Translational

Characterization of a Mouse Model With Complete RPE Loss and Its Use for RPE Cell Transplantation

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PURPOSE. Age-related macular degeneration (AMD) is a major leading cause of visual impairment and blindness with no cure currently established. Cell replacement of RPE is discussed as a potential therapy for AMD. Previous studies were performed in animal models with severe limitations in recapitulating the disease progression. In detail, we describe the effect of systemic injection of sodium iodate in the mouse retina. We further evaluate the usefulness of this animal model to analyze cell-specific effects following transplantation of human embryonic stem cell (hESC)-derived RPE cells.

METHODS. Morphologic, functional, and behavioral changes following sodium iodate injection were monitored by histology, gene expression analysis, electroretinography, and optokinetic head tracking. Human embryonic stem cell-derived RPE cells were transplanted 1 week after sodium iodate injection and experimental retinae were analyzed 3 weeks later.

RESULTS. Injection of sodium iodate caused complete RPE cell loss, photoreceptor degeneration, and altered gene and protein expression in outer and inner nuclear layers. Retinal function was severely affected by day 3 and abolished from day 14. Following transplantation, donor hESC-derived RPE cells formed extensive monolayers that displayed wild-type RPE cell morphology, organization, and function, including phagocytosis of host photoreceptor outer segments.

CONCLUSIONS. Systemic injection of sodium iodate has considerable effects on RPE, photoreceptors, and inner nuclear layer neurons, and provides a model to assay reconstitution and maturation of RPE cell transplants. The availability of an RPE-free Bruch’s membrane in this model likely allows the unprecedented formation of extensive polarized cell monolayers from donor hESC-derived RPE cell suspensions.

Keywords: retinal pigment epithelium, sodium iodate, age-related macular degeneration, transplantation, hESC-derived RPE

The RPE is a monolayer of hexagonal, highly polarized pigmented cells that are crucial for retinal survival and visual function.1 Several retinal degenerative diseases including dry AMD are characterized by progressive RPE atrophy, leading to cone photoreceptor degeneration and loss of central high-acuity vision.2 Importantly, AMD is the most common cause of blindness in developed countries,3 affecting 1 of 10 people in elderly populations (>60 years old).

The lack of endogenous regenerative capacity and effective treatments has led to an increased interest in the development of novel therapies, namely cell-based transplantation strategies for RPE cell replacement. A previously used animal model to test such approaches is the Royal College of Surgeons (RCS) dystrophic rat. RCS rats are characterized by a phagocytic defect in the RPE due to a mutation in the receptor tyrosine kinase Mertk gene1 that leads to accumulation of photoreceptor outer segment (POS) fragments in the subretinal space and fast photoreceptor degeneration. Several cellular populations have been transplanted into RCS rats and shown to protect host photoreceptors, including human embryonic stem cell (hESC)-derived RPE,5-7 induced pluripotent stem cell (iPSC)-derived RPE,8-9 HRPE,10 rat RPE,11,12 ARPE19,13-15 fibroblasts,16 neural progenitors,16,17 and Schwann cells,18,19 among others. Thus, rescue effects are observed regardless of donor cell population, suggesting that the RCS rat present major limitations as a model for AMD and for assessing cell-specific therapeutic effects. These limitations become even more apparent when considering the early onset and fast progression of photoreceptor degeneration in RCS rats despite the persistence of endogenous RPE cells for an extended period of time. In spite of the increased need to study AMD, this disease is still incompletely recapitulated in currently existing animal models.20 Complications for modeling the disease include its late onset, complex
RPE Cell Transplantation in a Mouse Model

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genetics, confounding associated environmental risks and the lack of a macula in rodents. In this study, we evaluated an alternative, chemically-induced RPE degeneration mouse model based on the toxic effect of intravenously administered sodium iodate (NaIO3), that was shown to cause severe retinal damage in several mammalian species. As a consequence of RPE cell death, POS renewal is arrested and photoreceptors degenerate. Further characterization of the effect of NaIO3 on the mouse retina, showing that profound alterations occur at the mRNA and protein level in outer as well as inner nuclear layers (ONL/INL), and that retinal degeneration is accompanied by behavioral and functional changes. Moreover, we assessed NaIO3-treated mice as an experimental model to study transplantation of hESC-derived RPE as a prospective AMD therapy. Our results show that transplanted RPE cells form extensive polarized monolayers on the Bruch’s membrane of host NaIO3-treated mice and phagocyte mouse POS.

