Lentiviral mediated RPE65 gene transfer in healthy hiPSCs-derived retinal pigment epithelial cells markedly increased RPE65 mRNA, but modestly protein level

Florian Udry¹, Sarah Decembrini¹-4, David M. Gamm², Nicole Déglon³, Corinne Kostic² & Yvan Arsenijević¹✉

The retinal pigment epithelium (RPE) is a monolayer of cobblestone-like epithelial cells that accomplishes critical functions for the retina. Several protocols have been published to differentiate pluripotent stem cells into RPE cells suitable for disease modelling and therapy development. In our study, the RPE identity of human induced pluripotent stem cell (hiPSC)-derived RPE (iRPE) was extensively characterized, and then used to test a lentiviral-mediated RPE65 gene augmentation therapy. A dose study of the lentiviral vector revealed a dose-dependent effect of the vector on RPE65 mRNA levels. A marked increase of the RPE65 mRNA was also observed in the iRPE (100-fold) as well as in an experimental set with RPE derived from another hiPSC source and from foetal human RPE. Although iRPE displayed features close to bona fide RPE, no or a modest increase of the RPE65 protein level was observed depending on the protein detection method. Similar results were observed with the two other cell lines. The mechanism of RPE65 protein regulation remains to be elucidated, but the current work suggests that high vector expression will not produce an excess of the normal RPE65 protein level.

Lying between the outer retina and the choroidal vessels, the retinal pigment epithelium (RPE) fulfils many crucial tasks for visual function such as phagocytosis, 11-cis retinal recycling, ion and water transports, and growth factor secretion. Deficiencies in RPE functions give rise to various diseases such as Leber congenital amaurosis (LCA) or retinitis pigmentosa, most of which result in drastic visual impairments or blindness. The generation of RPE in vitro is particularly valuable to understand pathophysiological mechanisms through disease modelling and to help answer the major challenge of developing therapies.

Numerous protocols to differentiate embryonic or induced-pluripotent stem cells (ESCs or iPSCs, respectively) have been published, providing researchers with a renewable source of RPE. Thanks to the protocols developed, the therapeutic potential of stem cell-derived RPE cells has been explored by transplantation in RPE degenerative diseases, first in animal models, and more recently in human patients suffering from age-related macular degeneration (AMD) or Stargardt’s disease.

In addition, iPSC-derived RPE (iRPE) has been used as an in vitro model for Best disease and choroideremia. It has proven to be an interesting alternative model for some diseases such as AMD, where no animal recapitulates all the cellular features of human AMD.

Worthy of note, more and more genetic diseases are tackled with a gene therapy approach and iRPE has also allowed to test the efficacy of adeno-associated virus (AAV)- or CRISPR-mediated gene correction.
Moreover, in many diseases that are candidates for gene augmentation therapy, it is not clear how many vector copies are necessary to re-establish a physiological level of gene expression. For instance, AAV-mediated gene augmentation therapy on RPE65-deficient dogs showed a significant improvement in visual function that appeared on electroretinogram readings, but this success could not be reproduced on LCA2 patients with similar doses. Therefore, studying the vector dose-response relationship in human iRPE may help to better estimate the vector dose required to attain optimal rescue in human in vivo.

In our study, we evaluated the suitability of iRPE cells as an in vitro model for lentiviral gene therapy, first through an extensive characterization of RPE features, followed by testing different lentiviral constructs on iRPE cells. We subjected healthy iRPE to an RPE65 gene augmentation therapy previously tested on RPE65 deficiency mouse models and healthy non-human primates to study RPE65 gene and protein expression.

Results

From hiPSCs to RPE. Differentiation protocol. We first tested 3 hiPSC lines for their potency to generate iRPE: two of them produced 10 to 50 pigmented foci during the differentiation protocol, whereas the one described by Singh and colleagues produced 50 to 200 foci. We thus decided to focus the iRPE characterization on this hiPSC line. The differentiation protocol was adapted from Singh et al., recapitulating its main steps with modifications in time points (plating at day 1) and minor changes in the media (addition of embryoid body media – EBM and retinal differentiation media cum vitamin A – RDMcA) (Fig. 1a). Human iPSCs were lifted and placed in free-floating condition to form embryoid body-like aggregates (EBs) that were plated one day later to expand (Fig. 1c) in Neural Induction Medium (NIM) which contains a high concentration of insulin potentially mimicking IGF1 action. The medium was switched 9 days later to a vitamin A-free RDM to prevent the neural retina fate that may arise detrimentally to RPE differentiation due to the trans-differentiation ability of RPE during development.

Pigmented foci (Fig. 1d,e) appeared around day 18-24 and were collected around day 30. Optic vesicle-like structures can also be observed (e, arrowheads). Plated PFs develop pigmented patches (PPs) that are collected, dissociated and seeded to obtain the pure hiPSC-derived RPE (iRPE) cell monolayer.

Expression of RPE gene markers. In order to confirm the RPE identity of the iRPE, we investigated the RPE gene markers' expression compared to iPSCs and cultured foetal RPE (fRPE). The RT-qPCR results (Fig. 2a) showed...
a marked and significant upregulation of RPE65, MITF-H (RPE expressed isoform H), PMEL, DCT and BEST1 gene expression in iRPE compared to iPSCs while there was no significant difference for KLF4, EZR, MERTK and MITF-M (melanocyte specific isoform M). A significant downregulation for the pluripotency markers POU5F1-A and NANOG was observed. Nonetheless, the iRPE was still positive for these markers (<30 Cq).

Surprisingly, a downregulation of LRAT expression in iRPE compared to iPSCs was observed whereas an increase similar to RPE65 was expected, as both are involved in the visual cycle. Nonetheless LRAT mRNA expression can be considered positive in iRPE (<30 Cq).

Despite this unexpected result, the iRPE showed an expression pattern close to fRPE with a strong significant correlation (R = 0.902) for the 10 genes investigated (Supp. Fig. 1b). Note that LRAT, EZR and MERTK in fRPE have similar expression levels in comparison to iRPE.

**Figure 2.** Expression of RPE markers at mRNA and protein levels. (a) Quantitative PCR investigating RPE marker expression in fRPE (n = 3) and iRPE (n = 6) normalized to iPSC (n = 3). Bars represent mean ± SD. Kruskal-Wallis tests were performed (iPS vs. iRPE and iPS vs. fRPE for each gene) followed by Dunn’s multiple comparisons test. Significance threshold: *< 0.05; **< 0.01. (b,e) Z-stack Maximum Intensity Projection of iRPE cells labelled with anti-BEST1 and -RPE65 antibodies, respectively. (c,d) iRPE cells immunolabelled for OTX2. (f–i) Z-stacks acquired over the whole iRPE cell monolayers to investigate RPE marker localizations. Orthogonal view confirmed the preferential basolateral or apical distributions of BEST1 (f) and ZO-1 proteins (g), respectively. RPE65 and CRALBP localizations were widespread in iRPE cells (h,i). (j) Violin plots corresponding to images (f–i) showing the distribution of the labelled proteins and DAPI staining throughout the Z-stacks. Images are representative of 1-2 observations per marker in at least 3 iRPE lines.
At the protein level, the RPE-specific proteins RPE65, RLBP1 (also known as CRALBP), BEST1, TJP1 (also known as ZO-1) and OTX2 were all expressed although with some heterogeneity (Fig. 1b–i). NANOG was not expressed. However, a few POU5F1-A positive cells were observed (Supp. Fig. 2). Quantification of OTX2 positive cells (Supp. Fig. 2) showed that more than 94% of cells express OTX2 confirming a homogeneous RPE identity. Moreover, the polarization of some critical proteins was confirmed by confocal microscopy, particularly the apical distribution of ZO-1 and the basolateral presence of BEST1 as assessed by orthogonal view (Fig. 1f–i). In order to better visualize the protein distribution in the cell in the Z-axis, the mean grey value of each Z-stack of Fig. 1f–i was measured to present the Z-profile of both the nucleus (labelled by DAPI) and the protein of interest (Fig. 1j).

