffer R, the cells must be processed within 15 min to avoid a reduction in cell viability and transfection efficiency.

2.3.9. Insert the head of the transfection pipette into the 10 µL transfection tip until the clamp fully picks up the mounting stem of the piston. Draw up the cell/plasmid solution into the transfection tip.

NOTE: Avoid generation of air bubbles and their aspiration into the transfection tip.

2.3.10. Provide 1 mL of DMEM/F12 supplemented with FBS, but without Pen/Strep and without AmphoB, into the required number of wells of a 24-well cell culture plate.

2.4. Electroporation process

2.4.1. Insert the transfection pipette into the buffer tube placed in the pipette station (see step 2.2.2) until a click sound is heard.

NOTE: Ensure the metal head of the transfection pipette is connected to the ball plunger inside of the pipette station and to the buffer tube.

2.4.2. Press **Start** on the touchscreen of the transfection device. Before application of the electric pulse, the device automatically checks whether the buffer tube and the transfection pipette are properly inserted. After delivery of the electric pulses, Complete is displayed on the touchscreen.

2.4.3. Carefully remove the transfection pipette from the pipette station and immediately release the electroporated cells from the 10 µL transfection tip by pipetting the cell/plasmid solution into the prepared wells of the cell culture plate (see step 2.3.10).

2.4.4. Maintain transfected RPE cell cultures at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Add Pen/Strep and AmphoB with the first medium exchange 3 days after electroporation.

3. Analyses of transfected primary human RPE cells

3.1. Sample preparation

3.1.1. After a cultivation time of 3 weeks, ultimately incubate the RPE cell cultures in a defined volume of 1.0 mL DMEM/F12 supplemented with FBS, Pen/Strep, and AmphoB for a defined time of 24 h.

3.1.2. Take the cell culture supernatants and store them at -20 °C until further near-time processing or at -80 °C for thermally unstable samples and long-term storage.

3.1.3. Trypsinize transfected RPE cell cultures with 0.5 mL of 0.05% trypsin-EDTA at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 10 min.

3.1.4. Stop trypsinization with 1 mL of DMEM/F12 supplemented with FBS. Take small aliquots for cell counting using a hemocytometer (see step 2.3.5). Centrifuge the RPE cell suspensions at 106 x *g* for 10 min.

3.1.5. Store the RPE cell pellets at -80 °C until further processing.

3.2. Evaluation of PEDF secretion by western blotting

NOTE: For a qualitative assessment, cell culture supernatants (see step 3.1.2) are analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent western blotting. Depending on the *PEDF* transposon plasmid DNA used, the supernatants have to be processed differently.

3.2.1. Purification of His-tagged PEDF fusion proteins from cell culture supernatants

3.2.1.1. Take 30 µL nickel-nitrilotriacetic acid (Ni-NTA) slurry per sample using a bevel-cut tip and pellet the Ni-NTA resin by centrifugation (2,660 x *g* for 30 s).

3.2.1.2. Carefully resuspend the Ni-NTA resin with 200 µL of 1x incubation buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and pellet it by centrifugation (2,660 x *g* for 30 s).

3.2.1.3. Repeat the previous step.

3.2.1.4. Carefully resuspend the Ni-NTA resin with 40 µL 4x incubation buffer (200 mM NaH_2PO_4, 1.2 M NaCl, 40 mM imidazole, pH 8.0) per sample.

3.2.1.5. Mix 900 µL of cell culture supernatant with 260 µL of 4x incubation buffer and 55 µL of pretreated Ni-NTA slurry (see step 3.2.1.4).

3.2.1.6. Incubate the mixture on a rocking shaker at room temperature for 60 min.

3.2.1.7. Pellet the Ni-NTA resin by centrifugation (2660 x *g* for 60 s).

3.2.1.8. Carefully resuspend the Ni-NTA resin with 175 µL of 1x incubation buffer and pellet resin by centrifugation (2,660 x *g* for 60 s).

3.2.1.9. Repeat the previous step.

3.2.1.10. Carefully resuspend the Ni-NTA resin with 30 µL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and incubate the mixture on a rocking shaker at room temperature for 20 min.

3.2.1.11. Pellet the Ni-NTA resin by centrifugation (2,660 x *g* for 30 s).

3.2.1.12. Carefully take the supernatant and mix it with 2x SDS sample buffer[47].

3.2.1.13. Heat the mixture at 95 °C for 5 min and separate the Ni-NTA purified protein on a 10% SDS-polyacrylamide gel.

3.2.1.14. Perform western blotting using anti-Penta-His antibodies (mouse monoclonal, 1:500) and horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (rabbit polyclonal, 1:1,000) as previously described[36,37].

3.2.2. Direct analysis of non-tagged PEDF proteins

3.2.2.1. Take 15 µL of cell culture supernatant and mix it with 2x SDS sample buffer[47].

3.2.2.2. Heat the mixture at 95 °C from 5 min and separate the proteins on a 10% SDS-polyacrylamide gel.

3.2.2.3. Perform western blotting using anti-human PEDF antibodies (rabbit polyclonal, 1:4,000) and HRP-conjugated anti-rabbit antibodies (goat polyclonal, 1:2,000) as previously described[38].

3.3. Quantification of PEDF secretion by ELISA

3.3.1. Analyze cell culture supernatants (see step 3.1.2) using a human PEDF ELISA kit according to the manufacturer's protocol.

3.3.2. Relate the amount of secreted PEDF to the time and the cell number determined for each transfection reaction (see step 3.1.4).

3.4. Analysis of *PEDF* gene expression in transfected RPE cells

3.4.1. Isolate total RNA from the RPE cell pellets (see step 3.1.5) using a commercially available kit according to the manufacturer's protocol.

3.4.2. Quantify RNA contents using a microvolume spectrophotometer.

3.4.3. Carry out reverse transcription on 0.1 µg RNA using a reverse transcription system according to the manufacturer's protocol.

3.4.4. Perform real-time qPCR as previously described[38].

3.5. Analysis of transgene insertion sites in transfected RPE cells

3.5.1. Isolate genomic DNA from the RNA cell pellets (see step 3.1.5) using a commercially available kit according to the manufacturer's protocol.

3.5.2. Quantify DNA contents using a microvolume spectrophotometer.

3.5.3. Generate insertion site libraries using a computation-assisted hemi-specific PCR scheme as previously described[38].

3.5.4. Perform computational analysis as previously described[38].

REPRESENTATIVE RESULTS:

Cultivation and electroporation of primary human RPE cells

We have shown that seeding of a sufficient number of primary RPE cells of animal origin allow for the cultivation and growth to an integrated monolayer of pigmented, hexagonally shaped cells[36,37,48]. Their capability to form tight junctions, to exhibit phagocytic activity, and to express specific marker genes in vitro[48] reflects substantial tasks of the retinal pigment epithelium in vivo. Cultivated primary RPE cells isolated from human donor eyes also showed the typical cobblestone morphology, regardless of the donor's age (65.3 ± 9.94 a, min: 49 a, max: 83 a, n = 12), the post-mortem time of isolation (37.3 ± 17.0 h, min: 16 h, max: 68 h, n = 12), and the cultivation time (27.6 ± 14.1 d, min: 13 d, max: 61 d, n = 12) (**Figure 2, left panel**). The application of short-term electrical pulses to primary human RPE cells using the capillary transfection system did not adversely affect the epithelial morphology (**Figure 2, right panel**). Testing of various electric parameters revealed that two pulses with a pulse voltage of 1,200 V and a pulse width of 20 ms yielded good and stable transfection efficiencies and the highest number of viable RPE cells (unpublished data).