Materials and Methods

Animals

Eight to 10-week-old C57BL/6j mice were restrained (rodent restrainer model #84; World Precision Instruments, Sarasota, FL, USA) and a single injection of sterile NaIO3 (70mg/Kg; Sigma-Aldrich, Munich, Germany) or 0.9% sodium chloride (NaCl, Brown) as control was administered in the tail vein. All animal experiments were carried out in strict accordance with European Union and German laws (Tierschutzgesetz) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the animal ethics committee of the TU Dresden and the Landesdirektion Dresden (approval number 24-9168.11-1/2010-23).

Immunohistochemistry

Paraffin sections, 5-μm thick and free-floating fixed eyecups were blocked 1 hour at room temperature (RT), incubated overnight at 4°C with the primary antibody (Supplementary Table S1) and 2 hours at RT with the appropriate secondary antibody (Jackson IR, Suffolk, UK). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Samples were imaged with a Z1-Imager fluorescence microscope with ApoTome (Zeiss, Göttingen, Germany).

Quantification of RPE Cell Number and ONL Thickness

The number of RPE cells was counted in a 200-μm region around the optic nerve head (minimum seven sections per animal (n), day 3: n = 4, day 7: n = 4, day 14: n = 4, day 21: n = 5, day 28: n = 3, controls: n = 4). Only cells with wild-type morphology (unaltered shape and pigment content) and/or DAPI-stained distinguishable nucleus, were included. The same sections were used to measure ONL thickness across the entire retina in 500-μm steps.

Evaluation of Retinal Cell Death

Cell death was evaluated by TUNEL assay (Roche, Mannheim, Germany). The number of TUNEL-positive cells per section was counted in four sections per animal. Calpain activity was analyzed in mixed cryosections from days 1 and 3, as previously described. Activated caspase-3 was detected by immunohistochemistry.

Transmission Electron Microscopy

Mice were perfused with modified Karnovsky buffer (2% glutaraldehyde, 2% paraformaldehyde). Small retinal pieces were post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences [EMS], Hatfield, PA, USA) 2 hours on ice, and contrasted with 1% uranyl acetate (Polysciences, Eppelheim, Germany) 2 hours on ice. Samples were embedded in epoxy resin (EPON 812; EMS). Epoxy resin embedding of paraffin sections previously mounted on glass slides required paraffin removal, section rehydration, fixation in Karnovsky buffer and post fixation in 1% osmium tetroxide. Fixed sections were infiltrated in EPON. Semithin sections were mounted on glass slides and stained with 1% toluidine blue, 0.5% borax. Ultrathin sections were collected on formvar-coated slot grids (EMS), stained with lead citrate (EMS, Hatfield, PA, USA) 2 hours on ice, and contrasted with 1% uranyl acetate (Polysciences, Eppelheim, Germany). 2 hours on ice. Samples were contrasted with 1% uranyl acetate (Polysciences, Eppelheim, Germany). The number of TUNEL-positive cells per section was counted in four sections per animal. Calpain activity was analyzed on a FEI Morgagni 268 (Eindhoven, The Netherlands) at 80 kV. Images were taken with an Olympus MegaViewIII camera (Olympus Soft-Imaging Solutions, Münster, Germany).

Electroretinography (ERG)

Mice were dark-adapted overnight and anesthetized by an intraperitoneal injection of medetomidine hydrochloride (0.01mg/10g body weight; Dormitor, Pfizer, Berlin, Germany) and ketamine (0.75mg/10g body weight; Ratiopharm, Ulm, Germany) in dim red light. Pupils were dilated using 0.5% tropicamide and 2.5% phenylephrinehydrochlorid (TU Dresden pharmacy, Dresden, Germany). Full-field ERGs were recorded using a Ganzfeld bowl (Roland Consult, Brandenburg an der Havel, Germany). Single-flash responses were obtained under dark and light-adapted conditions. For the analysis, a- and b-wave amplitudes from scotopic (0.005 cd.s.m-2), mesopic (3 cd.s.m-2), and photopic (10 cd.s.m-2) were obtained for a- and b-wave amplitudes from scotopic (0.005 cd.s.m-2), mesopic (3 cd.s.m-2), and photopic (10 cd.s.m-2) conditions. The threshold of visual acuity and contrast sensitivity was determined as the stimulus parameters when the OKT was not triggered anymore. After injection, six animals were tested for their thresholds of visual acuity (high Weber contrast) and contrast sensitivity (0.15 cyc/deg).

