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Guiding the morphogenesis of dissociated newborn mouse retinal cells and hES cell-derived retinal cells by soft lithography-patterned microchannel PLGA scaffolds

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Abstract

Embryonic stem (ES) cell-derived photoreceptors are a promising cell source for enhanced in vitro models of retinal degenerative diseases, but the more differentiated characteristics of retinal cells do not typically develop in dissociated cell cultures. Therefore, we have reconstructed organized retinal tissue by seeding dissociated cells into an array of aligned units that more faithfully mimics the retina. We solvent-processed poly(lactic-co-glycolic acid) (PLGA) into a microchannel scaffold format to achieve this geometric constraint. We compared the effect of PLGA concentration on channel morphology and, along with other culture conditions, on the infiltration of dissociated newborn mouse retinal cells into the channels. Culturing scaffolds at the gas-liquid interface with low serum media increased infiltrated rod photoreceptor viability 18-fold over submerged, high serum cultures when evaluated after seven days. Rod photoreceptors and Müller glia aligned processes parallel to the microchannel walls. Otx2+ and Pax6+ subpopulations recapitulated lamination behavior. Further, we constructed scaffold/retinal pigment epithelium (RPE) co-cultures and observed rods extending rhodopsin-positive processes toward RPE cells, mimicking normal rod polarization and morphology. Finally, human embryonic stem cell-derived photoreceptors exhibited infiltration and morphological characteristics similar to mouse retinal cells inside the scaffolds. These findings constitute an important advance in generating tissue-level retinal models from dissociated cells for use as drug screening platforms and in regenerative medicine.

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Keywords

retina; scaffold; micropatterning; stem cell; cell morphology; biomimetic material

1. Introduction

Photoreceptor degenerations, including age-related macular degeneration and retinitis pigmentosa, cause visual impairment for millions of patients in the United States and worldwide [1, 2]. Current investigations into gene, cell, and small molecule pharmaceutical therapies to treat photoreceptor degeneration, while promising, are limited by fundamental gaps in our understanding of the establishment, maintenance, and deterioration of retinal architecture during degeneration. Although a number of mouse retinal degeneration models are available, there are no ideal mouse models for macular degeneration or many of the mutations underlying retinitis pigmentosa [3]. The advent of human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) technology promises the possibility of screening patient-specific retinal cells in culture [4]; however, the creation of valid disease models for many of these retinal degenerations requires that the more highly differentiated aspects of the retina be recapitulated in vitro. While dissociated cultures of mammalian retinal cells typically display rudimentary aspects of morphological differentiation, explant cultures of mammalian retinas have shown that cells can acquire highly differentiated characteristics when maintained in their organized state [5–8]. Therefore, the development of a method to construct three-dimensional retina-like tissue from dissociated cells, like those generated from hESCs, may provide the characteristics necessary for an ideal in vitro disease model.

The use of biomaterial scaffolds to direct the in vitro organization of dissociated retinal cells in a morphologically relevant manner has not yet been reported. Studies to date have focused on the use of such devices as cell delivery vehicles to the subretinal space in efforts to restore visual function following photoreceptor loss. These studies have shown that a variety of polymeric biomaterials, including poly(lactic-co-glycolic acid) [9–11], poly(glycerol sebacate) [12, 13], poly(methyl methacrylate) [14], and poly(caprolactone) [15–17] are supportive of retinal cell attachment and viability. These materials can be molded into various architectures using freeze casting/solvent sublimation techniques [9, 10], pressure-assisted microsyringe patterning [11], or photolithography/microfabrication strategies [12–16]. Following surface modification with adhesion-promoting proteins such as laminin and collagen, the resultant scaffolds may be seeded with retinal cells and cultured in vitro prior to transplantation. Although many diverse scaffold patterns have been evaluated, recapitulating the columnar spatial arrangement of the in vivo retinal environment by engineering the appropriate microscale features has proven to be elusive.

Progenitor cell lineage-tracing in the rodent retina [18] has revealed continuously juxtaposed yet distinct radial-columnar clonal units approximately 15–20 μm in diameter, suggesting this as the ideal size for engineered microchannels. We hypothesized that microchannel scaffolds tuned to these spatial dimensions could organize dissociated neonatal mouse retinal cells by facilitating neural and Müller glial process alignment and polarity. Employing soft lithography techniques, we patterned a silicone negative mold with high aspect ratio micropillar features and optimized patterning of the resultant PLGA scaffolds. We measured the effects on cell survival and morphology under various polymer and culture conditions. By constraining the retinal cells in the micro-columnar space during culture, we aimed to achieve a basic level of retinal organization dictated by the physical constraints imposed by the scaffold architecture, thereby mimicking with dissociated cells what is observed in vivo or in organotypic explant cultures. We confirmed the advanced state of

differentiation of retinal cells grown in microchannel PLGA scaffolds by examining rod, bipolar, and Müller glia marker expression and inner/outer retinal cell lamination. In combination with retinal pigment epithelium (RPE) co-culture, mouse and human embryonic stem cell-derived retinal cells grown in the scaffolds exhibited several aspects of phenotypic maturation, including the laminar separation of photoreceptors from amacrine cells and rudimentary photoreceptor outer segment disc membrane assembly.

