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Small molecule mediated proliferation of primary retinal pigment epithelial cells

Jonathan G. Swoboda^{1,‡}, Jimmy Elliott^{2,‡}, Vishal Deshmukh¹, Lorenzo de Lichtervelde¹, Weijun Shen³, Matthew S. Tremblay³, Eric C. Peters², Charles Y. Cho², Bin Lu⁴, Sergej Girman⁴, Shaomei Wang^{4,*}, and Peter G. Schultz^{1,*}

¹Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

²Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, CA 92121, USA

³California Institute for Biomedical Research, 11119 North Torrey Pines Road, La Jolla, CA 92037, USA

⁴Regenerative Medicine Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA

Abstract

Retinal pigment epithelial (RPE) cells form a monolayer adjacent to the retina and play a critical role in the visual light cycle. Degeneration of RPE cells results in retinal disorders such as age-related macular degeneration. Cell transplant strategies have potential therapeutic value for such disorders; however, risks associated with and an inadequate supply of donor cells limit their therapeutic success. The identification of factors that proliferate RPE cells *ex vivo* could provide a renewable source of cells for transplantation. Here we report that a small molecule (WS3) can reversibly proliferate primary RPE cells isolated from fetal and adult human donors. Following withdrawal of WS3, RPE cells differentiate into a functional monolayer, as exhibited by their expression of mature RPE genes and phagocytosis of photoreceptor outer segments. Furthermore, chemically expanded RPE cells preserve vision when transplanted into dystrophic Royal College of Surgeons (RCS) rats, a well-established model of retinal degeneration.

Retinal pigment epithelial (RPE) cells form a tightly woven monolayer of cells positioned between the neural retina and Bruch's membrane.(1, 2) RPE cells serve many functions including maintenance of the blood-retinal barrier, homeostasis of ions/nutrients and contribution to the visual light cycle.(2) Loss of functional RPE cells leads to degenerative retinal disorders such as age-related macular degeneration (AMD).(3) Cell-based therapies using various stem cell origins (*e.g.*, embryonic, induced pluripotent) may preserve or ultimately restore vision.(4–7) Indeed, a recent clinical study on the first use of RPE cells derived from human embryonic stem cells suggests that transplantation of such cells could

*To whom correspondence should be addressed.

‡Author contributed equally

Contacts:

Peter G. Schultz; The Scripps Research Institute, 10550 North Torrey, Pines Road, La Jolla, CA 92037, USA; schultz@scripps.edu; Phone: (858), 784-9300; Fax: (858) 784-9440

Shaomei Wang; Regenerative Medicine Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA; shaomei.wang@cshs.org; Phone: (310) 248-7970; Fax: (310) 248-8066

Supporting Information: Additional evidence for *in vitro* proliferation and further characterization of differentiated RPE cells can be found in this section. This material is available free of charge via the Internet at <http://pubs.acs.org>.

be an effective treatment.(8) However, controlled differentiation protocols have not generated a pure RPE population, thereby increasing the risk of tumorigenesis from the remaining undifferentiated cell population.(9) Alternatively, freshly-isolated RPE cells from either adult or fetal sources could potentially restore vision.(4, 10–12) Unfortunately, primary RPE cells proliferate for a finite number of passages in culture and eventually enter senescence.(11, 13, 14) To overcome this, we demonstrate that a previously identified proliferative molecule for β -cells (15) (WS3; Figure 1A) can reversibly induce proliferation *in vitro* of primary fetal and adult human RPE cells. We also show that an expanded RPE population remains functional *in vitro* (upon differentiation) and *in vivo*, following subretinal transplantation into the dystrophic Royal College of Surgeons (RCS) rat.(16)

Previous screening efforts identified a diarylurea class of compounds capable of proliferating primary rodent and human β -cells.(15) We tested the ability of these compounds to induce proliferation in other primary cell types that could provide potential clinical utility from *ex vivo* expansion for transplantation. We demonstrate that WS3, the most potent diarylurea analog, proliferates primary RPE cells (rodent; human fetal; human adult). Briefly, cryopreserved primary human fetal RPE cells at passage one (ScienCell™ Research Laboratories) were cultured until the cell number leveled off at passage six due to senescence, as determined by viable cell counts. Cells were plated in 384-well plates and grown for three days in the presence of varying concentrations of WS3 (1.0 μ M to 1.0 nM) or DMSO (0.2% v/v) for an EdU proliferation assay. EdU-positive cells were quantified using high-content imaging (ImageXpress Ultra Confocal High Content Screening System). In non-proliferating RPE cells at passage six, 25 nM WS3 induced a dose-dependent incorporation of Edu, reaching its maximum with a four-fold increase over the DMSO control (Figure 1B). Both adult rat and adult human RPE cells responded similarly (Supplemental Figure 1). Analyses using Molecular Probes'™ Celltrace™ CFSE Cell Proliferation Kit provided additional evidence of increased cell division of fetal RPE cells in the presence of 25 nM WS3 (Supplemental Figure 2). Primary RPE cells need to be treated with WS3 prior to entering senescence (around passage 6) for the most effective expansion. However, initial expansion (passage 1–4) in the presence of WS3 does not increase the total number of cell doublings. This result suggests that the compound prevents cells from exiting the cell cycle. Since the vast majority of cells are actively dividing at early passages, addition of WS3 does not have much effect on proliferation, and therefore does not need to be present during initial expansion.