SB100X-mediated insertion of the *PEDF* gene into cultivated primary human RPE cells

We have tested plasmid DNA ratios of *SB100X*-to-*PEDF* transposon ranging from 250 ng (0.08 pmol) *SB100X* transposase plus 250 ng (0.052 pmol) *PEDF* transposon to 12.2 ng (0.0039 pmol) *SB100X* transposase plus 487.8 ng (0.1 pmol) *PEDF* transposon. This approach identified the two combinations 29.4 ng (0.0094 pmol) *SB100X* transposase plus 470.6 ng (0.098 pmol) *PEDF* transposon and 23.8 ng (0.0076 pmol) plus 476.2 ng (0.099 pmol) as the ones that obtained the best transposition efficiencies[37]. In cultivated primary human RPE cells, delivery of the *PEDF* transgene using the *SB100X* transposon system allowed for a continuously increased *PEDF* gene expression and PEDF protein secretion. For His-tagged recombinant PEDF, western blot analysis of cell culture media from transfected primary human RPE cells demonstrated PEDF secretion at constant levels without transgene silencing for more than 500 days (**Figure 3A**). For non-tagged PEDF, secretion in transfected primary human RPE cells was also shown to be significantly increased compared to non-transfected cells[38]. A representative western blot analysis of cell culture media from serially performed transfections clearly indicated the universally higher PEDF secretion rate at 21 days after transfection (**Figure 3B**), and in a pursued culture, long-term elevated PEDF secretion was proven for at least 165 days (**Figure 3C**). ELISA-based quantification demonstrated a 20-fold increase of total PEDF secretion in transfected primary human RPE cells compared to respective non-transfected control cells (**Figure 3D**). This increment was also affirmed on gene expression level, where the total *PEDF* expression was raised more than 30-fold (**Figure 3E**).

FIGURE LEGENDS:

Figure 1: Workflow for the electroporation-based insertion of the *PEDF* gene into primary human RPE cells by means of the *SB100X* transposon system. The scheme describes the chronological course of (**A**) the isolation of primary human RPE cells and their subsequent cultivation to a confluent monolayer, (**B**) the single steps of the electroporation process, including the purification of the *SB100X* transposase and the *PEDF* transposon plasmid DNA, the preparation of the *SB100X* transposase/*PEDF* transposon plasmid mixture, the set-up of the capillary transfection system, the preparation of the cultivated primary human RPE cells,

the electroporation, the seeding, and the cultivation of the transfected RPE cells, as well as (**C**) the analyses of the transfected primary human RPE cells, comprising the preparation of the cell culture supernatant and transfected cell samples, the evaluation and quantification of PEDF secretion, and the analysis of *PEDF* gene expression.

Figure 2: Phase contrast micrographs of RPE cells isolated from different donor eyes. Cultures of primary human RPE cells, differing in donor age, post-mortem time of isolation, and cultivation time prior to their application for electroporation (***left panel***), as well as cultures of transfected primary human RPE cells, exposed to the electric parameters 1,100 V (pulse voltage), 20 ms (pulse width), and 2 pulses (number of pulses) using the capillary transfection system (***right panel***), exhibited a confluent integrated monolayer of pigmented cells in a cobblestone-like pattern (scale bars: 500 µm).

Figure 3: PEDF secretion and *PEDF* gene expression after *SB100X*-mediated transfection of primary human RPE cells. (**A**–**C**) Immunoblot-based analyses of PEDF secretion stability in cultivated primary human RPE cells transfected with a mixture of 29.4 ng (0.0094 pmol) *SB100X* transposase plasmid plus 470.6 ng (0.098 pmol) *PEDF* transposon plasmid using the capillary transfection system. (**A**) RPE cells from a 26-year-old donor (female, post-mortem time of isolation: 26 h, cultivation time before electroporation: 14 days) were transfected and maintained in culture for more than 500 days. His-tagged recombinant PEDF (~48 kDa) was purified from cell culture media at different time points and evaluated by western blotting using anti-Penta-His antibodies. (**B**) For non-tagged recombinant PEDF, RPE cells from a 53-year-old donor (female, post-mortem time of isolation: 28 h, cultivation time before transfection: 19 days) were electroporated without plasmid DNA (control cultures #1 and #2) or with the addition of plasmid DNA (PEDF-transfected cell cultures #3 to #7) and maintained in culture for 21 days. Cell culture supernatants were not purified, but directly analyzed by immunoblotting using anti-PEDF antibodies. Western blot analysis of cell lysates showed the level of intracellular PEDF in controls (cultures #1 and #2) and transfected cells (cultures #3 and #4). Loading of similar protein amounts was indicated by the equal density of GAPDH protein bands (~36 kDa). (**C**) For the analysis of long-term PEDF secretion, RPE cells from a 63-year-old donor (male, post-mortem-time of isolation: 26 h, cultivation time before transfection: 15 days) were electroporated without plasmid DNA (control cultures #1 and #2) or with plasmid DNA (PEDF-transfected cell culture #3) and maintained in culture for at least 165 days. Cell culture media were not purified, but directly analyzed by immunoblotting using anti-PEDF antibodies. (**D**) ELISA-based quantification of total PEDF secretion in transfected primary human RPE cells (two samples) and non-transfected control cells (four samples). Secretion of the transfected cells was compared to the secretion of the non-transfected cells (*p = 0.0264, unpaired *t* test with Welch's correction). (**E**) Endogenous and total (endogenous plus recombinant) *PEDF* gene expression in transfected primary human RPE cells were related to the expression in non-transfected control cells, whose expression was set to 1 (dashed line). Data is presented as a box-and-whisker plot (whiskers: min to max). Total *PEDF* gene expression was compared to endogenous *PEDF* gene expression (not significant, unpaired *t* test with Welch's correction). The results for the non-tagged recombinant PEDF (**B**–**E**) represent two units of the entire data set of primary RPE cells from up to 27 donors transfected with a *SB100X*-to-*PEDF* transposon ratio of 29.4 ng (0.0094 pmol) plus 470.6 ng (0.098 pmol), which was described by Thumann et al. 2017[38].

DISCUSSION:

In our project, we aim for the non-viral production of genetically modified primary human RPE cells that continuously overexpress and secrete an effective factor in order to use the transfected cells as long-term therapeutic for the establishment and maintenance of a protective environment. We have established the introduction of the gene encoding PEDF, a ubiquitously expressed multi-functional protein with anti-angiogenic and neuroprotective functions. The protocol described here can be used to stably and reproducibly transfect primary human RPE cells using the *SB100X* transposon system. DNA delivery and introduction into the RPE cells is performed with a pipette-based capillary transfection system, which uses specific tips as electroporation chamber instead of cuvettes.

The enrichment of primary RPE cultures consisting of morphologically well-differentiated cells to be used for subsequent transfections is influenced by several factors related to the human donor (age and general health status) as well as the receipt of the eyes (post-mortem time of cell isolation). Freshly isolated and seeded primary RPE cells need at least 3 weeks to develop a monolayer of hexagonally shaped cells. As a general rule, cell cultures that need more time to reach confluence prior to transfection also showed a decelerated adherence and spreading after transfection. Cell adherence and spreading is also impeded by free pigment depositing from RPE cells damaged during cell isolation or the transfection process.