2. Materials and Methods

2.1. Scaffold fabrication

We used AutoCAD 2011 software (Autodesk, Inc., San Rafael, CA) to design a two-dimensional master file and the Heidelberg μ PG 101 (Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, Germany) to write the pattern directly into SU8-2035 photoresist (MicroChem Corp., Newton, MA) spun at 1200 RPM for 30 seconds onto a 4-inch silicon wafer (Wafer Works Corp./Helitek Company, Ltd., Fremont, CA). The pattern consisted of $6\text{ mm} \times 6\text{ mm}$ (36 mm^2) square areas containing cutout circular polygons, each $15\text{ }\mu\text{m}$ in diameter and spaced $5\text{ }\mu\text{m}$ apart (edge-to-edge). The two layers in the AutoCAD DXF output file were subtracted into a single layer DXF for writing using LinkCAD 5 software (Bay Technology, Burlington, IA). The direct-write was performed at energy mode $\times 4$, 70% of 18 mW power with fixed autofocus. The patterned wafer was developed according to the manufacturer's instructions and surface treated with vacuum deposition of (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane for 30 minutes. Sylgard 184 silicone elastomer (Dow Corning Corp., Midland, MI) with a base to curing agent ratio of 10:1 was mixed and defoamed using a THINKY Mixer (THINKY USA, Inc., Laguna Hills, CA) and then poured onto the treated wafer and cured at 70°C for 1 hour. The resultant PDMS negative mold, comprised of micropillar features, was subsequently used for casting microchannel features. A solution of 5–20% (wt/vol) in acetone poly(D,L-lactic-co-glycolic acid) with a lactic to glycolic ratio of 75:25 and an average molecular weight of 65,000 g/mol, polydispersity 1.8 (Polysciences, Inc., Warrington, PA) was pipetted onto the PDMS, excess solution was removed, and the acetone was allowed to evaporate in a chemical fume hood or under vacuum. Under 100% ethanol, the micropatterned PLGA was peeled away from the mold using forceps, rinsed with sterile PBS, and treated with 0.25% Matrigel (BD Biosciences, San Diego, CA) in DMEM/F12 media (Invitrogen Corp., Carlsbad, CA) for one hour before use in cell culture experiments.

2.2. Mouse tissue dissection and cell culture

Postnatal day 5 (P5) mouse pups of the *Nrl-GFP* strain (a gift of Dr. Anand Swaroop [19]), which express EGFP under the rod photoreceptor-specific *Nrl* (neural retina leucine zipper) promoter, were anesthetized under ice, sprayed with 70% ethanol and decapitated according to protocols approved by the University of Washington Institutional Animal Care and Use Committee. The eyes were enucleated, placed in ice-cold PBS, and the neural retina dissected free of the lens, RPE, and surrounding scleral tissues. Retinas from several pups were pooled and dissociated to a single cell suspension by papain (Worthington Biochemical Corp., Lakewood, NJ). Retinas were nutated in the papain solution with DNase at 37°C for 10 minutes, triturated vigorously 10 times with a P1000 tip, nutated at 37°C for an additional 10 minutes, and triturated 10 more times. The papain was then neutralized in ovomucoid and the cells were collected by centrifugation at $250 \times g$ for 3 minutes at 4°C , then resuspended in ice-cold calcium/magnesium-free PBS (CMF PBS) and kept on ice during seeding to prevent cell clumping. For RPE explants, freshly enucleated whole eyes were placed in a 0.0125% proteinase K (Invitrogen) solution in sterile CMF PBS and nutated at 37°C for 10 minutes, followed by neutralization in 10% FBS at 4°C . A 21-gauge needle was inserted into the corneal-scleral margin and this opening was exploited with forceps to

gently remove the cornea and sclera. The intact RPE, lens, and retina were then transferred to a Millicell-CM 30 mm culture plate insert (Millipore Corp., Billerica, MA) previously coated with a thin layer of undiluted Matrigel. The lens was removed with forceps and approximately 100 μ l of PBS was added to the membrane to facilitate separation of the retina from the RPE. After the retina was removed, the PBS was aspirated to flatten the RPE. Dissociated cells or scaffolds were then placed on top of RPE explants.