For chemically induced expansion of RPE cells to be a viable strategy for cell therapy, WS3 should reversibly induce cell proliferation over multiple passages without genetic alteration. To this end, we assayed serial passages of human fetal RPE cells expanded in the presence of 25 nM WS3 and quantified the doubling rate at each passage. Passage six cells were plated at 50% confluence and treated with 25 nM WS3 or DMSO (0.03% v/v). The cells were split every three days, viable cell numbers were determined, and cells were re-plated at the starting density. DMSO-treated cells immediately ceased dividing and eventually dropped to below 0.1 doublings per passage due to cell loss (Figure 1C). WS3-treated cells continued to double through passage fifteen with maximal doubling (1.95) at passage eleven. Following passage 14–16, cells entered senescence but did not appear to undergo cell death. Furthermore, these cells looked similar to those untreated at passage six. Importantly, we found that WS3-treated cells ceased dividing and cell number remained constant upon compound removal at passage eight or thirteen. Given the doubling rates observed through passage sixteen with WS3, a biopsy sample of 1000 RPE cells (17, 18) could theoretically provide 1×10^7 viable RPE cells for differentiation and/or transplantation (Supplemental Figure 3).

Since *ex vivo* expansion is often associated with a high incidence of karyotype aberrations, (19) providing a selective growth advantage to mutated cells, we analyzed WS3-expanded RPE cells for possible chromosomal rearrangements. Fetal RPE cells were grown to passage six, treated with 25 nM WS3 until passage ten, and then standard G-band karyotyping was performed. None of the 19 cytogenic analyses on metaphase cells revealed chromosomal abnormalities, suggesting that long-term culture of RPE cells in the presence of WS3 does not induce genetic alterations or tumorigenic events (Supplemental Figure 4).

At confluence *in vitro*, RPE cells can terminally differentiate over several weeks into a functional monolayer.(10, 20) During this process, cells become pigmented, adopt a typical RPE cobblestone morphology and are able to phagocytose photoreceptor outer segments (POS).(2) To confirm that WS3-expanded RPE cells can differentiate *in vitro*, passage ten human fetal RPE cells were plated at high confluence and cultured in the absence of WS3 for five weeks until terminal differentiation. During this period, RPE cells adopted a cobblestone appearance and became pigmented (Figure 2A–B).(4) Cellular staining indicated tight junctions as seen by positive staining for ZO-1 (Figure 2C, E and F). Expression of CRALBP and RPE65 further confirmed the formation of a mature RPE monolayer (Figure 2D and F).(21) Lastly, differentiated RPE cells possessed phagocytosis activity, as demonstrated by the internalization of POS. (Figure 2E). Undifferentiated cells did not express RPE cell markers or phagocytose POS (Supplemental Figure 5).

Similarly, WS3-expanded adult human RPE cells could also differentiate after compound withdrawal and acquire the pigmented, cobblestone phenotype of mature RPE cells (Data not shown). In addition, gene expression analyses of differentiated adult RPE cells via reverse transcriptase polymerase chain reaction (RT-PCR) confirmed the expression of known markers of mature RPE cells including *RPE65*, *RAX*, *MITF*, *BEST*, *PEDF* and *PMEL* (Supplemental Figure 6).(21, 22) Overall, these data show that WS3-proliferated fetal and adult human RPE cells retain their ability to differentiate into functional RPE monolayers *in vitro* upon WS3 removal. However, WS3-expanded RPE cells do not appear to differentiate as well as stem cell-derived RPE cells *in vitro*. Previous studies have shown that stem cell-derived RPE cells also differentiate better.(23, 24)