As a comparison, fRPE showed similar staining and protein localization (Supp. Fig. 3). However, after cell passages, the cells lose progressively their identity (Supp. Fig. 7). The iRPE coming from replicated differentiation experiment displayed consistent results (Supp. Fig. 4).

**Cell ultrastructure analysis reveals polarized distributions of certain organelles.** Vertical sections of iRPE cells were imaged by electron microscopy to reveal their ultrastructure (Fig. 3a). The presence of tight junctions between iRPE cells (Fig. 3b,c), apical microvilli (Fig. 3d), as well as a basal nucleus and melanosomes at different stages of maturation were observed. Nu, Nucleus; Ph, Phagosome; M, Mitochondria.

**Differentiated iRPE form an epithelium with high cohesion.** As part of the blood retinal barrier, RPE cells need to form a functional epithelium that can regulate ion, molecule and protein transport. By measuring the transepithelial resistance, we sought to investigate the epithelial integrity. The iRPE monolayer showed an increasing transepithelial resistance in parallel with the number of days in culture (Fig. 4a) with a mean transepithelial resistance of around 300 Ohm cm² at day 42 (when characterization is performed), which is in line with previous publications on iRPE and above the functional minimum required as stated by others. Foetal RPE displayed a limited transepithelial resistance reaching approximately 150 Ohm cm² at day 42, confirming results obtained by Takahashi’s lab with fRPE from the same source. Foetal RPE from a different source reached a higher transepithelial resistance, suggesting heterogeneity in iRPE lines that may stem from the age of the donor, isolation process or culture conditions.

**VEGF is preferentially secreted at the basal iRPE side.** Factor secretion is another important feature of RPE cells. Many proteins have been shown to be secreted by the RPE such as the vascular endothelial or pigment epithelium growth factor (VEGF and PEDF, respectively). To determine the secretive ability of the iRPE, the levels of both VEGF and PEDF were measured by ELISA (Fig. 4b) in the medium of the apical and basal compartments of transwells 24 hr after changing the medium. VEGF was preferentially secreted towards the basal compartments of the transwell, which corresponds to the choroid in vivo, as expected from previous publications on stem cell-derived
RPE14,38,41–43 or adult human RPE44. On the other hand, although PEDF secretion level was comparable to previous reports14,36,38,43, it was not observed in a polarized fashion.

**Figure 4.** After maturation, iRPE cells present typical biological functions of RPE cells. (a) TER was measured on iRPE and fRPE monolayers at 14, 28 and 42 days in culture on transwell. Bars represents mean ± SD. (b) PEDF and VEGF secretion levels were measured by ELISA after 42 days in culture for both apical (blue) and basal (grey) compartments of n = 5 iRPE lines. Comparison between compartments was done by paired Wilcoxon match-pairs signed-rank test. Bars represent mean ± SD. (c–f) FITC-conjugated POS were fed to iRPE and fRPE cells for 16 h and the RHODOPSIN protein was immunolabelled without permeabilization (red labelling). As a result, the internalized POS fluoresce in green only, and microvilli-bound POS fluoresce in green and red. *p*-value. *p*-value significance threshold for all tests: 0.05.

**RPE cells phagocytize bovine photoreceptor outer segments.** In vivo, the RPE phagocytizes debris coming from the daily partial renewal of photoreceptor outer segments (POS)45. The internalization mechanism of the POS has been identified and it has been shown that the integrins involved in POS internalization are highly conserved between bovine and human46, allowing to test this particular pathway and not only observe passive uptake of the POS. To assess the iRPE phagocytic capacity, we fed fRPE and iRPE with 5 million bovine POS over 16 hr and performed the same treatment with fRPE in parallel as a comparison point. Both fRPE and iRPE were able to phagocytize bovine POS (Fig. 4c–f), although POS uptake was heterogeneous.
Lentiviral-mediated transduction of iRPE cells. Lentiviral vectors (LV) are suitable vectors to efficiently deliver a transgene in the RPE47,48 and to carry long construct containing either long transgene or complex regulatory sequences49,50. We thus tested LV in the human iRPE to evaluate the bona fide RPE identity of the iRPE as well as confirm the tropism of the lentiviral vector for the human RPE as observed in vivo in rodent24,47. We thus transduced iRPE and fRPE with 6 different lentiviral vectors (Fig. 5a) each bearing a construct to express GFP driven by different promoters: either RPE-specific (RPE65 short sequence: R0.8)20, ubiquitous (EFS) or specific for glial (GFAP) or neural (SYNAPSIN and rat NSE) promoter. iRPE and fRPE cells were transduced using these vectors and the proportion of GFP-positive iRPE and fRPE cells was determined by FACS. In addition, the relative lentiviral genome copy number was quantified by qPCR. (b,c) EFS and R0.8 promoters showed a significant positive correlation between the percentage of GFP-positive cells (iRPE and fRPE together) and the relative lentiviral genome copy number whereas none of the aspecific promoters for RPE (d–g) did. R²: coefficient of determination of the linear regression. p: p-value from the F-test under the assumption of a null slope. P-value significance threshold: 0.05.
photoreceptor or neuronal cells (GFAP, RHO, SYN1 and rat Eno2 respectively). To ensure that the lentiviral vectors transduced the cells, we determined by qPCR the lentiviral genome copy number relative to the iPPE or iRPE ACTB gene copies. All the vectors successfully transduced the cell, as revealed by the positive qPCR for the lentiviral genome amplification. To assess the activity of the transduced promoters, the percentage of GFP<sup>+</sup> cells was linearly regressed to the lentiviral genome relative copy number. The ubiquitous EFS and specific R0.8 promoters showed a substantial prediction of the percentage of GFP<sup>+</sup> cells by the lentiviral genome relative copy number (Fig. 5b,c) whereas none of the nonspecific promoters for iPPE did, as assessed by the R<sup>2</sup> coefficient of determination. In addition, only the EFS and R0.8 promoters presented significantly positive slopes of the best-fit line equations.