2.3. Human embryonic stem cell culture

Retinal progenitor cells were derived from the H1 human embryonic stem cell line (hESC-RPCs) as previously reported [20]. Undifferentiated colonies of H1 cells were passaged and treated with media containing IGF-1, Dkk-1, and Noggin to promote the anterior neural (i.e., retinal) fate. Expression of eye-field transcription factors was verified by quantitative RT-PCR prior to use. Just before seeding, hESC-RPCs were gently dissociated with trypsin, the enzyme was neutralized in FBS, and the cells collected by centrifugation at $250 \times g$ for 3 minutes at 4°C. Cells were resuspended in DMEM/F12 media supplemented with 1% BSA, N2 supplement, B27 supplement, sodium pyruvate, non-essential amino acids, HEPES buffer, and penicillin/streptomycin (all from Invitrogen).

2.4. Cell seeding and culture in scaffolds

Cells were plated at a density of 75,000 cells/mm² in a volume of 5–10 μ l on scaffolds that had been positioned at the bottom of wells in 48-well plates (Corning) or onto 30 mm Millicell-CM culture plate inserts placed into 6-well plates (Corning). Culture media consisted of DMEM/F12 media supplemented with 10% or 0.5% FBS, N2 supplement, B27 supplement, sodium pyruvate, non-essential amino acids, HEPES buffer, and penicillin/streptomycin. Media (250 μ l per scaffold/well in 48-well plates or 1 ml between the insert and well for culture inserts) was changed daily.

2.5. Cryosectioning

After the desired culture period, scaffolds were immersed in a 4% paraformaldehyde/PBS solution for 30 minutes. The scaffolds were rinsed with PBS and then dehydrated in escalating sucrose concentrations from 10% to 30%, followed by a solution half comprised of OCT compound (Sakura, Torrance, CA) and half 30% sucrose, and then finally 100% OCT and snap-frozen at -80°C for 30 minutes. Ten-micron thick frozen sections were taken perpendicular to the scaffold surface and adhered to glass slides. The slides were allowed to dry on a slide dryer.

2.6. Immunohistochemistry and fluorescence microscopy

Prior to immunostaining, slides were rehydrated in PBS for 5 minutes. The rehydrated sections were blocked and permeabilized with 6% FBS, 0.5% Triton-X-100 in PBS for 2 hours. Sections were then incubated overnight at 4°C with the following primary antibodies: guinea pig anti-glutamate transporter GLAST (1:500, AB1782, Millipore; to mark Müller glia cells), rabbit anti-cleaved caspase 3 (1:200, 559565, BD Biosciences, San Jose, CA; to mark apoptotic cells), biotinylated goat anti-Otx2 (2.5 μ g/ml, BAF1979, R&D Systems, Minneapolis, MN; to mark the photoreceptor/bipolar cell lineage), mouse anti-Pax6 (1:25 supernatant, Developmental Studies Hybridoma Bank, Iowa City, IA; to mark retinal progenitors and the ganglion/amacrine cell lineage), and mouse anti-rhodopsin (1:10 supernatant, a gift of Robert Molday, University of British Columbia; to mark rod photoreceptors). Endogenously expressed mouse Nrl-GFP was bright enough that immunostaining for GFP was not necessary. Slides were rinsed and incubated with appropriate secondary antibodies for 1 hour at room temperature, counterstained with DAPI, and extensively washed with PBS. Images were acquired on a Nikon A1 Confocal Laser

Scanning Microscope. Quantitation of cell numbers was performed manually using the NIS Elements Software package.

2.7. Scanning electron microscopy (SEM)

Scaffolds and PDMS negative molds were prepared for imaging with SEM by immersion in 100% ethanol, followed by drying in a vacuum overnight and sputter coating with a 7 nm thin film of Au/Pd. Images were acquired on an FEI Sirion Field Emission SEM at a working distance of 5 mm with an accelerating voltage of 5 kV and a spot size of 3. Open channel density measurements were performed on SEM images using ImageJ.

2.8. Transmission electron microscopy (TEM)

To detect the development of rod outer segment (ROS) disc membranes, cell-seeded scaffolds were prepared for TEM imaging by fixation in half-strength Karnovsky's fixative [21] for several days. Fixative was rinsed away with sodium cacodylate buffer. The scaffolds were stained with 1% osmium tetroxide for 1 hour, rinsed, stained with an uranyl acetate solution for 20 minutes, and rinsed again with buffer before being taken through a 70% to 100% ethanol dehydration series. Dehydrated samples were immersed in propylene oxide and then embedded in EMbed 812 resin (Electron Microscopy Sciences, Hatfield, PA) before being cured for 24 hours at 60°C. Ultrathin (80 nm) sections were taken and contrasted with lead citrate before being placed on copper grids for imaging with a JEOL JEM 1200EXII at 80 kV and a spot size of 3.

3. Results

3.1. Scaffold fabrication

To design scaffolds capable of facilitating the self-assembly of organized retinal tissue from dissociated cells, we considered the anatomical arrangement of neurons and glia in the mammalian retina. Based on lineage tracing experiments performed in rodents that tracked clonal populations of dividing progenitor cells [18], we chose a scaffold pattern that consisted of high aspect ratio cylindrical pores arrayed in a highly packed, Z-oriented configuration. These microchannel scaffolds were thus made to mimic the many columnar units which are present in the retina.