The *SB* transposon system is a widely used genetic tool that enables the efficient and safe delivery of therapeutically relevant nucleotide sequences into various types of cells to be subsequently applied in gene-based cell therapies. Especially, its enhanced variant *SB100X* combines the advantages of viral vectors and non-viral plasmid DNA. The integrating mode of action ensures a stable genomic integration of the expression cassette into the host cell's genome and enables a sustained expression of the gene or sequence of interest. In contrast to retroviral vectors, which preferentially integrate into active transcription units with a high risk of potential mutagenesis and oncogenesis[27,28], the *SB*-based integration profile is random. This was demonstrated in a large number of studies using various cultured and primary mammalian cells, including human cells[49–52], as well as for primary rat and human RPE cells[38,46]. Moreover, application of the *SB* transposon system as plasmid DNA provides reduced immunogenicity and facilitates the manufacturing process.

Delivery of the genetic information of the *SB100X* transposase and the *PEDF* transgene on separate plasmids is an important aspect, as it allows for a precise adjustment of the optimal *SB100X*-to-*PEDF* transposon ratio. As described earlier, the *SB* transposon system is sensitive to an excess of transposase expression, which results in inhibition of the transpositional activity[53]. We also observed this effect for ARPE-19 cells, a spontaneously evolved cell line purified by selective trypsinization of a primary human RPE culture[54], and primary RPE cells. Here, transfection with *SB100X*-to-*PEDF* transposon ratios up to 55.6 ng (0.018 pmol) *SB100X* transposase plus 444.4 ng (0.093 pmol) *PEDF* transposon resulted in clearly diminished PEDF secretion rates[37]. The ideal transposase-to-transposon ratio has to be determined for every newly constructed transposon plasmid.

Non-viral plasmid-based vectors can be transferred via lipo-/polyplexes, nanoparticles, laser, ultrasound, or electroporation. We have already shown that lipofection-based transfection of primary PE cells was not efficient and thus switched to the electroporation-based gene

transfer[36]. Electroporation is defined as application of a short-duration electric field to cells, which results in an increase of the membrane's permeability and the formation of aqueous pores, through which plasmid DNA can enter the cell. In general, a mixture of cells, plasmid DNA, and conducting buffer is filled into a specific electroporation chamber between two electrodes, which are connected to the electroporation device generating the electrical pulses. In this so-called bulk electroporation setup, a conventional cuvette electroporation chamber is characterized by two parallel plate-type electrodes, whose distance is variable (0.1–0.4 mm), depending on the cell type and the number of cells used per electroporation reaction. Despite good transfection efficiencies, different side effects may negatively affect the survival of the transfected cells, e.g., pH changes, heat development, bubble generation, and turbulent flow. The capillary transfection system at least attenuates the cuvette-based side effects by possessing electrodes with a minimized surface area and a maximized gap size between them[42] as well as by using specific resuspension and electrolytic buffers; however, their compositions are not disclosed.

Despite the enhancements within the bulk electroporation set-up, which led to an increased cell survival, irreversible damage to a certain percentage of cells is unavoidable. Moreover, a precise control of the amount of plasmid integrated into the cells is not possible. The more recent development of miniaturized electroporation systems has allowed for additional improvements by further reducing the limitations associated with bulk electroporation. These new devices are based on microelectrodes, microfluidic, or nanostructures. They offer new application perspectives in the fields of gene therapy, regenerative medicine, and in situ intracellular investigation (reviewed by Chang et al. and Shi et al.)[55,56].

We have demonstrated that electroporation, which was initially performed using a cuvette-based system and subsequently established using a pipette-based capillary system, is a suitable and efficient method to transfect both the pigment epithelial cell line ARPE-19 as well as primary PE cells[36–38,57,58]. Depending on the cell type used, it is important to define appropriate electroporation protocols applying parameters that allow for both good transfection efficiencies and cell survival. Insufficient electric fields prevent the cells from damage but result in low transfection efficiencies. With the increase in electric field strength, enhanced transfection efficiencies are achieved. However, elevated electric parameters also lead to higher percentages of dead cells because of irreversible cell damage. For the ARPE-19 cell line, using the capillary transfection system, electroporation parameters of 1,350 V, 20 ms, and 2 pulses were shown to result in initial transfection efficiencies of 100%[37]. The application of these parameters for the transfection of primary RPE cells resulted in good efficiencies, too, but also in a lower number of cultivated cells after transfection when compared to electroporation parameters of 1,100 V, 20 ms, and 2 pulses (data not shown). Therefore, the decision was taken to choose milder electroporation parameters to prevent cell damage and accept less transfected cells.

The overall aim of this approach lies in the clinical application of PEDF-transfected cells for the treatment of patients suffering from neovascular AMD. The therapy comprises the stable introduction of the *PEDF* transgene into autologous pigment epithelial cells ex vivo based on the *SB100X* transposon system, followed by transplantation of the transfected cells to the subretinal space of the patient. Thus, it is important to ensure that the PEDF-transfected cells do not transdifferentiate and do not acquire a fibroblastic shape and growth behavior. It is

also important to prove that the transfected cells allow for a sustained and consistent secretion of PEDF. Moreover, it is crucial to know the precise amount of PEDF secreted by a particular number of cells for a specific period of time.

ACKNOWLEDGEMENTS:

This work was supported by the European Union's Seventh Framework Programme for research, technological development and demonstration, grant agreement no. 305134. Z. Izsvák was funded by the European Research Council, ERC Advanced (ERC-2011-ADG 294742). The authors would like to thank Anna Dobias and Antje Schiefer (Department of Ophthalmology, University Hospital RWTH Aachen) for excellent technical support, and the Aachen Cornea Bank (Department of Ophthalmology, University Hospital RWTH Aachen) for providing the human donor eyes.

DISCLOSURES:

Z. Ivics and Z. Izsvák are inventors on several patents on *SB* transposon technology.

REFERENCES:

1. James, S. L. et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. **392** (10159), 1789–1858 (2018).
2. Wong, W. L. et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Global Health*. **2** (2), e106–116 (2014).
3. Strauss, O. The retinal pigment epithelium in visual function. *Physiological Reviews*. **85** (3), 845–881 (2005).
4. Chan, C. K. et al. Differential expression of pro- and antiangiogenic factors in mouse strain-dependent hypoxia-induced retinal neovascularization. *Laboratory Investigation*. **85** (6), 721–733 (2005).
5. Gao, G. et al. Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization. *FEBS Letters*. **489** (2–3), 270–276 (2001).
6. Bhutto, I. A. et al. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. *Experimental Eye Research*. **82** (1), 99–110 (2006).
7. Holekamp, N. M., Bouck, N., Volpert, O. Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. *American Journal of Ophthalmology*. **134** (2), 220–227 (2002).
8. Kolomeyer, A. M., Sugino, I. K., Zarbin, M. A. Characterization of conditioned media collected from aged versus young human eye cups. *Investigative Ophthalmology & Visual Science*. **52** (8), 5963–5972 (2011).
9. Li, X. et al. The significance of the increased expression of phosphorylated MeCP2 in the membranes from patients with proliferative diabetic retinopathy. *Scientific Reports.* **6**, 32850 (2016).
10. Mohan, N., Monickaraj, F., Balasubramanyam, M., Rema, M., Mohan, V. Imbalanced levels of angiogenic and angiostatic factors in vitreous, plasma and postmortem retinal tissue of patients with proliferative diabetic retinopathy. *Journal of Diabetes and its Complications*. **26** (5), 435–441 (2012).