**RPE65 gene augmentation by lentiviral gene therapy.** Finally, we investigated the response of the iPPE to a RPE65 gene augmentation treatment that has shown rod and cone function restoration in mouse models of Rpe65 deficiency<sup>3,4</sup> and is also manageable to use in non-human primate retina<sup>25</sup>. We transduced iPPE cells with a multiplicity of infection (MOI) of 1 or 5 and measured RPE65 mRNA level and the lentiviral genome relative copy number by qPCR five days later (Fig. 6a). The RPE65 mRNA level was regressed against the lentiviral genome relative copy number (Fig. 6b), revealing a substantial prediction of the mRNA level by the lentiviral genome relative copy number. The expression of RPE65 mRNA was significantly upregulated after the gene augmentation treatment for both MOI 1 and 5 compared to untreated control (35.00 ± 21.27 and 137.34 ± 95.22 times more RPE65 mRNA compared to control, respectively; mean ± SD; Fig. 6c). The RPE65 protein level was also investigated by fluorescence immunoblotting densitometry (Fig. 6d,e) for MOI 5-transduced iPPE and showed an upregulation that was not significant (1.55 ± 0.63-fold of Ctrl, mean ± SD).

The same experiment was repeated with two other RPE lines, namely fRPE and iCell RPE line, a stem cell-derived RPE line commercially available. Instead of 5 days, RPE cells were collected 14 days post-transduction to leave more time for the transgene to be expressed (day 42).

Both iCell RPE and our iPPE line transduced with the RPE65 gene augmentation therapy presented a significant upregulation of RPE65 mRNA expression compared to their Ctrl (71.58 ± 32.63 and 7.94 ± 1.51-fold upregulation respectively; mean ± SD, Fig. 7a). fRPE showed a high but non-significant upregulation of RPE65 mRNA expression (280.73 ± 274.09-fold of Ctrl) probably due to its very low expression in the non-transduced Ctrl iPPE cells. While fRPE cells were in characterization experiments, they were P5 in this experiment and their characterization at the time of transduction (Supp. Fig. 7) suggests a certain loss of RPE identity at this higher passage. Although all RPE markers tested were expressed, their expression was substantially lower than for our iPPE or the iCell RPE (Supp. Fig. 7a). In addition, immunohistochemistry (Supp. Fig. 7b) showed an absence of GSTP1 protein, partial OTX2 expression and actin stress fibres that have been linked to RPE identity loss<sup>51</sup>. At the protein level, only the iPPE presented an upregulation (modest) of RPE65 by ELISA (1.17 ± 0.03-fold of Ctrl, 114.5 ± 6.11 vs 133.9 ± 3.56 pg/µg of total protein, Fig. 7b) and Western blotting (2.55 ± 0.66-fold of Ctrl Fig. 7c,d) whereas no upregulation was observed for iCell RPE (ELISA: 1.01 ± 0.06-fold of Ctrl, 118.7 ± 6.87 vs 120.5 ± 8.11 pg/µg of total protein; WB: 2.26 ± 1.67-fold of Ctrl) and fRPE (ELISA: 1.04 ± 0.05-fold of Ctrl, 99.9 ± 5.98 vs 103.9 ± 5.6 pg/µg of total protein). Worthy of note, RPE65 protein could not be quantified in fRPE due to a weak signal out of the range of the standard curve, which is consistent with the characterization results discussed previously. Although the outcomes were consistent between ELISA and WB, no correlation in RPE65 quantification was found between the two methods (R<sup>2</sup>=0.167, p-value = 0.116, n = 16).

**Discussion**

In this study, we modified a protocol to differentiate hiPSC into RPE and performed an extensive characterization of the resulting iPPE to validate its RPE identity. The majority of RPE marker genes investigated showed a significant upregulation after differentiation, whereas MERTK and EZR, two genes involved in the phagocytosis of POS<sup>52,53</sup> did not. Several publications confirmed MERTK expression in ESC-derived RPE or iPPE by conventional PCR, while only a few investigated the MERTK expression level by qPCR. However, none compared iPPE and iPSC MERTK expression<sup>4,7,44</sup>. Only Liao and colleagues<sup>54</sup> showed a conserved MERTK level after differentiation by microarray transcriptome analysis, suggesting that MERTK mRNA expression may play a role in pluripotent cells. Similarly, most publications investigated EZR protein expression by immunohistochemical staining, and none by RT-qPCR. Transcriptomic and proteomic stem cell data from repositories (respectively StemMapper<sup>55</sup>, and PepTracker<sup>56,57</sup>) confirmed EZR expression in stem cells suggesting also a role of this gene in the pluripotent cells function. Although little differences of gene expression for MERTK and EZR were observed, the mRNAs levels of these genes were substantial revealing, along with the other markers, a good differentiation of the hiPSC into RPE.

*In vivo,* the RPE is responsible for recycling the 11-cis retinal chromophore through the visual cycle, in which RPE65 and LRAT proteins are involved. While RPE65 expression was upregulated, LRAT exhibited a down-regulation in iPPE compared to iPSCs. To our knowledge, no publication investigated LRAT mRNA level by qPCR in iPPE compared to iPSCs, however conventional PCR results from most studies suggest a weak LRAT expression in iPPE and ESC-derived RPE<sup>15,38,49</sup>. In a previous work using RT-qPCR, LRAT expression was not detected in the iPSC whereas it was upregulated during RPE differentiation<sup>49</sup>. This different result in comparison to the present work may be related to the sensitivity of the method used and/or stem cell line used to derive RPE. Transcriptomic and proteomic data respectively from StemMapper and PepTracker<sup>®</sup> confirm LRAT expression in iPPE in stem cells. When examining the relative expression in iPSCs, it is interesting to note that LRAT is more expressed than KLF4 (Supp. Fig. 1a), a result that needs confirmation in other iPSCs lines. Thus, similarly to MERTK and EZR, LRAT upregulation in iPPE compared to iPSCs may not be indicative of RPE functions. A functional assay investigating the chromophore recycling capability of iPPE as performed by Maeda and colleagues<sup>49</sup> could confirm this hypothesis. Moreover, previous reports have shown that the LRAT protein is 25'000-fold more active<sup>41</sup> than the RPE65 one, supporting the hypothesis that LRAT does not need to be highly expressed to perform its
function, in contrast to RPE65. Although LRAT expression is positive, but weak, one cannot exclude that our culture conditions are not optimal to promote the full maturation of the iRPE. Carr and colleagues have shown that iRPE in contact with retinal explant have an increased microvilli length, suggesting an adaptation of iRPE to the culture environment.