Scaffolds with the desired dimensional features (40–50 μm thick with 15 μm diameter channels spaced 5 μm apart) were fabricated using direct-write SU8 photolithography for generation of a patterned silicon master, followed by PDMS negative mold casting from the master. High aspect ratio PDMS micropillars (Fig. 1A) were infused with PLGA solutions with concentrations ranging from 5–20% (wt/vol) in acetone and, following solvent evaporation, the molds were placed in 100% ethanol to enable removal of the patterned PLGA from the PDMS (Fig. 1B). While the scaffold “top-side” (the side originally abutting the bottom of the PDMS mold) exhibited a near-perfect channel morphology in all conditions (Fig. 1C–E), the scaffold “bottom-side” morphology was far more sensitive to the PLGA concentration and solvent evaporation technique employed. We found that if the solvent was evaporated in a chemical fume hood, low PLGA concentrations (5%) resulted in open channels but did not provide enough final deposited material to complete the formation of channel sidewalls (Fig. 2B,D,F). The use of vacuum to rapidly evaporate the solvent preserved channel sidewall morphology in 5% PLGA scaffolds but resulted in the closing off of most channel bottoms as polymer was left behind on top of the PDMS micropillars (Fig. 2A–A”, F). In contrast, 10% and 15% PLGA solutions with fume hood-aided solvent evaporation produced well-formed channel sidewalls with open channel pores (Fig. 2C,E,F). While 20% PLGA solution produced adequate channel sidewalls, the viscosity of the solution led to the closing off of many channel pores (Fig. 2F). Quantification of the number

of open channels per mm² for each of the PLGA concentrations and evaporation conditions is shown in Fig. 2F. The optimal conditions in terms of scaffold morphology were identified as 10% PLGA solution with fume hood-aided evaporation.

3.2. Optimization of culture conditions and dissociated mouse retinal cell morphology inside microchannels

Dissociated retinal cells were used to assay the effects of the engineered PLGA scaffolds on primary retinal neurons. We used cells from Nrl-GFP mice [19] (Fig. 3A) in order to trace rod photoreceptors directly by their bright GFP signal. To assess material toxicity of PLGA to rod photoreceptors, we plated the cells on PLGA films and immunostained for the activation of caspase 3 (AC3), a marker of apoptosis. Cells co-expressing Nrl-GFP fluorescence (Fig. 3D) and AC3 (Fig. 3B) were counted as apoptotic rods (Fig. 3C,F). Comparison to a poly-D-lysine (PDL)-coated glass coverslip control over a four day period revealed that PLGA preserved overall rod number as well as or better than PDL-coated glass did (Fig. 3E) and that apoptosis was observed in less than 2% of the rod population (Fig. 3F). After 4 days of culture, the percentage of cells co-expressing GFP and AC3 was significantly lower on PLGA than PDL-coated glass ($p = 0.001$, two-tailed unpaired t -test).

We next plated the dissociated mouse retinal cells into the patterned PLGA scaffolds and cultured them for 3 or 7 days. To examine the histological arrangement of cells inside the microchannels, scaffolds were fixed and cryosectioned perpendicular to the scaffold surface. Culturing the cell-seeded scaffolds submerged under even small amounts of media caused rapid cell death, with only 1.45 ± 0.40 cells per channel (10.9% of seeded) populating the 5% PLGA scaffolds after three days of culture, decreasing to 0.89 ± 0.08 cells per channel (6.72% of seeded) after seven days (Fig. 4A,D). This effect was exacerbated by higher PLGA concentrations (data not shown), suggesting compromised availability of dissolved oxygen.

To improve cell survival, we cultured scaffolds at the gas-liquid interface on Millicell culture plate inserts as is typically done for retinal explants. This resulted in robust survival and extension of processes from rods and Müller glia inside the channels (Fig. 4B–D). Nrl-GFP+ rods could be found aligning their processes along those of Müller glia immunolabeled by Glast (Fig. 4C). Quantification of retinal cell numbers in the scaffolds grown for 3 or 7 days with media containing 10% FBS (Fig. 4D) showed similar survival in 10% and 15% PLGA scaffolds at 3 days and in all PLGA concentrations evaluated after 7 days.

Because gas-liquid interface methods improved short-term survival of retinal cells cultured in the scaffolds but still resulted in the loss of approximately half the day 3 cells by day 7 (Fig. 4D), we also assessed the effects of serum concentration on cell survival in the scaffolds. We found that scaffolds cultured with low serum (0.5%) (Fig. 5B) demonstrated significantly greater rod (Nrl-GFP+) and total cell (DAPI) survival relative to those cultured with high serum (10%) (Fig. 5A). Quantification of cells per channel after 7 days (Fig. 5C) and percent cell survival per channel between 3 and 7 days (Fig. 5D) revealed that this gain was almost entirely attributable to the improved survival of rods alone: there were 4.12 more total cells per channel at 7 days in 0.5% FBS relative to 10% FBS, while rods alone increased by 3.95 cells per channel, accounting for 96% of this observed total cell increase.