11. Empeslidis, T. et al. How successful is switching from Bevacizumab or Ranibizumab to Aflibercept in Age-Related Macular Degeneration? A systematic overview. *Advances in Therapy*. **36** (7), 1532–1548 (2019).
12. Solomon, S. D., Lindsley, K., Vedula, S. S., Krzystolik, M. G., Hawkins, B. S. Anti-vascular endothelial growth factor for neovascular age-related macular degeneration. *Cochrane Database of Systematic Reviews*. **3**, CD005139 (2019).
13. Dugel, P. U. et al. HAWK and HARRIER: phase 3, multicenter, randomized, double-masked trials of brolucizumab for neovascular age-related macular degeneration. *Ophthalmology*. **127** (1), 72–84 (2020).
14. Falavarjani, K. G., Nguyen, Q. D. Adverse events and complications associated with intravitreal injection of anti-VEGF agents: a review of literature. *Eye*. **27** (7), 787–794 (2013).
15. Jaffe, D. H., Chan, W., Bezlyak, V., Skelly, A. The economic and humanistic burden of patients in receipt of current available therapies for nAMD. *Journal of Comparative Effectiveness Research*. **7** (11), 1125–1132 (2018).
16. Eghoj, M. S., Sorensen, T. L. Tachyphylaxis during treatment of exudative age-related macular degeneration with ranibizumab. *British Journal of Ophthalmology*. **96** (1), 21–23 (2012).
17. Forooghian, F., Cukras, C., Meyerle, C. B., Chew, E. Y., Wong, W. T. Tachyphylaxis after intravitreal bevacizumab for exudative age-related macular degeneration. *Retina - The Journal of Retinal and Vitreous Diseases*. **29** (6), 723–731 (2009).
18. Otsuji, T. et al. Initial non-responders to ranibizumab in the treatment of age-related macular degeneration (AMD). *Clinical Ophthalmology*. **7**, 1487–1490 (2013).
19. Zuber-Laskawiec, K., Kubicka-Trzaska, A., Karska-Basta, I., Pociej-Marciak, W., Romanowska-Dixon, B. Non-responsiveness and tachyphylaxis to anti-vascular endothelial growth factor treatment in naive patients with exudative age-related macular degeneration. *Journal of Physiology and Pharmacology*. **70** (5) (2019).
20. Campochiaro, P. A. et al. Lentiviral vector gene transfer of endostatin/angiostatin for macular degeneration (GEM) study. *Human Gene Therapy*. **28** (1), 99–111 (2017).
21. Constable, I. J. et al. Phase 2a randomized clinical trial: safety and post hoc analysis of subretinal rAAV.sFLT-1 for wet age-related macular degeneration. *EBioMedicine*. **14**, 168–175 (2016).
22. Rakoczy, E. P. et al. Three-year follow-up of phase 1 and 2a rAAV.sFLT-1 subretinal gene therapy trials for exudative age-related macular degeneration. *American Journal of Ophthalmology*. **204**, 113–123 (2019).
23. Kumar-Singh, R. The role of complement membrane attack complex in dry and wet AMD - from hypothesis to clinical trials. *Experimental Eye Research*. **184**, 266–277 (2019).
24. Campochiaro, P. A. et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Human Gene Therapy*. **17** (2), 167–176 (2006).
25. Campochiaro, P. A. Gene transfer for neovascular age-related macular degeneration. *Human Gene Therapy*. **22** (5), 523–529 (2011).
26. Ahi, Y. S., Bangari, D. S., Mittal, S. K. Adenoviral vector immunity: its implications and circumvention strategies. *Current Gene Therapy*. **11** (4), 307–320 (2011).
27. Hacein-Bey-Abina, S. et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *New England Journal of Medicine*. **363** (4), 355–364 (2010).

28. Howe, S. J. et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *Journal of Clinical Investigation*. **118** (9), 3143–3150 (2008).
29. Stieger, K. et al. Subretinal delivery of recombinant AAV serotype 8 vector in dogs results in gene transfer to neurons in the brain. *Molecular Therapy*. **16** (5), 916–923 (2008).
30. Tornabene, P., Trapani, I. Can adeno-associated viral vectors deliver effectively large genes? *Human Gene Therapy*. **31** (1-2), 47–56 (2020).
31. Ayuso, E. Manufacturing of recombinant adeno-associated viral vectors: new technologies are welcome. *Molecular Therapy - Methods & Clinical Development*. **3** 15049 (2016).
32. van der Loo, J. C., Wright, J. F. Progress and challenges in viral vector manufacturing. *Human Molecular Genetics*. **25** (R1), R42–52 (2016).
33. Ivics, Z., Hackett, P. B., Plasterk, R. H., Izsvak, Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell*. **91** (4), 501–510 (1997).
34. Mates, L. et al. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nature Genetics*. **41** (6), 753–761 (2009).
35. Izsvak, Z., Hackett, P. B., Cooper, L. J., Ivics, Z. Translating Sleeping Beauty transposition into cellular therapies: victories and challenges. *Bioessays*. **32** (9), 756–767 (2010).
36. Thumann, G. et al. High efficiency non-viral transfection of retinal and iris pigment epithelial cells with pigment epithelium-derived factor. *Gene Therapy*. **17** (2), 181–189 (2010).
37. Johnen, S. et al. Sleeping Beauty transposon-mediated transfection of retinal and iris pigment epithelial cells. *Investigative Ophthalmology & Visual Science*. **53** (8), 4787–4796 (2012).
38. Thumann, G. et al. Engineering of PEDF-expressing primary pigment epithelial cells by the SB transposon system delivered by pFAR4 plasmids. *Molecular Therapy - Nucleic Acids.* **6**, 302–314 (2017).
39. Abdul Halim, N. S., Fakiruddin, K. S., Ali, S. A., Yahaya, B. H. A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. *International Journal of Molecular Sciences*. **15** (9), 15044–15060 (2014).
40. Ahlemeyer, B., Vogt, J. F., Michel, V., Hahn-Kohlberger, P., Baumgart-Vogt, E. Microporation is an efficient method for siRNA-induced knockdown of PEX5 in HepG2 cells: evaluation of the transfection efficiency, the PEX5 mRNA and protein levels and induction of peroxisomal deficiency. *Histochemistry and Cell Biology*. **142** (5), 577–591 (2014).
41. May, R. D. et al. Efficient nonviral transfection of primary intervertebral disc cells by electroporation for tissue engineering application. *Tissue Engineering Part C - Methods*. **23** (1), 30–37 (2017).
42. Kim, J. A. et al. A novel electroporation method using a capillary and wire-type electrode. *Biosensors & Bioelectronics*. **23** (9), 1353–1360 (2008).
43. Johnen, S. et al. Antiangiogenic and neurogenic activities of Sleeping Beauty-mediated PEDF-transfected RPE cells in vitro and in vivo. *Biomed Research International*. **2015**, 863845 (2015).
44. Kuerten, D. et al. Transplantation of PEDF-transfected pigment epithelial cells inhibits corneal neovascularization in a rabbit model. *Graefes Archive for Clinical and Experimental Ophthalmology*. **253** (7), 1061–1069 (2015).