Regarding the expression of MITF-M, a melanocyte-specific isoform of MITF, an upregulation in iRPE compared to iPSCs was observed. Previous publications showed no expression of MITF-M in human immortalized RPE cell lines. However, our results suggest that MITF-M expression in iRPE and IRPE is possible, although at a very low level, confirming results obtained by Maruotti and colleagues. In comparison, the MITF-H isoform that is well expressed in RPE is approximately 300 times more expressed than MITF-M in our iRPE (Supp. Fig. 1a).
Lying at the interface between the choroid and the retina, the RPE helps to maintain the homeostasis of both tissues and is therefore a highly polarized epithelium. Immunohistochemical analyses showed a polarized distribution of some RPE marker proteins, as well as a polarized ultrastructure by electron microscopy. The iRPE displayed a tendency for a polarized secretion of VEGF but not PEDF, unlike previous reports have shown. The difference in PEDF secretion polarization with other works on iRPE and ESC-derived RPE may originate from the culture conditions. Indeed, Kamao and colleagues cultured their iRPE on type I-A collagen that contains a PEDF binding site which could potentially buffer PEDF and limit its secretion to the basal compartment resulting in a polarized secretion of PEDF towards the apical compartment. Our iRPE were cultured on MRF that does not contain type I but type IV collagen to which PEDF does not bind. The polarized secretion of PEDF by the RPE in vivo may also be the result of PEDF buffering by the Bruch’s membrane, composed of collagens type I and III, among many other proteins. However, other publications reported a polarized secretion of PEDF on substrate without type I collagen, suggesting that the RPE inherently secretes PEDF in a polarized manner. Interestingly, Singh and colleagues observed a polarized secretion with the same culture medium used in this study suggesting that the differentiation process may be responsible for this discrepancy. Improvement of the differentiation protocol by the addition of small molecules or proteins, such as Activin A, may help to promote a better differentiation and polarization. Moreover, supplementation of the culture medium with ROCK inhibitor has shown significant results on F-actin stress fibre morphology of RPE cells and could be beneficial to iRPE polarization.

Overall, the iRPE displayed many features of bona fide RPE. Its RPE identity was further confirmed by the transduction of lentiviral GFP reporter constructs bearing RPE specific promoters or promoters specific for other cell types. As the differentiation protocol we used promotes a neural and then retinal fate, we tested promoters of lineages that could have arisen during differentiation, namely glia (GFAP), retina (RHO), and neuronal (SYN1, rat Eno2) specific promoters. Notably, only the specific RPE65 and ubiquitous EFS promoters drove GFP expression and no leakiness from aspecific promoters for RPE was observed.
Finally, we transduced iRPE cells differentiated from healthy iPSCs with an RPE65 gene augmentation approach previously tested on RPE65 deficiency mouse models and healthy non-human primates. Interestingly, the RPE65 mRNA level could be substantially increased despite the iRPE already expressing RPE65 and the use of a partial RPE65 promoter. However, no marked increase of the protein level was observed. This experiment was repeated with two other RPE models, namely iRPE and iCell RPE cells (commercially available stem cell-derived RPE), and with a longer transgene expression period (5 vs 14 days). A significant upregulation of RPE65 mRNA expression was observed in iRPE and iCell RPE transduced cells, although the magnitude of the increase was lower in iRPE than in previous results for the same MOI (~10-fold vs ~130-fold). At the protein level, a significant but modest increase in RPE65 was observed by Western blotting and ELISA for the iRPE (in spite of the lower RPE65 mRNA upregulation), but none for the iCell RPE and iRPE. The longer transgene expression period might have helped to reach a significant RPE65 protein upregulation in iRPE although the magnitude in the increase remains modest, confirming a discrepancy between RPE65 mRNA and protein level increase. In total, three different batches of lentivirus were used (two for Fig. 6 and one for Fig. 7) which may explain the heterogeneity observed in RPE65 mRNA level expression post-transduction in iRPE. In addition, heterogeneity between iRPE batches may also be involved, although their characterization showed consistent results.

The difference of magnitude between the mRNA and protein upregulation in all 3 RPE models suggests a potential post-transcriptional regulation mechanism that has yet to be elucidated. Although inhibitory translational regulation of the RPE65 mRNA by miRNA has been described, this regulation was mediated by the 3' UTR that is absent from the lentiviral construct containing only the RPE65 coding sequence. To our knowledge, no other publication reported post-transcriptional regulation mechanisms of RPE65. This mechanism of regulation may be of great interest for gene augmentation therapy since it suggests no potential toxic effects due to high gene doses. Nonetheless, such observations need to be confirmed in iRPE deficient for RPE65 or expressing mutated forms of the protein.

Several clinical trials have been conducted to treat LCA2 caused by RPE65 deficiency, one of which led to the first AAV-based gene therapy to reach the market. Although AAV-mediated RPE65 gene augmentation therapy showed substantial vision improvements in the patients, none proved to prevent the progression of the photoreceptor degeneration observed, contrasting with LCA2 animal model results. This discrepancy may originate from the different levels of RPE65 protein needed to perform physiological functions between animals and human beings, which suggests that a higher concentration of RPE65 is necessary to provide an optimal rescue in human. Bainbridge and colleagues observed a limiting dose of 10^12 vector genomes inducing no toxicity, therefore precluding from using higher amounts to achieve higher RPE65 protein expression, and creating the need for a more potent gene therapy. Georgiadis and colleagues developed an AAV-based optimized RPE65 (OPTIRPE65) gene augmentation therapy 300 times more potent than their original work and were able to reach a plateau of rescue with only 10^8 vector genomes in the Rpe65 deficiency mouse model. Interestingly, they tested their OPTIRPE65 on wild-type mice they observed physiological expression of the RPE65 transgene, meaning a doubling of the overall cDNA Rpe65 expression. Although comparison with our in vitro results is tricky due to differences in vector doses, vector biodistribution, model (endogenous RPE vs iPSC-derived) and methodology (absolute vs relative mRNA quantification), the significant increase in RPE65 mRNA level (up to 135-fold at MOI 5) suggests a high potential of the LV-based gene therapy. Further studies on the RPE65-deficient iRPE model are needed to confirm that this strategy is able to rescue RPE65 mRNA and protein to a physiological level, and which vector dose is needed to attain such concentration.

Lentiviral vectors have long been used in gene therapy, but their use has mostly been restricted to ex vivo gene therapy in human. Studies of lentiviral integration revealed that more than 60% of the insertions occur in transcription regions, but rarely in promoter sequences, reducing the risk of tumorigenesis as the disruption of a promoter sequence generally impacts more genes than the disruption of a transcription region. Moreover, RPE is a post-mitotic tissue and adenomas of the RPE are seldom while adenocarcinomas are even rarer, suggesting the RPE is a safer target for LV integration than most tissues. In addition to safety studies in non-human primate, studying the safety of lentiviral-mediated gene therapy in vivo is expected to knockdown the RPE65 gene is an important preclinical step to elucidate untoward effect occurring in this cell type before considering its translation to clinic.

Conclusions and future directions. In conclusion, we modified an RPE differentiation protocol that produces iRPE bearing close features to bona fide RPE. The efficiency of this differentiation protocol should still be validated in other iPSC lines. The iRPE revealed to be a suitable in vitro model for lentiviral gene therapy tests after transduction of several different constructs, confirming the lentivirus tropism for RPE previously observed in vivo. RPE65 gene augmentation therapy in iRPE cells resulted in high RPE65 mRNA expression corroborating previous results obtained in animal models, but only elicited a modest RPE65 protein increase. Future directions include the production of an RPE65-deficient iRPE model to further test the lentiviral vector gene therapy and demonstrate its safety and efficacy in human cells.