3.3. Lamination of cell populations within the scaffolds

To ascertain whether tissue lamination was recapitulated by dissociated neonatal mouse retinal cells cultured in the 3D context of the scaffolds, we labeled sections of the scaffold cultures with antibodies to specific retinal cell types. Pax6 and Otx2 are expressed in non-

overlapping cells in the retina; Pax6 is expressed in amacrine and ganglion cells (inner retina), while Otx2 is expressed in bipolar cells and photoreceptor cells (rods and cones in the outer nuclear layer, ONL) (Fig. 6A). When we analyzed the cells grown in scaffolds for expression of these two markers, we found that they tended to form a non-overlapping laminar arrangement similar to that of normal retina. Otx2+ cells had a propensity to navigate more deeply (i.e., outer retina) into the scaffold than Pax6+ cells (Fig. 6B-B"). Binning the depth distribution into 10 micron zones (Fig. 6C) revealed that the shallowest 20 microns (i.e., inner retina) contained 86.2% of the Pax6+ cells in the sample and the deepest 20 microns contained 80.9% of the Otx2+ cells. The fraction of total Pax6+ cells residing in the first (innermost) 10 microns was significantly higher than the fraction of total Otx2+ cells residing in this same zone ($p = 0.005$, two-tailed unpaired t -test). Mid-way into the scaffold (depth of 10–20 microns), there was no significant difference between fraction of either population. In contrast, the fraction of total Otx2+ cells residing in the deepest (outermost) 10 microns (depth of 20–30 microns) was significantly higher than the fraction of total Pax6+ cells residing in this zone ($p = 0.001$, two-tailed unpaired t -test). The mean fraction of cells residing in given binned-depths was significantly different between bins for both Otx2 distributions ($p = 3 \times 10^{-5}$, oneway ANOVA) and Pax6 distributions ($p = 0.002$, one-way ANOVA).

3.4. Retinal cell-seeded scaffold/RPE co-cultures

Because the RPE is known to play a major role in photoreceptor morphogenesis, we built retina-like scaffold/RPE sandwich tissue cultures to further recapitulate ocular anatomy and promote more advanced stages of photoreceptor differentiation. RPE explants were made through a modification of the protocol of Caffé et al. [8], with the apical surface facing upward (Fig. 7A). Scaffolds were laid onto the RPE explant and seeded with dissociated Nrl-GFP mouse retinal cells. Staining for rhodopsin in cross sections (Fig. 7B,D) revealed rods extending processes towards the RPE monolayer, similar to their appearance in vivo.

To examine the ultrastructural characteristics of the scaffold/RPE co-cultures, we further analyzed them with transmission electron microscopy (TEM). Dissociated mouse retinal cells were either directly seeded onto the RPE or seeded onto scaffolds laid onto the RPE. The cultures were allowed to survive for 14 days in vitro. When cultured without scaffolds, photoreceptors lacked features of maturation, namely the formation of any outer segment disc material (Fig. 7F). In contrast, when the cells were grown in scaffolds we found sparse outer segment disc material that had been assembled by photoreceptors in apposition to the RPE monolayer (Fig. 7E,E'). While we were unable to detect fully organized outer segments, the partial assembly of these mature features suggests that the organization provided by the scaffolds in combination with RPE co-culture can promote photoreceptor maturation.

3.5. Human embryonic stem cell-derived retinal progenitor cell-seeded scaffolds

Although the scaffold design process was optimized on mouse retinal cells, the ideal application of the microchannel array is to construct embryonic stem cell-derived or induced pluripotent stem cell-derived retinal progeny into retina-like tissues for use in disease models, drug screening platforms, or transplantation. Using previously reported protocols, we directed hESCs toward retinal fates [20]. Briefly, hESCs are treated with a combination of Dkk1, Noggin, and IGF-1 for three weeks, directing their differentiation towards an "eye-field" fate; further culture of these eye-field cells in retinal progenitor medium leads to their production of differentiated retinal cells, including rod and cone photoreceptors. After retinal cells were derived from hESCs according to this protocol, the cells were dissociated and then plated onto scaffolds for further culture as described above for the mouse cells.

Like mouse retinal cells, hESC-derived retinal cells aligned their processes along the microchannels.