45. Garcia-Garcia, L. et al. Long-term PEDF release in rat iris and retinal epithelial cells after Sleeping Beauty transposon-mediated gene delivery. *Molecular Therapy - Nucleic Acids*. **9**, 1–11 (2017).
46. Hernandez, M. et al. Preclinical evaluation of a cell-based gene therapy using the Sleeping Beauty transposon system in choroidal neovascularization. *Molecular Therapy - Methods & Clinical Development*. **15**, 403–417 (2019).
47. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227** (5259), 680–685 (1970).
48. Johnen, S. et al. Presence of xenogenic mouse RNA in RPE and IPE cells cultured on mitotically inhibited 3T3 fibroblasts. *Investigative Ophthalmology & Visual Science*. **52** (5), 2817–2824 (2011).
49. Gogol-Doring, A. et al. Genome-wide profiling reveals remarkable parallels between insertion site selection properties of the MLV retrovirus and the piggyBac transposon in primary human CD4(+) T cells. *Molecular Therapy*. **24** (3), 592–606 (2016).
50. Holstein, M. et al. Efficient non-viral gene delivery into human hematopoietic stem cells by minicircle Sleeping Beauty transposon vectors. *Molecular Therapy*. **26** (4), 1137–1153 (2018).
51. Moldt, B. et al. Comparative genomic integration profiling of Sleeping Beauty transposons mobilized with high efficacy from integrase-defective lentiviral vectors in primary human cells. *Molecular Therapy*. **19** (8), 1499–1510 (2011).
52. Yant, S. R. et al. High-resolution genome-wide mapping of transposon integration in mammals. *Molecular and Cellular Biology*. **25** (6), 2085–2094 (2005).
53. Grabundzija, I. et al. Comparative analysis of transposable element vector systems in human cells. *Molecular Therapy*. **18** (6), 1200–1209 (2010).
54. Dunn, K. C., Aotaki-Keen, A. E., Putkey, F. R., Hjelmeland, L. M. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Experimental Eye Research*. **62** (2), 155–169 (1996).
55. Chang, L. et al. Micro-/nanoscale electroporation. *Lab on a Chip*. **16** (21), 4047–4062 (2016).
56. Shi, J. et al. A review on electroporation-based intracellular delivery. *Molecules*. **23** (11) (2018).
57. Johnen, S. et al. Endogenic regulation of proliferation and zinc transporters by pigment epithelial cells nonvirally transfected with PEDF. *Investigative Ophthalmology & Visual Science*. **52** (8), 5400–5407 (2011).
58. Pastor, M. et al. The antibiotic-free pFAR4 vector paired with the Sleeping Beauty transposon system mediates efficient transgene delivery in human cells. *Molecular Therapy - Nucleic Acids*. **11**, 57–67 (2018).