Optokinetic Reflex (OKT)

Behavioral analysis was performed as previously described. Before NaIO3 injection baseline visual acuity was evaluated at a high and low contrast (Weber contrast of 109 and 1.3, respectively), and contrast sensitivity was measured at 0.05, 0.15, and 0.30 cycles/degree. The contrast sensitivity was measured at 0.05, 0.15, and 0.30 cycles/degree. Threshold of visual acuity and contrast sensitivity was determined as the stimulus parameters when the OKT was not triggered anymore. After injection, six animals were tested for their thresholds of visual acuity (high Weber contrast) and contrast sensitivity (0.15 cyc/deg).

RNA Isolation and Real Time RT-PCR

Total retinal RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), following manufacturer’s instructions. Approximately 150 ng of RNA from each sample were used to produce cDNA, using Oligo(dT) primers (Biomers, Ulm, Germany) and Superscript II Reverse Transcriptase (Invitrogen). Retinase from NaIO3-treated animals were collected from three independent biological replicates (independent injections). A minimum of two retinase, usually from two or more animals, was used per sample. Genes of interest were amplified using specific primers (Supplementary Table S2). Expression was quantified using RT2 SYBR Green Fluor qPCR Mastermix (Qiagen, Hilden, Germany) following manufacturer’s instructions in a qPCR cycler DNA Engine Opticon 2 (formerly MJ Research, Waltham, MA, USA). The ΔΔCT method was used to calculate relative expression of mRNAs in control and NaIO3 treated retinase normalized to β-actin mRNA expression levels.
hESC-Derived RPE

Retinal pigment epithelial cells were generated from hESC cultures (line H9) using a recently established method. For the transplantation experiments, cells were harvested at day 35 of differentiation. Briefly, cells were incubated with 10 mg/mL trypsin (Life Technologies, Darmstadt, Germany) 15 minutes at 37°C and mechanically dissociated. Following addition of 10 mg/mL DNaseI (Sigma-Aldrich) and 20 mg/mL trypsin inhibitor (Roche) cells were centrifuged five minutes at 0.3g and resuspended in PBS to a final concentration of approximately 100,000 cells/μL.

Transplantation

Subretinal transplantation was performed as described elsewhere. Recipient mice were anesthetized, fixed in a head holder, and pupils were dilated. Of the cell suspension, 1 to 2 μL were transplanted into the subretinal space of untreated or NaCl-injected controls (n = 5) and NaIO3-injected (n = 2) 10- to 11-week-old C57BL/6J mice, 7 days after systemic tail vein injection. Control eyes were sham-injected (PBS) or remained nontransplanted.

Optical Coherence Tomography (OCT)

Transplanted animals were analyzed with a high-resolution dual-band custom built OCT system illuminated by a super-continuum laser source (SuperK Versa, Koheras AS, Cologne, Germany) and a fiber-coupled ophthalmic scanning unit. An OCT volume scan of the eye’s fundus consisting of 480 cross-sectional scans with 512 axial scans per cross-section was recorded.

Statistics

Results are presented as mean ± SD. Significance was calculated by two-tailed Student’s t-test or one-sample t-test and is represented in the figures by *P less than 0.05, **P less than 0.01, and ***P less than 0.001.

Results

Loss of RPE Cells Following NaIO3 Injection

The effect of a single, systemic NaIO3 injection on the RPE was quantitatively evaluated by counting the number of RPE cells around the optic nerve head. Three days after NaIO3 injection, the RPE was severely affected as shown by thinning of the RPE monolayer and significant reduction of DAPI-stained nuclei (Fig. 1A). Significant reduction of immunostain positivity for Otx2, a transcription factor expressed in mature RPE cells, was observed, specifically around the optic nerve head (Fig. 1B). Toward the periphery the eyecups retained more RPE, but the remaining RPE nuclei were equally affected and rarely Otx2-positive (Fig. 1B2). The number of RPE cells reduced to approximately zero by day 14 (Fig. 1A), leaving the Bruch’s membrane devoid of RPE cells (Fig. 1C). Also evident was the appearance of macrophages (Fig. 1C, from day 7). Fig. 1D shows the profound effect of NaIO3 at the cellular level: 3 days post injection RPE cells were much thinner, the nuclei were severely affected and typical morphologic characteristics of RPE cells were lost, for example, basal membrane infoldings (Fig. 1D, arrowheads), apical microvilli (Fig. 1D, arrows) and tight junctions. Given the extent of damage observed at day 3, expression of typical mature RPE proteins was analyzed at earlier time points. As shown in Figure 2, the expression of Otx2 (Fig. 2A2), RPE65 (Fig. 2B2), ZO-1 (Fig. 2C2), and ATPase (Fig. 2D2), essential for RPE function and/or structural integrity, was already affected at day 1 following NaIO3 injection.