Analysis of Pax6+ and Otx2+ staining revealed a more striking stratification than that observed in dissociated mouse cell cultures. When cultured on filter inserts alone, hESC-derived cells formed neural rosettes but otherwise appeared to lack organization in cross-section (Fig. 8A). When cultured in scaffolds on filter inserts, however, there was a distinct separation observed between a region containing presumptive photoreceptors (Otx2+ cells) and a region containing presumptive ganglion and amacrine cells (expressing Pax6, but not Otx2) (Fig. 8C). Binning the depth distribution into 10 micron zones showed that cells grown on the filter insert alone (Fig. 8B) did not show significant difference in the distribution of photoreceptor cells between bins ($p = 0.86$, one-way ANOVA). In contrast, for scaffold cultures (Fig. 8D), the mean fraction of cells residing in given binned-depths was significantly different between bins for both photoreceptor cells ($p = 8 \times 10^{-12}$, one-way ANOVA) and amacrine cells ($p = 8 \times 10^{-4}$, one-way ANOVA). The fraction of total photoreceptor cells was significantly higher than amacrine cells in the deepest (outermost) 10 microns ($p = 7 \times 10^{-6}$, two-tailed unpaired *t*-test). Between 30 and 40 microns, there was no significant difference between cell distributions. However, there were significantly more amacrine cells than photoreceptor cells in the 20–30 micron depth ($p = 0.03$, two-tailed unpaired *t*-test), 10–20 micron depth ($p = 2 \times 10^{-7}$, two-tailed unpaired *t*-test), and 0–10 micron depth (no Otx2+ cells present).

4. Discussion

We have shown the construction of retina-like tissues from dissociated mouse retina and hESC-derived cultures in a manner that considers relevant aspects beyond scaffold fabrication, basic biocompatibility, and attachment of cells to the biomaterial substrate. Scaffold design must address the ultimate goals for its use, and in this study we aimed to advance important aspects of retinal tissue engineering, including evaluation of the specific toxicity of the biomaterial to confirmed rod photoreceptors, fabrication of anatomically relevant, bio-inspired morphological features, examination of the appropriate rod, bipolar, and Müller glia markers, and optimization of tissue culture conditions to promote survival and the establishment of advanced tissue properties.

Our scaffold pattern was chosen in order to maximize the density of useable high aspect ratio channels, rather than two-dimensional, spaced out pore patterns such as those that have been designed to act as thin patches for cell transplantation. Optimization of the microfabrication process for a commonly used, FDA-approved, biodegradable material such as PLGA ensured that the morphological characteristics of the scaffold were appropriate for the intended purpose of assembling retinal cells but that, should the device be used for transplant applications, the synthetic component could serve its purpose in organization and delivery of the cells and then degrade away in the implant site. The monomers of PLGA degradation, lactic and glycolic acid, are metabolites frequently processed in high concentrations by the pigment epithelium for clearance from the subretinal space [22]. Therefore, resorption of these scaffolds would not be expected to significantly lower microenvironmental pH, and the relatively immune-privileged status of the retina would reduce foreign body response.

Survival of cells cultured inside of scaffolding materials is an obvious, but nontrivial prerequisite that must be satisfied before more advanced tissue properties, especially those requiring long culture periods to establish, can be investigated. Although the material, PLGA, was itself nontoxic to rods as assayed by immunolabeling for a marker of apoptosis in cells cultured on films, preserving survival of the fragile retinal neurons inside of

microchannels made of the same material proved more complicated than a simple analysis of material toxicity. Initial cell-seeding experiments highlighted the importance of the diffusion of gases and nutrients to retinal progeny for their survival, since increased PLGA concentrations resulted in fewer surviving cells in a media-submerged context. The observation that 5%, 10%, and 15% PLGA scaffolds exhibited small differences in number of cells surviving after 7 days of culture (Fig. 4D) suggests that gas-liquid interface culture can circumvent survival differences due to PLGA concentration. Further, a common element of cell culture media, serum, was shown to have detrimental effects on the survival of retinal cells, specifically rods, cultured inside the scaffolds. In combination with reduced media serum concentration, gas-liquid interface culture was able to reduce rod loss to only ~10% between 3 and 7 days in culture, a 6-fold reduction in cell death compared to the high serum condition and which resulted in an 18-fold higher number of surviving rods compared to submerged cultures. Collectively, these data suggest that the scaffolded cell cultures possess “hybrid” needs representative of both dissociated cells (low serum, submergible) and retinal explants (high serum, gas-liquid interface).

The observation that rod processes (Nrl-GFP) aligned along those of Müller glia (Glast) along the channel pores suggests that this scaffold design promotes the recapitulation of retinal anatomy in a relevant 3D context. Müller glia are known to play several roles in supporting photoreceptors, including their provision of an important substrate onto which rods and cones attach, extend neurites, and establish polarity [23]. In 2D dissociated cultures, this occurs in a less useful orientation which cannot be juxtaposed onto RPE monolayer co-cultures to generate complex, retina-like tissues. We found that this aligned, 3D organization could be exploited in combination with RPE co-culture to promote maturation of photoreceptors as evidenced by rudimentary outer segment disc morphogenesis. This is a significant development for the field of regenerative medicine and drug screening because mature photoreceptors will function and respond in a manner more like their in vivo counterparts than immature photoreceptors.