Figure 1

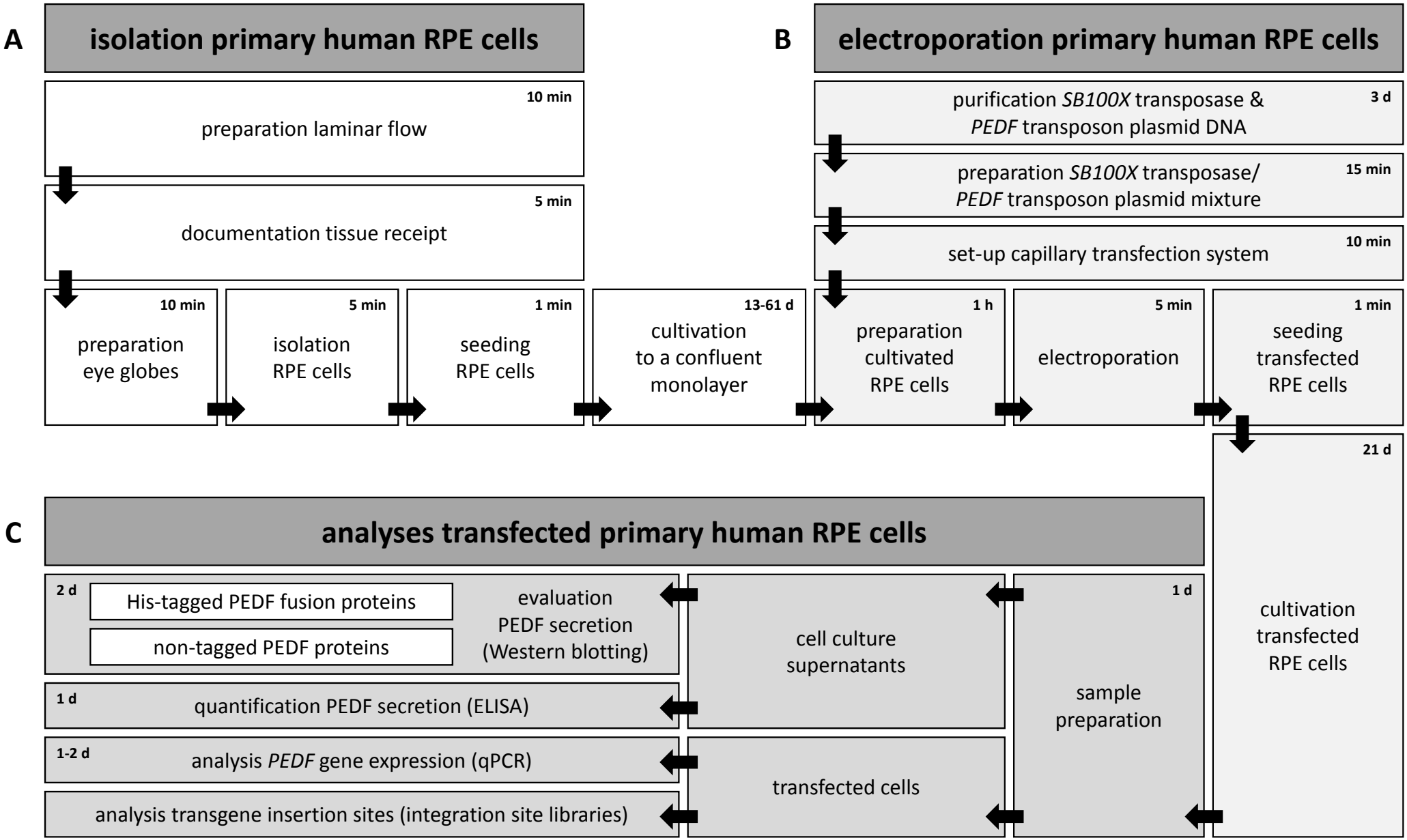

Figure 2

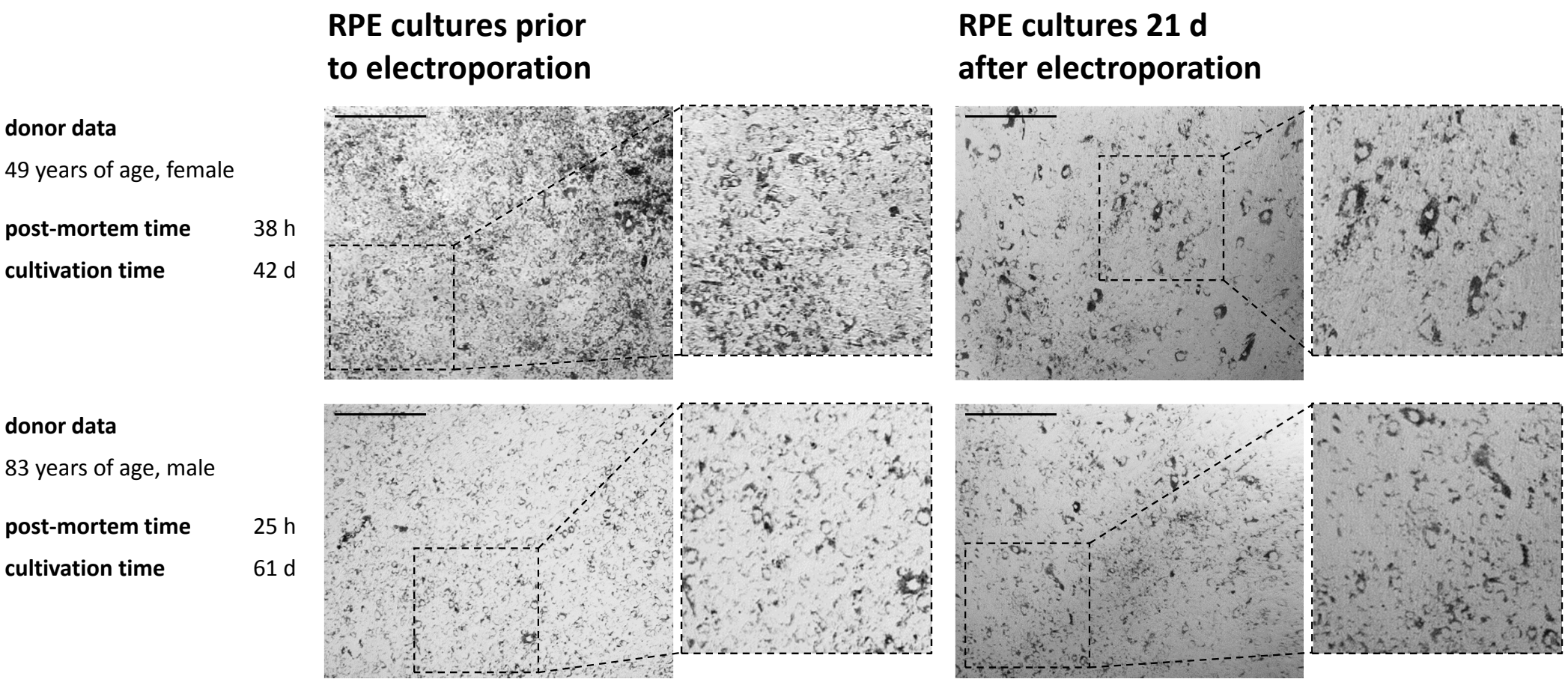

Figure 3

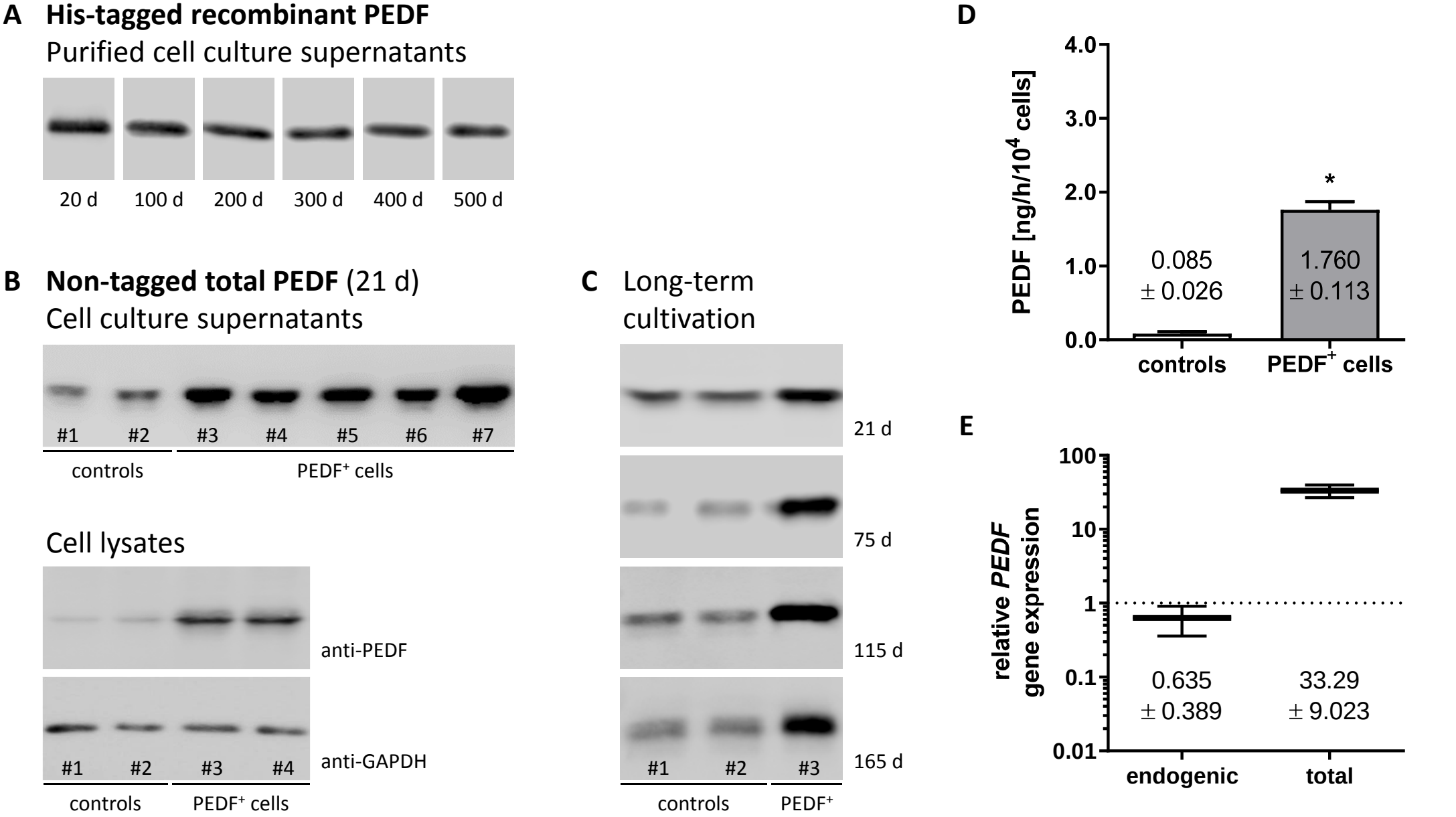

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Isolation of primary human RPE cells		
24-Well Cell Culture Plate	Eppendorf, Hamburg, Germany	0030722019
Amphotericin B [250 µg/mL] (AmphoB)	Merck, Darmstadt, Germany	A2942
Colibri Forceps	Geuder, Heidelberg, Germany	G-18950
Curved Iris Forceps	Geuder, Heidelberg, Germany	G-18856
Disposable Scalpel (No. 11)	Feather, Osaka, Japan	
Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12)	PAN-Biotech, Aidenbach, Germany	P04-41150
Extra Fine Pointed Eye Scissor	Geuder, Heidelberg, Germany	G-19405
Fetal Bovine Serum [0.2 µm Sterile Filtered] (FBS)	PAN-Biotech, Aidenbach, Germany	P40-37500
Glass Pasteur Pipettes	Brand, Wertheim, Germany	747715
Penicillin [10,000 units/mL] and Streptomycin [10 mg/mL] (Pen/Strep)	Merck, Darmstadt, Germany	P0781
Pipette Tips (1000 µL)	Starlab, Hamburg, Germany	
Single Channel Pipette (100-1000 µL)	Eppendorf, Hamburg, Germany	
Sterile Drape	Lohmann & Rauscher, Rengsdorf, Germany	
Sterile Gauze Compress	Fink-Walter, Merchweiler, Germany	321063
Sterile Gloves	Sempermed, Wien, Austria	
Sterile Petri Dish (Falcon 60 mm x 15 mm)	Corning, Corning, NY	351007
Sterile Surgical Gown	Halyard Health, Alpharetta, GA	
Straight Iris Forceps	Geuder, Heidelberg, Germany	G-18855
Electroporation of primary human RPE cells		
10 mM Tris-HCl (pH 8.5)		
12-Well Cell Culture Plate	Thermo Fisher Scientific, Waltham, MA	150628
24-Well Cell Culture Plate	Eppendorf, Hamburg, Germany	0030722019
Amphotericin B [250 µg/mL] (AmphoB)	Merck, Darmstadt, Germany	A2942
Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12)	PAN-Biotech, Aidenbach, Germany	P04-41150
Safe-Lock Microcentrifuge Tubes (1.5 mL)	Eppendorf, Hamburg, Germany	
Fetal Bovine Serum [0.2 µm Sterile Filtered] (FBS)	PAN-Biotech, Aidenbach, Germany	P40-37500
Inverted Microscope	Leica Mikrosysteme, Wetzlar, Germany	Leica DMi8
Microvolume Spectrophotometer (NanoDrop Spectrophotometer)	Thermo Fisher Scientific, Waltham, MA	
Capillary Transfection System (Neon Transfection System)	Thermo Fisher Scientific, Waltham, MA	MPK5000
Neon Transfection System 10 µL Kit	Thermo Fisher Scientific, Waltham, MA	MPK1096
Hemocytometer (Neubauer Chamber)	Paul Marienfeld, Lauda-Königshofen, Germany	0640110
PBS Dulbecco w/o Ca^{2+} w/o Mg^{2+}	Biochrom, Berlin, Germany	L182-50
Penicillin [10,000 units/mL] and Streptomycin [10 mg/mL] (Pen/Strep)	Merck, Darmstadt, Germany	P0781
Pipette Tips (10 µL)	Starlab, Hamburg, Germany	
Pipette Tips (1000 µL)	Starlab, Hamburg, Germany	
Pipette Tips (200 µL)	Starlab, Hamburg, Germany	
Plasmid Maxi Kit	Qiagen, Hilden, Germany	12163
Single Channel Pipette (0.1-10 µL)	Eppendorf, Hamburg, Germany	
Single Channel Pipette (100-1000 µL)	Eppendorf, Hamburg, Germany	
Single Channel Pipette (10-200 µL)	Eppendorf, Hamburg, Germany	
Trypan Blue Solution	Merck, Darmstadt, Germany	T8154
Trypsin-EDTA (0,05 %)	Thermo Fisher Scientific, Waltham, MA	25300054
Analyses of transfected primary human RPE cells		
10% SDS-Polyacrylamide Gel		
1x Incubation Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0)		
2x SDS Sample Buffer		
4x Incubation Buffer (200 mM NaH_2PO_4, 1.2 M NaCl, 40 mM imidazole, pH 8.0)		
Amersham Protran Supported 0.2 µm Nitrocellulose Blotting Membrane	Cytiva, Marlborough, MA	10600015
Amphotericin B [250 µg/mL] (AmphoB)	Merck, Darmstadt, Germany	A2942
Anti-PEDF Antibodies (Rabbit Polyclonal)	BioProducts, Middletown, MD	AB-PEDF1
Anti-Penta-His Antibodies (Mouse Monoclonal)	Qiagen, Hilden, Germany	34660
Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12)	PAN-Biotech, Aidenbach, Germany	P04-41150
Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0)		
Fetal Bovine Serum [0.2 µm Sterile Filtered] (FBS)	PAN-Biotech, Aidenbach, Germany	P40-37500