Methods
RPE differentiation protocol and iRPE culture. A detailed protocol is available in Supplementary Methods. Briefly, hiPSCs described in Singh et al. were cultured in feeder-free conditions in either mTeSR-1 or E8 media kit (StemCell) on Matrigel Embryonic Stem Cell grade (Corning). hiPSC clumps (between P18 and P30) were lifted using type IV collagenase (LifeTechnologies) and cultured overnight in free floating conditions in Embryoid Body Medium (EBM, see SM for composition). Resulting Embryoid Body-like aggregates (EBs) were plated on Matrigel Reduced Factor (MRF, Corning) in a neural induction medium (NIM also described in SM) changed every 3 days. On day 10, medium was switched to a Retinal Differentiation Medium with vitamin A (RDMa). On day 13, medium was switched to a version without vitamin A (RDMsa). Pigmented foci (PF)
RtEGM® Bulletkit® (Ref. 00195409, Lonza). Human iCell induced pluripotent stem cell-derived RPE (iCell RPE) cells were cultured on MRF-coated transwells (CLS3460, Sigma-Aldrich) for 42 days in RDMsA (unless stated otherwise). All tests were performed with the same iRPE or iCell RPE line and, for iRPE, several maturation tests were done independently.

RNA extraction and analysis of gene expression. RNA isolation was performed using TRI Reagent® (T9424, Sigma-Aldrich) according to the manufacturer’s protocol. Retro-transcription PCR was performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Ref. 4368814) according to manufacturer’s protocol with a Biometra Tadvanced thermocycler from Analytik Jena AG. For each RNA sample, a negative control lacking the retrotranscriptase in the retrotranscription step was used in qPCR. Gene expression was assessed by qPCR using the LightCycler®96 and the FastStart Essential DNA Green Master mix (Ref. 06402712001) from Roche. Experimental design and gene expression quantification are explained in SM. **ATP5F1B, SRP72 and GPP** reference genes were chosen after having reviewed literature42,84 and tested their stability in iPS, IRPE, iRPE and iCell RPE. Primer sequences, primer efficiencies, qPCR programmes and additional information on qPCR data processing are available in SM.

Immunohistochemistry and imaging. iRPE, iRPE and iCell RPE cells were cultured on 12 mm diameter transwells, (CLS3460, Sigma-Aldrich) coated with MRF (at a 1:30 dilution) in RDMsA for 42 days (unless stated otherwise) before processing for immunohistochemistry (see SM for detailed protocol). iPS on coverslips were processed for immunohistochemistry at around 60% confluence. Briefly, cells were fixed in 4% paraformaldehyde for 10-20 min at room temperature (RT), rinsed with PBS and blocked for 3-5 h in 5% foetal bovine serum + 5% normal goat serum + 0.1% Triton X-100 in PBS 1×. Primary antibody was incubated overnight at 4 °C in blocking solution (see SM for antibody references and dilutions). Primary antibody was washed with PBS 1X before secondary antibody incubation (see SM) 1 h at RT in PBS 1×. After wash with PBS 1×, the transwell membrane was cut and mounted in Mowiol. Z-stacks were acquired using a LSM700 confocal microscope from Zeiss at the EPFL Bioimaging & Optics Core Facility (EPFL-BIOP). Other images were acquired with a BX60 microscope from Olympus equipped with a DP72 camera or a Leica DM6B microscope equipped with a DFC9000GT camera.

Electron microscopy. iRPE cells cultured on 6.5 mm transwells (CLS3470, Sigma-Aldrich) for 45 days were washed with PBS 1×, fixed in 2.5% glutaraldehyde for 90 min and left in PBS 1X until processing and imaging by the Electron Microscopy Facility of the University of Lausanne (EMF-UNIL). Details of processing and acquisition are available in SM.

Transepithelial resistance measurements. Transepithelial resistance measurements were performed with an EVOM2 Epithelial Voltohmmeter (World Precision Instrument) on iRPE and iRPE cells grown on 12 mm diameter transwells. One transwell with only MRF coating was used as control. Transepithelial resistance was determined as the mean of three iRPE/iRPE transwell resistance measurements minus the control resistance times the transwell surface (1.12 cm²).

POS isolation and phagocytosis assay. Photoreceptor outer segments (POS) isolation and phagocytosis assay protocol were adapted from88 (see SM for detailed protocol). Briefly, fresh bovine eyes were collected from a local slaughterhouse and dissected in dim red light. Retinas were collected in a pre-chilled homogenization Tris buffer-based solution and ultraconcentration was performed in a sucrose gradient (113’000 g during 48 min at 4 °C). The resulting pink band - characteristic of rhodopsin - was collected and washed three times. POS concentration was determined using a Neubauer chamber and aliquots of 5·10⁶ POS were stored at -80 °C until use. Thawed POS were incubated with an FITC solution to obtain FITC-conjugated POS and fed to iRPE or IRPE cells for 16 hours (5·10⁶ POS/transwell). iRPE or IRPE cells were washed to remove the non-internalized and unbound POS, immunolabelled for rhodopsin without the permeabilization step, and fixed in 4% paraformaldehyde before mounting on microscope slides for imaging.

PEDF and VEGF quantifications. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) concentrations in medium were quantified using ELISA kits (RD191114200R from Bivendor, Brno, Czech Republic and RAB0507 from Sigma-Aldrich, respectively). iRPE cells cultured on MRF-coated transwells for 42 days were rinsed with HBSS twice and fresh RDMsA was added. Twenty-four hours later, the RDMsA was collected from the apical and basal chambers and stored at -80 °C. ELISAs were performed maximum one week after sample collection. For each iRPE cell line tested, triplicate samples (i.e. iRPE cell wells) were quantified in ELISA technical duplicates (i.e. ELISA wells). Mean of triplicate samples was calculated and Wilcoxon match-pairs signed-rank tests comparing contents of basal and apical chambers for VEGF and PEDF were performed using GraphPad Prism 6 software.
Lentiviral vector production and titration. Lentiviral vector and titration protocols have already been described in23, however detailed protocols are available in SM. Briefly, HEK293T cells were transiently transfected with a second generation lentiviral packaging system, culture supernatants were collected two days later and underwent two rounds of ultracentrifugation (70’000 g, 90 min, 4°C) to wash and pellet the lentivirus that was resuspended, aliquoted and stored at -80°C until use.

Lentiviral transduction of iRPE, iCell RPE and fRPE cells with GFP constructs driven by different promoters. The 6 different lentiviral constructs were originally designed in the following publications: hLox-Ro.8-GFP-WPRE33, hLox-EGFP-WPRE85, hLox-GEAP-GFP-WPRE (Unpublished – details in SM), hLox-Rhodopsin-GFP-WPRE85, SIN-ratNSE-AcGFPMuc-WPRE86, and SIN-Synapsin-GFP-WPRE (Unpublished – details in SM). iRPE and fRPE cells were cultured on transwells for 42 days and transduced with one of the aforementioned lentiviral vectors at a multiplicity of infection (MOI) of 1. Five days later, iRPE and fRPE cells were collected, fixed and one part of the cells were analyzed at the Flow Cytometry Facility of the University of Lausanne (on a BD FACSCalibur® cell analyser, BD Biosciences) to determine the proportion of GFP-positive cells while genomic DNA (gDNA) was extracted from the other part to quantify lentiviral genome relative copy number by qPCR (details available in SM).