Photoreceptor Degeneration in NaIO3-Injected Mice

Despite the absence of gross morphologic changes at day 3, retinal detachment and disorganization was evident from day 7. DAPI-stained retinal cross-sections revealed progressive thinning of the ONL from day 3 to 28 (Figs. 3A1-A3). For a quantification of the effect on photoreceptors, the ONL thickness was measured. Three days post injection central retina showed reduced thickness, matching the more pronounced effect of NaIO3 on RPE cells located around the optic nerve head (Fig. 3B). By day 7 the ONL thickness was more uniformly reduced, also affecting peripheral retina. From day 7 to day 28 the ONL thickness remained mainly constant (for simplicity only day 28 is shown in Fig. 3B). However, analysis of retinae 13 weeks after NaIO3 injection showed that the ONL was significantly reduced, with one row of photoreceptor nuclei left (P < 0.001 for all retinal regions; Figs. 3A4, 3B).

Subsequently, we analyzed the number of retinal cells undergoing cell death at all time points. TUNEL-positive cells were detected exclusively in the ONL, with a peak at day 3 (Figs. 3C, 3D). From this time point on the number of TUNEL-positive photoreceptors remained constant. These results are in agreement with the reduction of ONL thickness, which was stronger from day 3 to 7 and then remained constant, in line with previous reports. To clarify the cell death mechanism underlying photoreceptor degeneration, calpain activity, and presence of activated caspase3 were evaluated 1 and 3 days after NaIO3 injection (Fig. 3E). As evidenced by the TUNEL assay, cell death was already induced at day 1 (Fig. 3F). Since the number of photoreceptors positive for calpain activity increased significantly (P < 0.05) while the number of activated caspase3-positive cells in the ONL remained at control levels (Fig. 3F), a nonapoptotic cell death mechanism is suggested.

Impaired Retinal Function Following NaIO3 Injection

Retinal responses to light stimuli were evaluated by ERG. As shown in Figures 4A to 4E, ERG responses were significantly affected at day 3, with the rod-driven response abolished (Fig. 4A, P < 0.001) and both mesopic (Figs. 4B, 4C) and photopic responses (Fig. 4D, 4E) showing decreased a- and b-waves amplitudes (mesopic a-wave P < 0.05, for the other waves P < 0.001) and increased implicit time (Figs. 4F-N). All amplitudes decreased progressively until day 14, the time point from which no response was elicited anymore.

Visual function capability of NaIO3-injected mice was further analyzed by assessment of the OKT. Three days after treatment the contrast threshold was significantly increased (Fig. 4O, P < 0.05) and visual acuity significantly decreased (Fig. 4P, P < 0.05). Similarly to what happened when retinal function was assessed with ERG, there was a progressive deterioration of visual performance over time, which did not recover (Figs. 4O, 4P). By day 21, no OKT tracking responses were elicited anymore in any experimental animal.

NaIO3 Differentially Affects Gene Expression in Photoreceptors

Despite the relatively slow degeneration rate of the photoreceptors, retinal function was already significantly affected at
Systemic injection of NaIO₃ caused severe RPE cell loss. (A) Number of RPE cells present in the 200-μm region around the optic nerve head before and after NaIO₃ injection. Massive reduction in RPE numbers was observed already 3 days after treatment (2.16 ± 1.59 vs. 11.81 ± 1.7 cells in controls, P < 0.001). (B) Drastic reduction of Otx2 staining on RPE flatmounts of day 3 animals in comparison with an untreated control. One and two correspond to a higher magnification of boxed areas. (C, D) Electron microscopic analysis of RPE before and after treatment, following standard EPON embedding. (C) Overview of the RPE monolayer in control and injected animals. Complete loss of RPE cells led to a free Bruch's
day 3. Although the lack of 11-cis retinal upon RPE degeneration might be responsible for impaired retinal function, it is not known what gene expression changes in the photoreceptors might also influence ERG amplitudes. Hence, the expression of genes involved in the phototransduction cascade and visual cycle was analyzed up to day 28 after NaIO 3 administration. Interestingly, as revealed by real time RT-PCR, the expression of some genes was downregulated upon NaIO 3 injection (e.g., rhodopsin, m-opsin, arrestin3, pde6b, transducin), whereas for others it remained unaltered (e.g., s-opsin, arrestin1, cnga1, IRBP, rdh8, recoverin; Fig. 5).