Hemocytometer (Neubauer Chamber)	Paul Marienfeld, Lauda-Königshofen, Germany	0640110
Horseradish Peroxidase-Conjugated Anti-Mouse Antibodies (Rabbit Polyclonal)	Agilent Dako, Santa Clara, CA	P0260
Horseradish Peroxidase-Conjugated Anti-Rabbit Antibodies (Goat Polyclonal)	Abcam, Cambridge, United Kingdom	ab6721
Human PEDF ELISA Kit	BioProducts, Middletown, MD	PED613
LAS-3000 Imaging System	Fujifilm, Minato, Japan	
LightCycler 1.2 Instrument	Roche Life Science, Penzberg, Germany	
LightCycler FastStart DNA Master SYBR Green I	Roche Life Science, Penzberg, Germany	12239264001
LightCycler Capillaries (20 µl)	Roche Life Science, Penzberg, Germany	4929292001
Microvolume Spectrophotometer (NanoDrop Spectrophotometer)	Thermo Fisher Scientific, Waltham, MA	
Mini-PROTEAN Tetra Cell Casting Module	Bio-Rad Laboratories, Feldkirchen, Germany	1658015
Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels, 4-gel	Bio-Rad Laboratories, Feldkirchen, Germany	1658004
Ni-NTA Superflow	Qiagen, Hilden, Germany	30410
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific, Waltham, MA	26616
Penicillin [10,000 units/mL] and Streptomycin [10 mg/mL] (Pen/Strep)	Merck, Darmstadt, Germany	P0781
Pipette Tips (10 µL)	Starlab, Hamburg, Germany	
Pipette Tips (1000 µL)	Starlab, Hamburg, Germany	
Pipette Tips (200 µL)	Starlab, Hamburg, Germany	
PowerPac Basic Power Supply	Bio-Rad Laboratories, Feldkirchen, Germany	1645050
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany	51304
Reverse Transcription System	Promega, Madison, WI	A3500
RNase-Free DNase Set	Qiagen, Hilden, Germany	79254
RNeasy Mini Kit	Qiagen, Hilden, Germany	74104
Rocking Shaker	Cole-Parmer, Staffordshire, United Kingdom	SSM3
Safe-Lock Microcentrifuge Tubes (1.5 mL)	Eppendorf, Hamburg, Germany	
Safe-Lock Microcentrifuge Tubes (2.0 mL)	Eppendorf, Hamburg, Germany	
Single Channel Pipette (0.1-10 µL)	Eppendorf, Hamburg, Germany	
Single Channel Pipette (100-1000 µL)	Eppendorf, Hamburg, Germany	
Single Channel Pipette (10-200 µL)	Eppendorf, Hamburg, Germany	
Trans-Blot Turbo Transfer System	Bio-Rad Laboratories, Feldkirchen, Germany	1704150
Trypan Blue Solution	Merck, Darmstadt, Germany	T8154
Trypsin-EDTA (0,05 %)	Thermo Fisher Scientific, Waltham, MA	25300054

UNIKLINIK RWTH AACHEN
Klinik für Augenheilkunde

Uniklinik RWTH Aachen · Klinik für Augenheilkunde
Pauwelsstraße 30 · 52074 Aachen

Vineeta Bajaj
Review Editor
JoVE

Aachen, December 10th, 2020

Reply to 61987 Editorial Review

Dear Dr Bajaj:

Thank you for the review of our research article titled “Electroporation-Based Genetic Modification of Primary Human Pigment Epithelial Cells Using the Sleeping Beauty Transposon System” (manuscript no.: 61987).

We have revised the manuscript according to the editorial comments, which are detailed below.

Sincerely,
Sandra Johnen, PhD

Klinikdirektor
Univ.-Prof. Dr. med. Peter Walter

Universitätsklinikum Aachen
Anstalt öffentlichen Rechts (AöR)
Pauwelsstraße 30
52074 Aachen

www.augenklinik.ukaachen.de

Sekretariat des Klinikdirektors
Stefanie von Atens
Tel.: 0241 80-88191
Fax: 0241 80-82408
svonatens@ukaachen.de

Privatsprechstunde
Ana Paula Freire
Tel.: 0241 80-88192
Fax: 0241 80-82408
afreire@ukaachen.de

Oberärzte
Vorderer Augenabschnitt
PD Dr. med. Matthias Fuest
Dr. med. Hannah Schellhase

Hinterer Augenabschnitt
Dr. med. Yassin Djalali Talab
Dr. med. Antonis Koutsonas

Orthoptik
Dr. med. Kathi Hartmann

Oberarzt-Sekretariat
Ferhan Saydam
Tel.: 0241 80-88227
Fax: 0241 80-82490
fsaydam@ukaachen.de

Studiensekretariat
Birgit Wauben
Tel.: 0241 80-88192
Fax: 0241 80-82408
bwauben@ukaachen.de

Experimentelle Ophthalmologie
Dr. rer. nat. Sandra Johnen
Tel.: 0241 80-85431
sjohnen@ukaachen.de

Hornhautbank
Sabine Salla
Tel.: 0241 80-88185
Fax: 0241 80-82438
au-hornhautbank@ukaachen.de

Anmeldung – Poliklinik
Tel.: 0241 80-88552

Orthoptik
Martina Leisten
Tel.: 0241 80-88196

Editorial comments:

Lane 480, “Figure 3”: Please include blots for loading control as well.