One of the most daunting barriers to the use of hESC-derived specialized cell types in transplantation or in vitro disease modeling applications is the failure of these cells to progress to a fully differentiated state or organize into structures resembling tissues. While the attainment of a significant conversion of hESCs to multipotent progenitors has been extensively shown for a variety of tissue types, the yield of terminally differentiated, functional cells has been far less successful. This problem currently faces investigators across the field of regenerative medicine, and the retina is no exception. Our patterned biomaterial scaffolds show evidence for arranging the appropriate three dimensional contexts that allow hESC-derived retinal cells to assemble inside the microchannels into stratified layers appropriate for specific cell subtypes. Taken into context with the significant but less striking lamination of inner/outer retinal cells observed in the neonatal mouse cell cultures, this observation may reflect the relatively earlier developmental status of these cells, a property which may convey superior morphogenetic competence. Collectively, these findings suggest that appropriately micropatterned scaffolds can promote later stages of differentiation and direct the assembly of tissue into complex architectures that are required for function.

5. Conclusions

We present a bio-inspired pattern for the generation of microchannel scaffolds that direct the morphogenesis and differentiation of newborn retinal cells derived from mice or human embryonic stem cells. The scaffolds support retinal cell viability and facilitate the morphologically relevant expression of rod and Müller glia markers of differentiation. We conclude that inner/outer retinal lamination and polarization of photoreceptors are facilitated

by these scaffolds following the optimization of fabrication methods and cell culture parameters. These findings further efforts to generate tissue-level retinal models from dissociated cells for use in a variety of applications.

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Fig. 1.

Fabrication of PLGA microchannel scaffolds using soft lithography. (A) Scanning electron micrograph of micropillar negative mold in PDMS patterned from SU8/silicon master. (B) Patterning and removal of PLGA from PDMS negative mold using forceps under ethanol. (C,D,E) Scanning electron micrographs of top side of resultant patterned PLGA microchannel scaffolds, showing channel features approximately 40 μm tall, 15 μm in diameter and spaced 5 μm apart (edge-to-edge). (C) Overhead view. (C') Higher magnification of (C). (D) Alternate view at 45 degree tilt angle with cutaway showing inside walls of channels. (E) Alternate view at 45 degree tilt angle showing the edge of a scaffold. (E') Higher magnification of (E). Scale bars in (A) and (C) = 50 μm , (C') and (D) = 10 μm , (E) = 100 μm , and (E') = 20 μm .

Fig. 2.

Optimization of channel morphology of scaffold bottom side. (A) Scanning electron micrographs reveal morphology of channel bottom side when vacuum is applied to aid solvent evaporation in order to enhance channel morphology of 5% PLGA scaffolds, showing mostly closed-off channels (A') except at the corners (A'', 45 degree tilt angle). (B) Overhead view and (D) 45 degree tilt angle view of 5% PLGA scaffold channel bottoms produced without vacuum deposition. (C) Overhead view and (E) 45 degree tilt angle view of 10% PLGA scaffold channel bottoms produced without vacuum deposition. Note the open channels throughout the scaffold. (F) Quantification of open channels per square millimeter for the various percentage PLGA concentrations and solvent evaporation methods ($n = 3$ scaffolds per condition; error bars represent standard deviation). Scale bars in (A), (B), and (C) = 200 μm , (A') = 10 μm , (A'') = 20 μm , and (D) and (E) = 100 μm .

Fig. 3.

Dissociated newborn mouse rod photoreceptor survival on PLGA compared to poly-D-lysine-coated glass coverslips. (A) In cross-section, the Nrl-GFP retina shows GFP expression only in the rod photoreceptors of the outer nuclear layer (ONL). DAPI counterstain is shown in blue. GCL = ganglion cell layer; INL = inner nuclear layer. (B) Cultures stained for activated caspase 3 (AC3, red) and expressing (D) Nrl-GFP (green) to reveal (C) apoptotic rods (yellow, with white arrowheads). (E) Quantification of rods as a percentage of the total cells (DAPI, not shown) and (F) AC3+ rods on the different surfaces ($n = 6$ coverslips or scaffolds per condition; error bars represent standard deviation; ** $p = 0.001$ (two-tailed unpaired t -test)).

Fig. 4.

Dissociated neonatal mouse retinal cells cultured in microchannel scaffolds with rods marked by Nrl-GFP, visualized with DAPI counterstain and DIC overlay. (A) Typical scaffold cross section after 3 days of culture submerged under media. (B) Typical scaffold cross section after 3 days of culture atop filter inserts at the gas-liquid interface, showing cell infiltration, viability, and process outgrowth from rods. Scale bar = 20 μ m. (C) Nrl-GFP + rod photoreceptors (green) and Glst+ (red) Müller glia aligning and extending processes parallel to the channel walls, sometimes making endfoot-like processes at the ends of channels (white arrowheads). (D) Quantification of retinal cells in the microchannel scaffolds, revealing significantly enhanced infiltrated cell viability in all PLGA concentrations compared to that observed in submerged scaffold cultures at 3 and 7 days in vitro (div) (10% FBS culture media, $n = 3$ scaffolds per condition; * $p < 0.05$ relative to scaffolds cultured on filters at equivalent timepoints (two-tailed unpaired t -test); error bars represent standard deviation).