Since previous reports have shown that upregulation of neurotrophic factors might be related with preservation of photoreceptors in a degenerating retina, 33 we analyzed gene expression of basic fibroblast growth factor (FGF2). Indeed, as shown in Figure 5, FGF2 showed a tendency (although not statistical significant) for upregulation after NaIO 3 injection.

**Injection of NaIO 3 Causes Retinal Remodeling**

For cellular therapies to be successful, the retinal circuitry needs to be intact. 34 Because significant changes have been shown to occur in second order neurons of retinal degeneration animal models, 15,35,36 an extensive characterization of the retinal protein profile in NaIO 3-treated mice was performed.

Proteins that in the controls are restricted to the POS were mislocalized to the cell body after NaIO 3 administration. Interestingly, as revealed by real time RT-PCR, the expression of some genes was downregulated upon NaIO 3 injection (e.g., rhodopsin, m-opsin, s-opsin at the time point when such changes first occurred: day 21 for rhodopsin, day 14 for m-opsin (Fig. 6B’), and day 3 for s-opsin (Fig. 6C’). Interestingly, s-opsin immunoreactivity decreased significantly over time, with almost no s-cones detectable at day 28, and simultaneous loss of cone arrestin-positive cells in s-cone-dominated regions in accordance with the reduced arrestin3 expression seen with real time RT-PCR (Fig. 5).

Two possibly interconnected alterations at the synaptic level were observed: bassoon was dramatically downregulated from day 3 (Figs. 6F–F’’) and the dendrites of bipolar cells were hypertrophic from day 14 (Figs. 6G’’, H’’). Additional alterations in the INL included reduced number of Pax6-positive amacrine cells from day 14 (Figs. 6I–I’’) and temporary

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**FIGURE 2.** Decreased RPE marker expression following NaIO3 injection. (A–D) Representative images of RPE flatmounts analyzed for typical mature RPE proteins, in control and in day 1 and 2 retinae following NaIO3 injection. One day after NaIO3 injection expression of Otx2 (A2), RPE65 (B2), ZO-1 (C2), and ATPase (D2) was already affected, with further reduction at day 2 (A3–D3).
upregulation of glial fibrillary acidic protein (GFAP) in Müller glia cells, a well-established sign of stress and degeneration in the retina (Fig. 6J–J’). In summary, these results demonstrate that a single NaIO₃ injection led to complete loss of RPE cells, followed by significant retinal changes affecting both ONL/INLs, as well as the synaptic contacts between them.

Transplantation of hESC-Derived RPE Into NaIO₃-Treated Mice Yields Monolayer Engraftment of RPE

Next, the NaIO₃-treated mouse was investigated as an experimental model to study RPE cell transplantation as a therapeutic strategy. Human embryonic stem cell–derived RPE cells were transplanted as cell suspensions into wild-type saline- or NaIO₃-injected mice, 7 days post injection. The presence of donor cells was assayed by immunofluorescence staining for human specific markers (antihuman nuclei and β2 microglobulin) at 3 weeks post transplantation. In sham-injected control mice, the majority of donor RPE cells formed clusters in the subretinal space (Figs. 7A, 7B) and only few cells integrated within the intact host RPE monolayer (Fig. 7C). In contrast, transplantation into NaIO₃-treated animals yielded engraftment of donor RPE cells as highly organized monolayers attached to the host Bruch’s membrane for lengths up to 1650 μm (Figs. 8A–C), also evident in OCT images (Supplementary Fig. S1A5, arrows). Electron-microscopic images showed correct organization and apical-basolateral polarity of RPE cells within such monolayers, with basally located nuclei (Fig. 8D), apically distributed melanosomes at different stages of maturation (Fig. 8E) and rudimentary apical microvilli (Fig. 8F). Transplanted cells further showed polarized location of ATPase (Figs. 7D, 8G), collagen IV (Figs. 7E, 8H) and

**FIGURE 3.** Effect of NaIO₃ injection on photoreceptors. (A) DAPI-stained representative images of control (A1) and NaIO₃-treated retinae (A2–A4). (B) Quantification of ONL thickness. Three days after NaIO₃ injection the ONL was reduced only in central retina (≈2000 μm: not significant (n.s.), ≈1500 μm: n.s., ≈1000 μm: P < 0.01, ≈500 μm: P < 0.01, 1000 μm: n.s., 1500 μm: n.s., 2000 μm: n.s.), but by day 28 it was significantly reduced in all retinal regions. Only one row of photoreceptor nuclei remained 13 weeks after treatment. (C) Detection of cell death via the TUNEL assay. Representative images of control (C1) and injected retinae (C2–C4). (D) Number of TUNEL-positive cells in the ONL of control and injected animals. A peak of cell death was observed 3 days after NaIO₃ injection (168 ± 30.86 cells at day 3 versus 5.25 ± 2.66 in control retinae); however, from day 7 on the number of TUNEL-positive cells was reduced and remained constant up to day 28 (P < 0.001 for all time points). (E) Representative images of retinae analyzed for calpain activity (E1, E2, arrowheads) and activated caspase3 (E3, E4). (F) Percentage of photoreceptors positive for activated caspase3, TUNEL, and calpain. While the number of activated caspase3-positive cells remained at control levels, the number of both TUNEL- and calpain-positive cells was significantly increased three days following NaIO₃ injection. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
NaIO₃ injection caused impaired retinal function and blindness. (A–E) Effect of NaIO₃ on a- and b-wave amplitudes in scotopic (0.003 cd.s.m⁻² [A]), mesopic (3 cd.s.m⁻² [B, C]) and photopic (10 cd.s.m⁻² [D, E]) conditions. (F–N) Representative ERG curves in scotopic (F–H), mesopic (I–K), and photopic (L–N) conditions for controls (F, I, L), day 3 (G, J, M), and day 7 (H, K, N) post NaIO₃ injection. (O, P) Optokinetic reflex measurements for the analysis of contrast threshold (O) and visual acuity (P) before and after treatment. Injection of NaIO₃ significantly reduced ERG waves (A–E), increased contrast threshold (O), and reduced visual acuity (P), leading to functional and behavioral unresponsiveness from day 14. Optokinetic tracking behavior was not measurable anymore at day 21. *P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test.
FIGURE 5. Altered gene expression following systemic injection of NaIO₃. Real time RT-PCR analysis revealed decreased expression of some genes involved in the phototransduction cascade and regeneration of the photopigment upon NaIO₃ injection, including rhodopsin, m-opsin, arrestin3, pde6b, and transducin, whereas expression of other genes of the same categories remained unchanged (e.g., arrestin1, cnga1, IRBP, rdh8, and recoverin). Slight upregulation was also observed for the neurotrophic factor FGF2. *P < 0.05, **P < 0.01, one sample t-test.
FIGURE 6. Retinal remodeling caused by NaIO3-induced retinal degeneration. Representative pictures of control and NaIO3-treated retinae stained with several retinal markers. (A–C) Mislocalization of rhodopsin (A'''), m-opsin (B'', B'''), and s-opsin (C--C''') to the cell body, and loss of s-opsin-positive cells (C'', C'''). (D, E) Mislocalization of rod (D'', D''') and cone (E'', E''') arrestin. (F) Loss of bassoon in the OPL and IPL (F--F'''). (G, H) Sprouting of bipolar cell dendrites toward the ONL (G'', G''', H'', H'''). (I) Reduction of Pax6-positive amacrine cells (I'', I'''). (J) Reactive gliosis visualized by GFAP (J', J'').
ZO-1 (Fig. 8I). Furthermore, donor RPE cells contained rhodopsin-positive particles (Figs. 7F, 8J), suggestive of POS phagocytosis. Indeed, ultrastructural analysis revealed the presence of POS fragments within donor RPE cells (Fig. 8K). However, despite the apparent preservation of the ONL thickness and upregulation of GFAP to a lower extent within regions containing donor-derived RPE monolayers (Figs. 8L, 8M), functional recovery analyzed by ERG was not observed.

**DISCUSSION**

Recent achievements in ex vivo generation of RPE from pluripotent stem cells prompted investigations toward their use in cell replacement approaches. Although some clinical trials in human patients are already ongoing, multiple questions remain. Preclinical animal models used to investigate RPE transplantation show severe limitations. For instance, they do not sufficiently recapitulate diseases characterized by RPE cell loss and thus might not be optimal to evaluate transplantation of RPE cells. Therefore, we investigated in detail an animal model for chemically induced RPE loss and analyzed changes of the host retina as well as hESC-derived RPE transplants by various criteria. Our results show that systemic NaIO₃ injection leads to complete degeneration of mouse RPE cells within days and, over the following weeks, to the thinning of the ONL as a consequence of photoreceptor degeneration. We further extended the characterization of the effects of NaIO₃ on mouse retina, showing profound alterations at the mRNA and protein level in the ONL/INL, as well as behavioral and functional changes that accompany retinal degeneration. Upon transplantation of hESC-derived RPE cells into NaIO₃-injected animals, donor cells adhered correctly to the host Bruch’s membrane and formed extensive monolayers that phagocytosed mouse POS.

Previous reports characterizing the effect of NaIO₃ in pigmented mice followed the loss of RPE cells by assessing the loss of autofluorescence. Here, we performed a quantitative analysis for a better understanding of the dynamics of RPE cell degeneration after NaIO₃ injection. We show that by day 3 there was more than 80% RPE cell loss and that, in agreement with previous reports, cellular structures were profoundly affected. Importantly, expression of several mature RPE proteins was already affected by day 1. For instance, ZO-1 is essential for the assembly of tight junctions, and its downregulation might be partially responsible for the epithelial barrier breakdown previously reported. By day 14, RPE cells had completely disappeared, possibly due to the phagocytotic activity of macrophages.
FIGURE 8. Monolayer formation following transplantation of hESC-derived RPE cells into the NaIO₃ degeneration model. Three weeks after transplantation donor cells were identified by the human-specific markers human nuclei (A, B, G, H) and β2 microglobulin (C, J). Transplanted hESC-derived RPE cells formed extensive monolayers (arrows; the separation between RPE and retina is a technical artefact) in close contact with the host Bruch’s membrane, and displayed a polarized morphology (B, C). (D–F) Electron microscopic analysis of Epon embedded paraffin sections. Transplanted donor cells showed a characteristic RPE morphology (D), with a basal nucleus, apical melanin pigments at different stages of development (E, arrows) and rudimentary apical microvilli (F, arrows). Transplanted hESC-derived RPE cells expressed ATPase (G), collagen IV (H), and ZO-1 (I) in a correct polarized manner. Rhodopsin-positive particles (J, green) were detected inside transplanted cells (J, red) suggesting phagocytic activity of donor cells. Ultrastructural analysis revealed POS fragments within donor RPE cells (K, boxed area; the black bar is a technical artifact). Analysis of GFAP expression in the same retinal section in regions without (L) and with (M) transplanted cells (not displayed because of the artificial detachment also seen in [A]). Presence of donor cells correlated with lower upregulation of GFAP and with apparent better preservation of the ONL (M).
A direct effect of NaIO₃ on photoreceptors after systemic injection has been recently suggested, based on the observation of ERG suppression and downregulation of phototransduction-related genes without visible effects on the RPE. However, the authors did observe a dramatic loss of RPE65 expression within 24 hours. They further support their claim of a direct effect based on the in vitro observation that ARPE19 cells seemed less vulnerable to NaIO₃-induced cytotoxicity than did 661W cells. However, ARPE19 cells have been shown to have a remarkable resistance to oxidative stress, higher than that of primary RPE cells. Hence, photoreceptor cell loss might also be a consequence of altered RPE metabolism, gene, and protein expression and, ultimately, degeneration. Our data indicates that there was a first wave of photoreceptor cell death at day 3, seen as a peak of TUNEL-positive cells, as previously observed. It was followed by a period of ONL reduction in the periphery from day 3 to day 7 and by a period of stable ONL thickness from day 7 to day 28, probably caused by the upregulation of neurotrophic factors. Even though apoptosis was long thought to be the mechanism responsible for photoreceptor cell death during retinal degeneration, increased calpain activity was later reported in several animal models, suggesting a caspase-independent cell death mechanism. By demonstrating increased calpain activity and reduced caspase activity within the first 7 days following NaIO₃ injection, we provide additional evidence that NaIO₃ may trigger a nonapoptotic cell death mechanism.

We observed downregulation of some genes involved in the phototransduction cascade in the retina of NaIO₃-injected animals, whereas expression of others remained unchanged. Similar observations were reported in other retinal degeneration mouse models, (e.g., rpe65−/−51,52 and enga3−/−). Importantly, injection of 9- or 11-cis retinal into rpe65−/− animals significantly increased cone opsin mRNA levels and slowed cone degeneration, suggesting that the absence of chromophore could be responsible for cone cell death. Thus, it seems that photoreceptor degeneration, irrespective of cell death cause, results in altered gene expression for the same phototransduction-related genes.

The effect of NaIO₃ on the ERG is, most likely, multifactorial. For instance, analysis of mice with nonfunctional bassoon showed impaired synaptic transmission, with a-waves comparable with the wild-type and b-waves significantly reduced. Furthermore, leukemia inhibitory factor (LIF) was shown to decrease the isomerase activity of RPE65 through gp130 and STAT3 activation, suggesting the existence of a mechanism coordinately regulating the visual cycle in photoreceptors and RPE. It would be very interesting to analyze the effect of NaIO₃ on the ERG in a mouse model with this signaling pathway blocked. The slight upregulation of the neurotrophic factor FGF2 upon injection of NaIO₃ might contribute not only to a protective effect on photoreceptors, but also to a repression of the ERG curves, since it was shown that FGF2 has an impact on photoreceptor function. Finally, exposure to light would contribute to further exhaustion of 11-cis retinal, since rod photoreceptors even when saturated in bright light conditions were shown to consume recycled chromophore at high rates. Thus, it would be interesting to investigate whether retinal function is better preserved in NaIO₃-injected dark-reared animals. We propose that early, simultaneous downregulation of phototransduction- (e.g., rhodopsin, m-opsin, arrestin3, transducin) and synaptic- (e.g., bassoon) components would induce loss of rod-derived b-wave, decreased sensitivity and smaller rod-cone mixed b-wave, respectively. Further exhaustion of the 11-cis retinal pool by light exposure beside upregulation of neurotrophic factors (e.g., FGF2) would additionally contribute to ERG suppression.

Following transplantation of hESC-derived RPE cell suspensions, monolayers of considerable size were detected, suggesting that the great extension of free Bruch’s membrane in wild-type animals provides a permissive substrate for donor cells, fostering survival, and self-organized generation of extensive RPE monolayers. Hence, re-establishment of cell polarity and cell–cell connections, as well as maturation processes can be studied in vivo in this model. Indeed, we observed that some degree of maturation was achieved in the host tissue, with expression of important RPE-related proteins (e.g., ATPase and ZO-1), production of rudimentary apical microvilli and phagocytosis of POS. However, the amount of POS material within donor RPE cells was lower than in untreated wild-type mice and, thus, might not resemble physiological diurnal phagocytosis. Dry AMD is characterized by macular RPE cell loss and a successful cell-based strategy would require donor cells to integrate focally. Our data shows that, despite the majority of RPE cells formed clusters in the subretinal space of transplanted control animals, some donor cells integrated within the host RPE monolayer and correctly polarized.

The dramatic morphologic and functional alterations in the RPE and neuroretina seen early following NaIO₃ injection might impede functional recovery of photoreceptors upon transplantation of hESC-derived RPE cells. Indeed, 3 weeks after transplantation ERGs were not recordable in NaIO₃-treated animals. Thus, before clinical applications are considered, demonstration of functional restoration would be advantageous. Since transplantation of hESC-derived RPE 7 days after NaIO₃ injection might be too late to rescue photoreceptors, earlier time points should be evaluated. Furthermore, dry AMD represents a complex condition with several implications beside loss of RPE and consequent photoreceptor degeneration (e.g., drusen and lipofuscin formation), complement activation, and structural alteration of Bruch’s membrane. Thus, it would be interesting to perform transplantation experiments in mouse models that more closely mimic AMD, like humanized ApoE mice and NaIO₃-treated aged mice, to evaluate the performance of donor cells under more AMD-related conditions. Actually, NaIO₃-treated mice showed an essentially intact Bruch’s membrane and it is likely that transplanted RPE cells would not easily adhere to altered Bruch’s membrane, hence alternative techniques (e.g., injection of RPE sheets grown on carriers) might be considered. However, as RPE cells produce part of Bruch’s membrane, transplantation of healthy RPE cells might support the reconstitution of Bruch’s membrane in a diseased environment; thus, contributing to regenerate the blood–retina barrier and re-establish immune privilege besides generating their own substrate for proper attachment. Indeed, hESC-derived RPE cells produced considerable amounts of collagen IV, a component of Bruch’s membrane, at the basal side following transplantation into control as well as NaIO₃-treated hosts.

In conclusion, the availability of extensive patches of RPE-free Bruch’s membrane in the NaIO₃ mouse model likely constitutes a permissive environment for donor RPE cells to survive, adhere, and self-organize as extensive monolayers. Therefore, this model represents an excellent transplantation system to study in detail the cellular and molecular mechanisms required for the in vivo formation of donor-derived RPE. Detailed knowledge about the conditions that allow proper RPE reconstitution will be an important prerequisite for distinct modulations of the host tissue and/or donor cell population to foster functional repair in retinal diseases characterized by RPE loss.
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