Lentiviral-mediated RPE65 augmentation therapy. The Good Manufacturing Practices (GMP)-compliant construct pCCL-R0.8-RPE65-WPRE425 was used to produce lentivirus delivering the RPE65 protein cDNA under the control of the partial RPE65 promoter R0.8. RPE cells were transduced with this lentivirus at targeted MOI of 1 and 5. Five (Fig. 6) or 14 (Fig. 7) days later, RPE cells were collected, RNA and gDNA extracted using TRI reagent® and protein isolated using a custom lysis buffer (details available in SM). RPE65 mRNA expression and lentiviral genome relative copy number were determined by qPCR (details available in SM).

RPE65 protein level was determined by an RPE65 ELISA kit according to the manufacturer’s protocol (XPEH2516, XpressBio, see SM for details) or Western blotting. For Western blotting, 40 to 50 µg total protein lysates were run on a 10% acrylamide gel, transferred to a PVDF membrane, the membrane was then blocked in Odyssey® TBS Blocking Buffer (P/N 927-50100, Licor), incubated overnight at RT with RPE65 and α-Actin or GAPDH primary antibodies, washed in TBS containing 0.1% Tween-20, incubated with near infra-red (680 nm and 800 nm emitting) conjugated fluorophore secondary antibodies and washed again. Blot imaging was performed with an Azure c600 imaging system and densitometry analysis was performed with the Image Studio Lite software from Licor. Detailed Western blotting procedure is available in SM as well as original blots in Supp. Fig. 6.

Statistical analysis. All statistical analyses were performed and graphs realized with Graphpad Prism 6 software. The number of biological replicates is stated on figures and in their caption, as well as statistical tests used and the p-value significance level. For the characterization experiments, one biological replicate corresponds to one iRPE line differentiated from hiPSCs. For RPE65 gene augmentation therapy experiments, one biological replicate corresponds to one transwell.

Data availability
The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Received: 20 May 2019; Accepted: 8 May 2020;
Published online: 01 June 2020

References
1. Strauss, O. The Retinal Pigment Epithelium in Visual Function. Physiol. Rev. 85, 845–881 (2005).
2. den Hollander, A. I., Roepman, R., Koenekoop, R. K. & Cremers, F. P. M. Leber congenital amaurosis: Genes, proteins and disease mechanisms. Prog. Retin. Eye Res. 27, 391–419 (2008).
3. Hartong, D. T., Berson, E. L. & Dryja, T. P. Retinitis pigmentosa Prevalence and inheritance patterns. Lancet 368, 1795–1809 (2006).
4. Leach, L. L. & Clegg, D. O. Concise Review: Making Stem Cells Retinal: Methods for Deriving Retinal Pigment Epithelium and Implications for Patients with Ocular Disease. Stem Cells 33, 2363–2373 (2015).
5. Vugler, A. et al. Elucidating the phenomenon of HESC-derived RPE: Anatomy of cell genesis, expansion and retinal transplantation. Exp. Neurol. 214, 347–361 (2008).
6. Meyer, J. S. et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc. Natl. Acad. Sci. 106, 16698–16703 (2009).
7. Haruta, M. et al. In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. Investig. Ophthalmol. Vis. Sci. 45, 1020–1025 (2004).
8. Soin, E. H. et al. Allogeneic iPSC-derived RPE cell transplants induce immune response in pigs: A pilot study. Sci. Rep. 5, 1–10 (2015).
9. Schwartz, S. D. et al. Embryonic stem cell trials for macular degeneration: A preliminary report. Lancet 379, 713–720 (2012).
10. Song, W. K. et al. Treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium: Preliminary results in Asian patients. Stem Cell Reports 4, 860–872 (2015).
11. Mandai, M. et al. Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration. N. Engl. J. Med. 376, 1038–1046 (2017).
12. Da Cruz, L. et al. Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. Nat. Biotechnol. 36, 1–10 (2018).
13. Schwartz, S. D. et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: Follow-up of two open-label phase 1/2 studies. Lancet 385, 509–516 (2015).
14. Singh, R. et al. Functional analysis of serially expanded human iPSC-derived RPE cultures. Investig. Ophthalmol. Vis. Sci. 54, 6767–6778 (2013).
15. Cereso, N. et al. Proof of concept for AAV2/5-mediated gene therapy in iPSC-derived retinal pigment epithelium of a choroideremia patient. Mol. Ther. - Methods Clin. Dev 1, 14011 (2014).
16. Galloway, C. A. et al. Drusen in patient-derived hiPSC-RPE models of macular dystrophies. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E8214–E8223 (2017).
17. Pennesi, M. E., Neuringer, M. & Courtney, R. J. Animal models of age related macular degeneration. *Mol. Aspects Med.* **33**, 487–509 (2012).
18. Li, Y. et al. Gene therapy in patient-specific stem cell lines and a preclinical model of retinitis pigmentosa with membrane frizzled-related protein defects. *Mol. Ther.** **22**, 1688–1697 (2014).
19. Le Meur, G. et al. Restoration of vision in RPE65-deficient Bliard dogs using an AAV serotype 4 vector that specifically targets the retinal pigmented epithelium. *Gene Ther.** **14**, 292–303 (2007).
20. Acland, G. M. et al. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol. Ther.** **12**, 1072–1082 (2005).
21. Annear, J. M. et al. Successful Gene Therapy in Older RPE65-Deficient Dogs. *Hum. Gene Ther.** **24**, 883–893 (2013).
22. Bainbridge, J. W. B. et al. Long-Term Effect of Gene Therapy on Leber's Congenital Amauropia. *N. Engl. J. Med.* **372**, 1887–1897 (2015).
23. Benemans, A. P. et al. Lentiviral gene transfer of RPE65 rescues survival and function of cones in a mouse model of leber congenital amaurosis. *Plos Med.* **3**, 1982–1903 (2006).
24. Kostic, C. et al. Gene therapy regenerates protein expression in cone photoreceptors in Rpe65R91W/R91W mice. *Plos One** **6** (2011).
25. Matet, A. et al. Evaluation of tolerance to lentiviral LV-RPE65 gene therapy vector after subretinal delivery in non-human primates. *Transl. Res.** **188**, 40–57, e4 (2017).
26. Nieto-Estevéz, V., Deferali, C. & Vicario-Abejón, C. IGF-I: A key growth factor that regulates neurogenesis and synaptogenesis from embryonic to adult stages of the brain. *Front. Neurosci.* **10**, 1–9 (2016).
27. Versteyhe, S. et al. IGF-I, IGF-II, and insulin stimulate different gene expression responses through binding to the IGF-I receptor. *Front. Endocrinol. (Lausanne)** **4**, 1–13 (2013).
28. Duester, G. Keeping an eye on retinoic acid signaling during eye development. *Chem. Biol. Interact.* **178**, 178–181 (2009).
29. Harpavat, S. & Cepko, C. L. Thyroid Hormone and Retinal Development: An Emerging Field. *Thyroid** **13**, 1013–1019 (2003).
30. Cvekl, A. & Wang, W. L. Retinoic acid signaling in mammalian eye development. *Exp. Eye Res.* **89**, 280–291 (2009).
31. Martinez-Morales, J. R., Rodrigo, I. & Bovolenta, P. Eye development: A view from the retina pigmented epithelium. *BioEssays** **26**, 766–777 (2004).
32. Klimanskaya, I. et al. Derivation and Comparative Assessment of Retinal Pigment Epithelium from Human Embryonic Stem Cells Using Transcriptomics. *Cloning Stem Cells** **6**, 217–245 (2004).
33. Idelson, M. et al. Directege Differentiation of Human Embryonic Stem Cells into Functional Retinal Pigment Epithelium Cells. *Cell Stem Cell** **5**, 396–408 (2009).
34. Saari, J. C. Vitamin A Metabolism in Rod and Cone Visual Cycles. *Annu. Rev. Nutr.* **33**, 125–145 (2012).
35. Feeney, L. Lipofuscin and melanin of human RPE. *Chem. Biol. Interact.* **178**, 1–9 (1978).
36. Zhu, D. et al. Polarized secretion of PEDF from human embryonic stem cell-derived RPE promotes retinal progenitor cell survival. *Invest. Ophthalmol. Vis. Sci.* **52**, 1573–1585 (2011).
37. Zhu, Y. et al. Three-Dimensional Neuroepithelial Culture from Human Embryonic Stem Cells and Its Use for Quantitative Conversion to Retinal Pigment Epithelium. *Plos One** **8** (2013).
38. Kamo, H. et al. Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. *Stem Cell Reports** **2**, 205–218 (2014).
39. Sonoda, S. et al. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nat. Protoc.* **4**, 662–673 (2009).
40. Blenkinsop, T. A., Salero, E., Stern, J. H. & Temple, S. The Culture and Maintenance of Functional Retinal Pigment Epithelial Monolayers from Adult Human Eye. *In Journal of the American Medical Association LXIII*, 45–65 (2012).
41. Kokkinaki, M., Sahbrada, N. & Golestan, N. Human induced pluripotent stem-derived retinal pigment epithelium (RPE) cells exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE. *Stem Cells** **29**, 825–835 (2011).
42. Maroutti, J. et al. A Simple and Scalable Process for the Differentiation of Retinal Pigment Epithelium From Human Pluripotent Stem Cells. *Stem Cells Transl. Med.* **2**, 341–354 (2013).
43. Plaza Reyes, A. et al. Xeno-Free and Defined Human Embryonic Stem Cell-Derived Retinal Pigment Epithelial Cells Functionally Integrate in a Large-Eyed Preclinical Model. *Stem Cell Reports** **6**, 9–17 (2016).
44. 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. *Exp. Eye Res.* **82**, 99–110 (2006).
45. Bosch, E., Horwitz, J. & Bok, D. Phagocytosis of Outer Segments by Retinal Pigment Epithelium: Phagosome-Lysosome Interaction. *J. Histochem. Cytochem.* **41**, 253–263 (1993).
46. Mao, Y. & Finnemann, S. C. Analysis of photoreceptor outer segment phagocytosis by RPE cells in culture. *Methods Mol. Biol.* **935**, 285–95 (2013).
47. Miyoshi, H., Takahashi, M., Gage, F. H. & Verma, I. M. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10319–23 (1997).
48. Benemans, A. P. et al. Retinal cell type expression specificity of HIV-1-derived gene transfer vectors upon subretinal injection in the adult rat: Influence of pseudotyping and promoter. *J. Gene Med.* **7**, 1367–1374 (2005).
49. Hashimoto, T. et al. Lentiviral gene replacement therapy of retinias in a mouse model for Usher syndrome type 1B. *Gene Ther.** **14**, 584–594 (2007).
50. Holmgaard, A. et al. In Vivo Knockout of the Vegfa Gene by Lentiviral Delivery of CRISPR/Cas9 in Mouse Retinal Pigment Epithelium Cells. *Mol. Ther. - Nucleic Acids** **8**, 99–99 (2017).
51. Müller, C., Charniga, C., Temple, S. & Finnemann, S. C. Quantified F-Actin Morphology Predictive of Phagocyotic Capacity of Stem Cell-Derived Retinal Pigment Epithelium Cells. *Stem Cell Reports** **10**, 1075–1087 (2018).
52. Nandrot, E. et al. Homozygous deletion in the coding sequence of the c-myc gene in RCS rats unravels general mechanisms of physiological cell adhesion and apoptosis. *Neurobiol. Dis.* **7**, 586–599 (2000).
53. Finnemann, S. C. & Nandrot, E. F. Mertk Activation During RPE Phagocytosis in Vivo Requires αV35 Integrin. In *Advanced Experimental Medical Biology* **572**, 499–503 (Springer US, 2006).
54. Reichmann, S. et al. From confluent human iPSC cells to self-forming neural retina and retinal pigmented epithelium. *Proc. Natl. Acad. Sci.** **111**, 8518–8523 (2014).
55. Liao, J. L. et al. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. *Hum. Mol. Genet.** **19**, 4229–4238 (2010).
56. Pinto, J. P. et al. StemMapper: A curated gene expression database for stem cell lineage analysis. *Nucleic Acids Res.* **46**, D788–D793 (2018).
57. Brenes, A., Afsal, V., Kent, R. & Lamond, A. I. The Encyclopedia of Proteome Dynamics: A big data ecosystem for (prote)omics. *Nucleic Acids Res.* **46**, D1202–D1209 (2018).
58. Carr, A. J. et al. Protective effects of human iPSC-derived retinal pigment epithelium cell transplantation in the dystrophic retinal rat. *Plos One** **4** (2009).
94. Synnergren, J.
92. Ronen, K.
93. Shields, J., Shields, C., Gündüz, K. & Eagle, R. Neoplasms of the Retinal Pigment Epithelium.
96. Delzor, A.
95. Kostic, C.
91. Berry, C., Hannenhalli, S., Leipzig, J. & Bushman, F. D. Selection of Target Sites for Mobile DNA Integration in the Human Genome.
90. Naldini, L.
88. Mowat, F. M.
87. Cideciyan, A. V.
86. Smalley, E. First AAV gene therapy poised for landmark approval.
85. Georgiadis, A.
84. Jacobson, S. G.
83. Bainbridge, J. W. B.
82. Hershey, C. L. & Fisher, D. E. Genomic analysis of the Microphthalmia locus and identification of the MITF-J/Mitf-J isoform.
81. Kozaki, K. I.
80. Koster, W. S. S.
79. Liu, S. Y. & Redmond, T. M. Role of the 3′-untranslated region of RPE65 mRNA in the translational regulation of the RPE65 gene: Identification of a specific translation inhibitory element. Arch. Biochem. Biophys. 357, 37–44 (1998).
78. Meyer, C., Notari, L. & Becerra, S. P. Mapping the type I collagen-binding site on pigment epithelium-derived factor: Implications for its antiangiogenic activity. J. Biol. Chem. 277, 45400–45407 (2002).
77. Sekiya, A., Okano-Kosugi, H., Yamazaki, C. M. & Koide, T. Pigment epithelium-derived factor (PEDF) shares binding sites in collagen with heparin/heparan sulfate proteoglycans. J. Biol. Chem. 286, 26364–26374 (2011).
76. Kozaki, K. I. et al. Isolation, purification, and characterization of a collagen-associated serpin, caspin, produced by murine colon adenocarcinoma cells. J. Biol. Chem. 273, 15125–15130 (1998).
75. Karwatowski, W. S. S. et al. Preparation of Bruch’s membrane and analysis of the age-related changes in the structural collagens. Br. J. Ophthalmol. 79, 944–952 (1995).
74. Samuel, W.
73. Jacobson, S. G.
72. Samuel, W.
71. Liu, S. Y. & Redmond, T. M. Role of the 3′-untranslated region of RPE65 mRNA in the translational regulation of the RPE65 gene: Identification of a specific translation inhibitory element. Arch. Biochem. Biophys. 357, 37–44 (1998).
70. Booti, J. C., Baas, D. C., Beisekeeva, J., Gorgels, T. G. M. F. & Bergen, A. A. B. The dynamic nature of Bruch’s membrane. Prog. Retin. Eye Res. 29, 1–18 (2010).
69. Karwatowski, W. S. S. et al. Preparation of Bruch’s membrane and analysis of the age-related changes in the structural collagens. Br. J. Ophthalmol. 79, 944–952 (1995).
68. Kozaki, K. I.
67. Sekiya, A., Okano-Kosugi, H., Yamazaki, C. M. & Koide, T. Pigment epithelium-derived factor (PEDF) shares binding sites in collagen with heparin/heparan sulfate proteoglycans. J. Biol. Chem. 286, 26364–26374 (2011).
66. Meyer, C., Notari, L. & Becerra, S. P. Mapping the type I collagen-binding site on pigment epithelium-derived factor: Implications for its antiangiogenic activity. J. Biol. Chem. 277, 45400–45407 (2002).
65. Hershey, C. L. & Fisher, D. E. Genomic analysis of the Microphthalmia locus and identification of the MITF-J/Mitf-J isoform.
64. Takeda, K.
63. Amae, S.
62. Carr, A.-J.
61. Berry, C., Hannenhalli, S., Leipzig, J. & Bushman, F. D. Selection of Target Sites for Mobile DNA Integration in the Human Genome.
60. Maeda, T.
59. Maeda, A. & Palczewski, K. Retinal degeneration in animal models with a defective visual cycle. Drug Discov. Today Dis. Model 10, e163–e172 (2013).
58. Maeda, T. et al. Retinal Pigmented Epithelial Cells Obtained from Human Induced Pluripotent Stem Cells Possess Functional Visual Cycle Enzymes in Vitro and in Vivo. J. Biol. Chem. 288, 34484–34493 (2013).
57. Jin, M., Li, S., Moghrabi, W. N., Sun, H. & Travis, G. H. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 122, 449–459 (2005).
56. Carr, A.-J. et al. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol. Vis. 15, 283–95 (2009).
55. Amae, K. & Ishii, K. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol. Vis. 15, 283–95 (2009).
54. Boosti, J. C., Baas, D. C., Beisekeeva, J., Gorgels, T. G. M. F. & Bergen, A. A. B. The dynamic nature of Bruch’s membrane. Prog. Retin. Eye Res. 29, 1–18 (2010).
53. Liu, S. Y. & Redmond, T. M. Role of the 3′-untranslated region of RPE65 mRNA in the translational regulation of the RPE65 gene: Identification of a specific translation inhibitory element. Arch. Biochem. Biophys. 357, 37–44 (1998).
52. Samuel, W. & et al. Translational Regulation of RPE65 Expression by microRNA. Invest. Ophthalmol. Vis. Sci. 54, 6322 (2013).
51. Jacobson, S. G. et al. Safety of Recombinant Adeno-Associated Virus Type 2-RPE65 Vector Delivered by Ocular Subretinal Injection. Mol. Ther. 13, 1074–1084 (2006).
50. Bainbridge, J. W. B. et al. Effect of gene therapy on visual function in Leber’s congenital amaurosis. N. Engl. J. Med. 358, 2231–9 (2008).
49. Maguire, A. M. et al. Age-dependent effects of RPE65 gene therapy for Leber’s congenital amaurosis: a phase 1 dose-escalation trial. Lancet 374, 1597–1605 (2009).
48. Smalley, E. First AAV gene therapy poised for landmark approval. Nat. Biotechnol. 35, 998–999 (2017).
47. Cideciyan, A. V. et al. Human retinal gene therapy for Leber congenital amaurosis shows advancing retinal degeneration despite enduring visual improvement. Proc. Natl. Acad. Sci. USA 110, ES17–ES25 (2013).
46. Moveat, F. M. et al. RPE65 gene therapy slows cone loss in Rpe65-deficient dogs. Gene Ther 20, 545–555 (2013).
45. Georgiadis, A. et al. Development of an optimized AAV2/5 gene therapy vector for Leber congenital amaurosis owing to defects in RPE65. Gene Ther 23, 857–862 (2016).
44. Naldini, L. Ex vivo gene transfer and correction for cell-based therapies. Nat. Rev. Genet. 12, 301–315 (2011).
43. Berry, C., Hannenhalli, S., Leipzig, J. & Bushman, F. D. Selection of Target Sites for Mobile DNA Integration in the Human Genome. PLoS Comput. Biol. 2 (2006).
42. Ronen, K. et al. Distribution of Lentiviral Vector Integration Sites in Mice Following Therapeutic Gene Transfer to Treat β-thalassemia. Mol. Ther. 19, 1273–1286 (2009).
41. Shields, J., Shields, C., Gündüz, K. & Eagle, R. Neoplasms of the Retinal Pigment Epithelium. Arch. Ophthalmol. 117, 601 (1999).
40. Symergren, J. et al. Differentiating Human Embryonic Stem Cells Express a Unique. Stem Cells 25, 473–480 (2007).
39. Kostic, C. et al. Activity analysis of housekeeping promoters using self-inactivating lentiviral vector delivery into the mouse retina. Gene Ther 10, 818–821 (2003).
38. Delzor, A. et al. Restricted transgene expression in the brain with cell-type specific neuronal promoters. Hum. Gene Ther. Methods 254, 1210176303000 (2012).

Acknowledgements
We would like to thank the EPFL BioImaging & Optics Core Facility (EPFL-BIOP) staff for their availability and expertise, and particularly Dr. Luigi Bozzo for his assistance in imaging, and the UNIL Electron Microscopy Facility (UNIL-EMF) staff for their availability and expertise, particularly Dr. Jean Daraspe for the processing and imaging of our samples. We also would like to thank the Genethon. This work was supported by the SwissTransmed program from the CRUS, the Fondation Provisu, the Swiss National Foundation (#31003A_169492), and Sarah E. Slack Prevention of Blindness Fund, a component Fund of the Muskingum County Community Foundation.

Author contributions
F.U. performed all the experiments and analysed the data. S.D. participated in the development of the differentiation protocol. D.M.G. derived the iPSCs line. C.K. and N.D. designed the vectors. F.U., C.K. and N.D. produced the vectors. F.U. and Y.A. wrote the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-65657-y.
Correspondence and requests for materials should be addressed to Y.A.
Reprints and permissions information is available at www.nature.com/reprints.
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