Fig. 5.

Effect of serum concentration on survival of dissociated mouse retinal cells cultured inside microchannel scaffolds. (A,B) Representative section showing cells cultured at the gas-liquid interface for 7 days with (A) 10% FBS media and (B) 0.5% FBS media. (C) Reducing the media serum concentration to 0.5% FBS rescues cells at the 7 day timepoint relative to 10% FBS ($n = 3$ scaffolds per condition; ** $p = 0.001$ (two-tailed unpaired t -test); error bars represent standard deviation). (D) Low serum improves percentage cell survival per channel between 3 and 7 days when considering all cells (DAPI) and rods (Nrl-GFP) ($n = 3$ scaffolds per condition; ** $p = 0.001$ (two-tailed unpaired t -test); error bars represent standard deviation). The increased total cell survival is almost exclusively due to improved rod survival (see text).

Fig. 6.

Lamination of dissociated mouse retinal cells cultured in microchannel PLGA scaffolds. (A) Cryosection of a P6 mouse retina with the outer (apical) retinal surface down, showing lamination of Pax6+ (red, inner retina) and Otx2+ (green, outer retina) cell populations. (B,B',B'') Cross-sections of dissociated neonatal mouse retinal cells cultured in microchannel scaffolds for 3 days. (C) Binned-depth distribution of Pax6+ and Otx2+ cells in the scaffolds plotted as a fraction of all the cells expressing the marker of interest ($n = 6$ scaffolds; $p = 3 \times 10^{-5}$ for Otx2 distribution between bins (one-way ANOVA) and $p = 0.002$ for Pax6 distribution between bins (one-way ANOVA); * $p = 0.005$ (two-tailed unpaired t -test), n.s. = not significant, ** $p = 0.001$ (two-tailed unpaired t -test); error bars represent standard deviation).

Fig. 7.

Dissociated neonatal Nrl-GFP mouse retinal neurons cultured in scaffolds layered on retinal pigment epithelium (RPE) explants. (A) Following treatment with proteinase K, the sclera is removed from the RPE/choroid and the RPE/choroid sheet is flat-mounted with the apical surface facing upward. The neural retina is then dissociated to single cells by papain and seeded into a microchannel scaffold placed on top of the RPE explant. (B) Cross-section of a scaffold showing Nrl-GFP+ rods, stained with anti-rhodopsin antibody (red) and DAPI (blue). DIC overlay shows pigment in the RPE explant below the scaffold. (C) Transmission electron micrograph of an ultrathin section of the scaffold/RPE culture. (D) Higher magnification of a single microchannel in a portion of a scaffold cross-section after 7 days in vitro, showing a rhodopsin+/GFP+ double-labeled rod elaborating a process toward the RPE cells (visible with DIC overlay). (E) Electron micrograph of a region of interest at the interface between rods cultured in the scaffold and the RPE after 14 days in vitro, showing the genesis of rod outer segment disc material. (E') Magnification of the highlighted region in (E) shows outer segment disc material in greater detail. (F) Electron micrograph of dissociated neonatal mouse retinal cells cultured atop filter inserts without scaffolds for 14 days. Note the lack of organization and absence of rod outer segment disc material. Scale bar in (E) = 5 μm and (E') = 0.5 μm .

Fig. 8.

Human embryonic stem cell (hESC)-derived retinal cells cultured in microchannel scaffolds at the gas-liquid interface. (A) Cross-section of hESC-derived cells cultured on a filter insert at the gas-liquid interface shows neural rosette formation in cells co-expressing Pax6 and Otx2, but no other organization. (B) Binned-depth distribution of Otx2+ and Pax6+ cells cultured without scaffolds ($n = 6$ filter cultures; $p = 0.86$ between bins (one-way ANOVA); error bars represent standard deviation). (C) Cross-section of hESC-derived cells cultured in scaffolds on filter inserts at the gas-liquid interface shows stratification of Otx2 expressing cells into a layer distinct from one with cells expressing Pax6 alone. (D) Binned-depth distribution of Otx2+ and Pax6+ cells in the scaffolds ($n = 6$ scaffolds; $p = 8 \times 10^{-12}$ for Otx2+ distribution between bins (one-way ANOVA) and $p = 8 \times 10^{-4}$ for Pax6+ distribution between bins (one-way ANOVA); * $p = 0.03$ (two-tailed unpaired t -test), n.s. = not significant, ** $p < 10^{-5}$ (two-tailed unpaired t -test); error bars represent standard deviation).