To determine if *ex vivo* WS3-expanded RPE cells remain functional *in vivo*, they were transplanted into RCS rats.(16, 25) RCS rats harbor an inactivating mutation in the *MERTK* gene, resulting in RPE cells that are incapable of POS phagocytosis.(26) Visual acuity, as tested by an optokinetic response (OKR), decreases with age – from 0.52 cycles/degree (c/d) at postnatal (P) day 30 to 0.30 c/d at P90 (as compared with 0.55–0.60c/d in wild type rat). (27, 28) Another test for functional photoreceptors is luminance threshold (LT) recording from the superior colliculus (SC). In the RCS rat, LT elevates from 1.2 log units at P30 to 2.8 log units at P90 (as compared to less than 0.4 log units in wild type rats). As this assay measures functional sensitivity across the visual field, it provides a topographic indication of the magnitude and the area of photoreceptor rescue across the whole retina.(29, 30)

In order to assess preservation of visual acuity and LT, RCS rats transplanted with WS3-expanded rat RPE cells were analyzed at P90. OKR analysis revealed a significant difference between eyes injected with RPE cells and control eyes (p -value< 0.001, Student's *t* test) (Figure 3A). An average of 0.49 ± 0.02 c/d was recorded in RPE injected eyes ($n=16$), whereas 0.34 ± 0.02 c/d in BSS injected ($n=8$) and 0.31 ± 0.04 c/d in untreated controls ($n=8$) were recorded (Figure 3A). LT recordings correlated well with visual acuity and revealed that RPE grafted eyes produced thresholds less than 1.7 log units over 35% of the SC area. In contrast, controls produced thresholds less than 1.7 log units over 2% of the SC area (Figure 3B). Thus, RPE grafted eyes have significantly lower thresholds than control eyes, indicating greater photoreceptor preservation.

To further support retinal lamination and photoreceptor preservation, histological examination indicated that RPE treated eyes had 4–6 layers of photoreceptors compared with a single layer in controls at P90 (Figure 3C vs. 3D). Visual function was comparable to previously published data using human embryonic stem (hES) cell-derived RPE cells in the same animal model.⁽⁵⁾ Human ES-RPE cells survived for longer and yielded better photoreceptor rescue, suggesting that further studies are required to improve survival and engraftment of WS3-expanded RPE cells. The number of cells injected, differentiation stage, and creation of new supports for fully differentiated RPE monolayers could improve graft integration. Long-term studies will be required to compare the effectiveness of freshly isolated, WS3-expanded and hES-derived RPE cells in the RCS model.

We next performed target identification experiments to determine if WS3 acts upon the same target as that previously identified in β -cells. Again Erb3 binding protein 1 (Ebp1) was found to be the target of WS3 in RPE cells on the basis of an affinity-based strategy using a biotin-conjugated analog of WS3 (WS9; Figure 1A). Targeted Ebp1-knockdown and transcriptional analyses of Ebp1 targets following WS3 treatment confirmed a role for WS3 in RPE cell expansion (Supplemental Figure S7A–E), consistent with previous studies performed in β -cells.⁽¹⁵⁾ However, β -cell target identification also detected IKK ϵ as a target of WS3 based on affinity chromatography and kinase profiling (at 500nM WS3). This target was not identified in RPE cells, and further kinase profiling must be done at a lower concentration to eliminate the possibility of alternate kinase targets. WS3 appears to act through Ebp1 as a non-cell specific proliferative molecule, and it may be useful for the *ex vivo* expansion of other primary cell types for transplantation. Additional studies are ongoing to further characterize the mechanism of action for WS3 and to identify other potential targets. Further optimization of WS3 or other such molecules may ultimately allow the expansion of small RPE biopsies to provide an adequate number of cells for autologous treatment of AMD and other visual degenerative disorders without the risks of teratoma formation associated with stem cell-derived RPE cells.

Methods

Cell culture

RPE cells were grown in EpiCM (ScienCell™ Research Laboratories) containing 2% Fetal Bovine Serum and growth supplements. Fetal RPE cells were purchased at passage one (ScienCell™ Research Laboratories). Both adult rat and adult human RPE cells were prepared as described in the Supplemental Information. All cells were grown at 37°C, 5% CO₂.

RPE proliferation assays and growth curves

Human RPE cells were grown to passage six, or otherwise indicated, in EpiCM. Cells were plated in tissue-culture treated 384-well plates at a density of 1500 cells per 50 μ L. Compound was immediately added to the cells in a volume of 100 nL DMSO in a three-fold dilution series and incubated for three days. On day three, cells were pulsed with 5 μ M 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen™ Life Technology) for 2 hours, fixed with 4% paraformaldehyde, and then washed three times with Phosphate Buffered Saline (PBS). The azide/alkyne cycloaddition reaction was performed per the manufacturer's protocol (Invitrogen™ Life Technology, Click-iT® EdU Alexa Fluor® 488 Imaging Kit). Cells were then stained with 2 μ g mL⁻¹ of Hoechst dye in PBS. Plates were imaged on an ImageXpress Ultra Confocal High Content Screening System (Molecular Devices). Built-in algorithms for multi-wavelength cell scoring were used to count total nuclei and EdU positive cells.

Growth curves were obtained by growing RPE cells at a density of 500,000 viable cells per 10 mL in a T75 flask either with 25 nM WS3 or an equivalent volume of DMSO (<1% v/v). Cells were passaged twice a week, and cell number was determined either by hemacytometry or ViCell® automated cell counter (Beckman Coulter).

Immunofluorescence

Cells in 96- or 384-well plates were washed with PBS, fixed with 4% paraformaldehyde, rinsed, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin in PBS containing 0.05% Tween 20. Antibodies used for immunostaining: anti-opsin (Abcam catalog #ab3267) Anti-ZO1, (Invitrogen™ Life Technology catalog #40-2200), anti-RPE65 (Millipore catalog #MAB5428), Anti- CRALBP (Santa Cruz biotechnology Inc. catalog #sc-59487)

RPE transplantation experiment in RCS rats

All animals were housed and handled with the authorization and the supervision of the Institutional Animal Care Committee. Experiments were carried out in accordance with the NIH guidelines regarding the care and use of animals for experimental procedures and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The RCS rats at P22 received subretinal injection of adult rat undifferentiated RPE cells (passage nine; grown in 25 nM WS3 for seven passages; n=16); control rats received Balanced Salt Saline (BSS) (n=8); and untreated rats (n=8) using a published protocol.(5) Adult rat RPE cells were used in all transplant experiments to avoid rejection. Additionally, all rats were maintained on cyclosporine A (CyA group) (Novartis) in the drinking water (210 mg L^{-1}) from one day before transplantation until sacrifice. A RPE cell suspension containing approximately 3×10^4 cells in 2 μL BSS was injected into the subretinal space through a small scleral incision from a fine glass pipette (internal diameter, 50–150 μm) attached by tubing to a 25 μL syringe (Hamilton). The cornea was punctured to reduce intraocular pressure and to limit the efflux of cells. A sham-surgery group was treated similarly, except only BSS was injected. Immediately after injection, the fundus was examined to assess retinal damage or vascular distress. Animals exhibiting such problems were removed from the study and not included in the animal counts described herein. Pigmented RCS rats received daily intraperitoneal injections of dexamethasone (1.6 mg kg^{-1}) for two weeks following transplantation to control acute inflammatory responses.

Measurement of visual acuity following RPE transplantation

Animals were tested for spatial visual acuity at P90 using an Optometry testing apparatus (CerebraMechanics), as previously described.(31) Luminance threshold (LT) provided parallel data to the visual acuity. LT was evaluated by recording single and multi-unit activity close to the surface of the superior colliculus (SC) from P90-P95 using previously described procedures.(29) Data are expressed as a graph of percentage of SC area with a LT below defined levels and as raw results. Statistical analyses were performed using GraphPad Prism. All variables were expressed as mean $\pm$ standard error of the mean. Differences between groups were compared by either Student's two tailed unpaired *t* test or analysis of variance. Newman-Keuls procedure was used for multiple comparison analysis. Differences were considered to be significant at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proliferation of fetal human RPE cells grown in the presence of WS3. (A) Chemical structures of WS3 and WS9. Synthetic schemes have been previously published.(15) (B) WS3 shows a dose-dependent increase in EdU incorporation at passage six (Mean±SD, n=4). (C) Fetal human RPE cells grown in the presence of 25 nM WS3 demonstrate that WS3-induced proliferation increases cell number and is reversible.

Figure 2.
(A–B) WS3-expanded fetal human RPE cells grown in the presence of WS3 and differentiated for five weeks. (C–F) Staining of differentiated fetal RPE cells show the formation of tight junctions, expression of mature RPE markers and function *in vitro* as determined by phagocytosis of internalized opsin.

Figure 3.

Restoration of a functional RPE monolayer in RCS rats at P90 following transplantation of rat RPE cells expanded with WS3. (A) Visual acuity (**p-value<0.001, Student's *t* test). (B) Luminescence threshold. (C & D) Histology of rat eyes transplanted either with untreated controls (C) or WS3-expanded RPE cells (D). Arrows indicate residual photoreceptor layer. These data show that WS3-expanded RPE cells are capable of preserving photoreceptors in RCS rats.