For the experiment shown in Figure 3B (termination of the cultures after 21 days), we added Western blots of respective cell lysates analyzed with anti-PEDF, using anti-GAPDH as loading control. The resulting changes within the figure legend are written in blue.

The experiments shown in Figure 3A and 3C comprise the long-term cultivation of non-transfected and transfected primary cells. The cultures did not undergo passaging so that a collection of cells during the cultivation was not possible. Here, only the supernatants were collected at defined times and either purified using Ni-NTA resin (3A) or directly analyzed via immunoblotting (3C).

Lane 512, “which was described by Thumann et al. 2017[38]”: Please include reprint permission for reusing this data.

A permission request was completed and sent to Elsevier. A reply is awaited within the next days.

Lane 514, “Discussion”: Please expand on the limitations.

Within the first revision, we already included additional limitations of the protocol regarding the choice of the appropriate electroporation parameters, crucial facts concerning the cultivation of primary human RPE cells, and the electroporation process.

We have now added some more details concerning the cultivation of freshly isolated cells, the determination of the ideal transposase-to-transposon ratio, and the bulk electroporation set-up (written in blue).

Johnen, Sandra

Von: David Barrett <dbarrett@asgct.org>
Gesendet: Dienstag, 15. Dezember 2020 02:21
An: Johnen, Sandra
Betreff: FW: Molecular Therapy Permissions Request

Priorität: Hoch

You have permission to use the material as requested.

Thank you,

David Barrett, JD
Chief Executive Officer
American Society of Gene and Cell Therapy
414.278.1341
asgct.org

From: Info <info@asgct.org>
Sent: Monday, December 14, 2020 7:38 AM
To: David Barrett <dbarrett@asgct.org>
Subject: FW: Molecular Therapy Permissions Request
Importance: High

From: Johnen, Sandra <sjohnen@ukaachen.de>
Sent: Monday, December 14, 2020 12:16 AM
To: Info <info@asgct.org>
Subject: Molecular Therapy Permissions Request
Importance: High

Dear Sir/Madam,

regarding the reuse of already published data, I contacted the Permissions Granting Team of Elsevier (see e-mail conversation below) and got the answer to refer all permissions to The American Society of Gene and Cell Therapy. Therefore, I now contact you regarding my request.

In 2017, the article *Engineering of PEDF-Expressing Primary Pigment Epithelial Cells by the SB Transposon System Delivered by pFAR4 Plasmids* (Authors: G. Thumann, N. Harmening, C. Prat-Souteyrand, C. Marie, M. Pastor, A. Sebe, C. Miskey, L. D. Hurst, S. Diarra, M. Kropp, P. Walter, D. Scherman, Z. Ivics, Z. Izsvák, S. Johnen) was published in the journal *Molecular Therapy — Nucleic Acids* (Volume: 6, Pages: 302-314, https://doi.org/10.1016/j.omtn.2017.02.002). I am the last author of this article and was involved in the conceptualization, methodology, investigation, writing of the original draft and in reviewing and editing of the manuscript.

For a method description within the *Journal of Visualized Experiments*, I want to reuse two units of the entire data set of transfected primary human RPE cells (summary of 27 single experiments) that were described in the above

mentioned article. In fact, I have extracted two Western blot images that are shown in Figure S4 A (lanes 7 & 8). Also for the data shown in the Figures 4, 5, and 6, I have extracted the results from one single experiment out of a set of 27 experiments.

I kindly ask you to forward me a respective reprint permission.

In case of questions please do not hesitate to contact me.

Kind regards,
Sandra Johnen

Priv.-Doz. Dr. rer. nat. Sandra Johnen
Arbeitsgruppenleiterin

Experimentelle Ophthalmologie
Klinik für Augenheilkunde

UNIKLINIK RWTH AACHEN

Tel.: +49 241 80-35945
Fax: +49 241 80-82490
sjohnen@ukaachen.de

Universitätsklinikum Aachen
Anstalt des öffentlichen Rechts (AöR)
Pauwelsstraße 30
D-52074 Aachen
Telefon Call- & Service-Center: 0241 80-84444
info@ukaachen.de
www.ukaachen.de

Aufsichtsratsvorsitzender: Herbert Pfennig
Vorstandsvorsitzender: Professor Dr. Thomas H. Ittel
Kaufmännischer Direktor: Dipl.-Kfm. Peter Asché

Sitz Aachen
USt-Id-Nr: DE 813100566

Von: Rights and Permissions (ELS) <Permissions@elsevier.com>
Gesendet: Sonntag, 13. Dezember 2020 18:03
An: Johnen, Sandra <sjohnen@ukaachen.de>
Betreff: Re: Obtain permission request - Journal (1102083) [201210-009319]

Dear Sandra Johnen

Please refer all permissions to The American Society of Gene and Cell Therapy at info@asgct.org. Requestors should include the following subject line: Molecular Therapy Permissions Request

THanks
Anita

From: Administrator
Date: Thursday, December 10, 2020 10:30 AM GMT

Dear Sandra Johnen,

Thank you for contacting the Permissions Granting Team.

We acknowledge the receipt of your request and we aim to respond within seven business days. Your unique reference number is 201210-009319.

Please avoid changing the subject line of this email when replying to avoid delay with your query.

Regards,
Permission Granting Team

From: Sandra Johnen
Date: Thursday, December 10, 2020 10:30 AM GMT

Submission ID: 1102083
Date: 10 Dec 2020 10:30am

Name: PD Dr. rer. nat. Sandra Johnen
Institute/company: Department of Ophthalmology / University Hospital RWTH Aachen
Address: Pauwelsstraße 30
Post/Zip Code: 52074
City: Aachen
State/Territory:
Country: Germany
Telephone:
Email: sjohnen@ukaachen.de

Type of Publication: Journal

Title: Molecular Therapy — Nucleic Acids
Auhtors: Gabriele Thumann, Nina Harmening, Cécile Prat-Souteyrand, Corinne Marie, Marie Pastor, Attila Sebe, Csaba Miskey, Laurence D. Hurst, Sabine Diarra, Martina Kropp, Peter Walter, Daniel Scherman, Zoltán Ivics, Zsuzsanna Izsvák, Sandra Johnen
Year: 2017
From page: 302
To page: 314
ISSN: 2162-2531
Volume: 6
Article title: Engineering of PEDF-Expressing Primary Pigment Epithelial Cells by the SB Transposon System Delivered by pFAR4 Plasmids

I would like to use: Figure(s)

Quantity of material: Reuse of 2 units of the data set of transfected primary human RPE cells (summary of 27 single experiments). Extraction of 2 Western blot images shown in Figure S4 A, lanes 7 & 8. Also for the data shown in the Figures 4, 5, and 6, we have extracted the results from one experiment out of a set of 27.
I am the author of the Elsevier material: Yes
Involvement: Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing – Review & Editing

In what format will you use the material: Print and Electronic
Translation: No

Proposed use: Reuse in a journal/magazine

Publisher of new work: JoVE
Title of new work: Electroporation-Based Genetic Modification of Primary Human Pigment Epithelial Cells Using the Sleeping Beauty Transposon System

Authors of new work: Sandra Johnen, Nina Harmening, Zsuzsanna Izsvák, Zoltán Ivics, Peter Walter, Gabriele Thumann
Material can be extracted: No

Additional Comments / Information:
