Supplementary Information

Supplemental Methods

Materials

Human embryonic stem cells (hESC), WA09, were obtained from WiCell, (Madison, WI).

Human induced pluripotent cells (hiPSC), Y6 and IMR90-4, were obtained from the Yale Univ.

Stem Cell CORE (New Haven, CT) and WiCell, respectively. Human fetal RPE was a gift of A.

Mamaniskis and S. Miller (National Eye Institute, Bethesda, MD). Chondroitin sulfate, >90%,

was obtained from Alfa Aesar (Ward Hill, MA), gelatin type A from fish skin from J.T. Baker

(Phillipsburg, NJ), and hyaluronic acid from Calbiochem/Millipore (M.W. 50,000; San Diego,

CA). Ammonium persulfate, O.T.C. compound, and 4,6-diamidino-2-phenylindole (DAPI) were

obtained from Thermo-Fisher Scientific (Fair Lawn, NJ) and glutaraldehyde, 50%, from Merck

(Solon, OH). Tri-Buffer-saline with 1% Tween 20, phosphate buffer saline (PBS) (pH 7.4, 1.4M

NaCl, 0.1M phosphate, 0.03M KCl), and TEMED were purchased from American BIO (Natick,

MA). Milli-Q-grade water was used in all experiments except for gene expression in which

nuclease free-water from Bio-Rad i-Script cDNA synthesis kit was used. iTaq® Universal SYBR

Green Supermix, and custom PCR arrays were manufactured by Bio-Rad (Hercules, CA).

AlamarBlue was obtained from Accurate Chemical (Westbury, NY). Unless indicated

otherwise, all other chemicals and solvents were used without further purification, were

purchased from Sigma-Aldrich (St. Louis, MO).

Fabrication of the GCH-521 scaffold

The original method for fabricating the scaffolds29 was modified by the addition of laminin-521.

Briefly, gelatin (500 mg), chondroitin sulfate (250 mg) and hyaluronic acid (500 mg) was
dissolved in 10 ml double-distilled, degassed water and cross-linked with 20 μL ammonium persulfate (25 mg/ml), 20 μL tetramethylethlenediamine, and 50 μL glutaraldehyde, 0.5%. The solution was frozen in a 5-ml syringe barrel at -20°C for 16-18 hrs, and vacuum dried in a lyophilizer. The 3D scaffold monolith (GCH) was embedded in OCT and sectioned at -25°C to create 60 μm thick planar sheets with a 1.0 cm-diameter. The sheets were sterilized in a series of graded alcohols (20% to 80%) and stored at 4°C. Scaffolds were placed in a 48-well plate washed three times with PBS, treated with penicillin-streptomycin for 30 mins and finally left overnight at 37°C in a humidified incubator in neural induction medium (NIM): 50% Dulbecco’s modified Eagle’s medium, high glucose (DMEM)/50% F-12 nutrient medium (Gibco/Life technologies, Grand Island, NY) supplemented with 15% knockout serum (Invitrogen/Thermo-Fischer, Waltham, MA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1% N2 supplement, 1 mM glutamax, 0.1 M nonessential amino acids (Invitrogen), 2 μg/ml heparin, 50 U/ml penicillin, and 50 μg/ml streptomycin. The NIM was removed and the scaffolds dried by incubating them at 37°C. Laminin-521 (Stem cell technologies, Vancouver, CA) was diluted to 1.0 μg/ml in SFM (70% high glucose DMEM and 30% F-12 supplemented with 2% B27, and penicillin/streptomycin), and 20 μl was dropped onto each scaffold. These scaffolds were incubated at 37°C for 30 mins to allow complete absorption of the laminin-521.

**Differentiation and culture of retinal progenitor cells**

RPC were generated from hESC and hiPSC. Undifferentiated stem cells were maintained and differentiated as described previously.\(^9\) Briefly, RPC were differentiated by dissociating the stem cells with 1 U/ml dispase, suspended in mTeSR-1 (Stemcell technologies, Vancouver, CA) that
included 10 μm blebbistatin and 5 mM ROCK inhibitor (10 μm dihydrochloride, Y-27632, Enzo Life Sciences, Farmington, NY) and cultured in low-attachment six-well plates (Corning, NY) to generate embryoid bodies at day 0 (D0). The next day, mTeSR-1 was gradually exchanged for NIM. The ratio of mTeSR-1/NIM was 3:1 on D1, 1:1 on D2, and complete NIM from D3 to D7. On D7, embryoid bodies were seeded on Matrigel coated 6-well plates and cultured in NIM until D21. On D21, retinal vesicles were picked and cultured in suspension or dissociated and seeded on the GCH-521 scaffold. The cultures were maintained in serum free medium (SFM) comprised of 70% DMEM (high glucose) and 30% F-12, and supplemented with 2% B27, 2 mM glutamax, 1 mM pyruvate (Invitrogen), and penicillin/streptomycin. The RPCs were dissociated using papain (Worthington Biochemical Corp, Lakewood, NJ), according to the manufacturer’s protocol. The dissociated RPC were seeded on the GCH-521 scaffolds (5×10^5 cell/cm^2). Medium was changed 3× per week for rest of the experiment.

**RPE and co-culture**

RPE was derived from IMR90-4 cells, as described earlier. Briefly, stem cells were cultured and passaged on Matrigel-coated dishes (BD Biosciences). Embryoid bodies were formed by treating undifferentiated stem cell colonies with 5 mg/ml dispase (StemCell Technologies) and cultured as floating clusters in knockout serum replacement medium (KSR), composed of DMEM/F12 (1:1) medium, 14% knockout serum replacement (Invitrogen), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and 10 mM nicotinamide (Sigma-Aldrich) in six-well ultralow-attachment cluster plates (Costar, Corning) for 1 week. The embryoid bodies were plated on laminin-coated culture dishes (10 μg/ml) for 6 weeks. During the third and fourth weeks, KSR
was supplemented with 140 ng/ml actin A (Peprotech, Rocky Hill, NJ). After the sixth week, pigmented epithelial cells were isolated by trypsinization and expanded on laminin-coated dishes. RPE, passage 1-3, was seeded (1.3 × 10^5 cells per well) on Transwell culture inserts (1.12 cm² growth area, 0.4 μm pores; (Corning/ Sigma-Aldrich, St. Louis, MO) that were coated with 10 μg Synthemax (Corning). KSR was supplemented with 2 mM glutamax, 1 mM pyruvate (Invitrogen) and 10 μm ROCK inhibitor. After one week, the ROCK inhibitor was removed, and after two more weeks, the cultures were adapted to SFM over the course of 4 weeks.

The hfRPE were cultured, as described earlier. Briefly, hfRPE were plated on Transwell culture inserts in medium containing 5% FBS. For the first week the medium was supplemented with 10 μm ROCK inhibitor. Confluent cultures were adapted to SFM over the course of 4 weeks.

The transepithelial electrical resistance (TER) was monitored using an EVOM2 resistance meter with Endohm electrodes (World Precision Instruments, Sarasota, FL). Cultures with a TER > 150 Ω×cm² were used for further experimentation. One week after plating on GCH-521, RPC cultures were layered cell-side down onto differentiated RPE (Fig. S1). Metabolic activity was monitored by washing the cultures two times with HBSS and adding AlamarBlue (Biorad) to 10% (v/v) for 20 min at 37°C. The medium was collected, and fluorescence was measured at 560 nm excitation/590 nm emission using a Synergy H1 plate reader (Biotek, Winooski, VT). Co-cultures were maintained up to 9 months.

**Quantitative Real Time RT-PCR (qRT²-PCR):**

Total RNA was extracted using TRIZOL reagent. GCH-521-RPC was collected in 1.5 ml Eppendorf tubes and centrifuged to remove the culture medium. Trizol (700μl) was added to the tubes and sonicated for 3 secs at 4°C. Samples were incubated at room temperature for 5 min,
and 200 µl of chloroform was added and vortexed vigorously for 10 to 15 seconds. Samples were centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was removed to fresh tubes without disturbing the interface. Isopropanol was used to precipitate the RNA, and the precipitate was washed with 70% ethanol. The RNA was resuspended in RNase-free water and quantified using a nanodrop spectrophotometer.

RNA with A₂₆₀/A₂₈₀ between 1.8 to 2.0 was used for cDNA synthesis. cDNA was transcribed using 1 µg of total RNA using i-Script cDNA advance transcription kit (BioRad). Genes tested included: stem cell pluripotency markers (NANOG and OCT4), early eye field markers (PAX6, SIX3, LHX2, SIX6, and VSX2), and retinal progenitor markers (BRN3, RCVRN and NEUROD1). Primers were synthesized by the Keck center (Yale University) (Table S1). Alternatively, cDNA was analyzed using custom made RT-PCR microarrays, (BioRad) (Fig. S2). Relative mRNA expression was normalized to housekeeping genes, ACTB and GAPDH, and the 2^-ΔΔCt method was used for analysis.³⁹

**Immunocytochemistry:**

Samples were fixed in 4% paraformaldehyde for 5 mins, washed and incubated in graded sucrose solutions up to 30%. Samples were incubated overnight at 4°C in a 1:1 mixture 30% sucrose and OCT (Fisher Healthcare™, Pittsburg, PA). Sections, 12 µm, were made with a Leica CM1950 cryostat (Buffalo Grove, IL) at -25°C and mounted on poly-lysine coated slides. Slides were room dried for 48hrs before immunocytochemistry. Slides were washed in cold PBS, permeabilized with 0.1% Triton-X100 in PBS (PBST) and blocked with PBST containing 10% donkey serum. The sections were incubated overnight at 4°C with primary antibodies (Table S2).
The next day the slides were washed 3x with PBST before incubation at room temperature with secondary antibodies conjugated with Cy2, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). DAPI (4,6-diamidino-2-phenylindole) was used to label the cell nucleus. Finally, slides were washed 3 more times with PBS. Fluorescence images were captured with an LSM 410 spinning-disc confocal microscope and processed using Zen software (Carl Zeiss, Inc, Thornwood, NY). Images used are representative of 3 or more experiments. The windows for color channels were determined by the sample with the greatest signal intensity and applied uniformly to all related images in that Figure.

**Immunoblotting**

Protein extracts were prepared using a lysis buffer composed of complete Protease Inhibitor Cocktail (Sigma-Aldrich), 1.0% NP-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0. Equal amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting. Actin was used as an internal standard to normalize each sample. Details of the antibodies used are in Table S2. The immunoblots were developed using HRP conjugated secondary antibodies and ECL plus chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL). Blots were imaged using a ChemiDoc MP imaging system (BioRad).

**Statistical analysis:**

All statistical data are presented as the mean ± standard error (SE) unless otherwise stated. Independent cultures from each cell line were analyzed in triplicate. Comparisons were made using one-way ANOVA and p values <0.05 were considered statistically significant.
Supplemental Data

A

1) 60 µm thick disc of GCH scaffold is cut from 1 cm diameter cylinder and
   a) coated with laminin-521
   b) seeded with purified RPC (green) and
   c) cultured alone or cultured with RPE (red)

2) RPE and RPC signal back and forth to foster each other’s maturation

3) Concurrently, signaling among RPC fosters maturation and lamination of retinal layers

4) The scaffold gradually degrades

B

Figure S1. RPC culture and RPC/RPE co-culture. RPE and co-culture. A) Experimental scheme and hypotheses tested. Co-culture with RPE is shown; initial experiments were conducted with RPC alone. B) Method for measuring transepithelial resistance. RPE was cultured on Transwell inserts, and the RPC culture was layered on top. Following maturation, the resistance was measured using EndOhm electrodes. Panels (A 2-4) and (B) reproduced from Fields et al, 202048 with permission.
Figure S2. Maps for PCR arrays. Related to Experimental procedures: Quantitative Real Time RT-PCR (qRT2-PCR).

**RPE maturation**
Red: maturation genes (Peng et al., 2013)
Blue: signature genes (Strunnikova et al., 2010)

**RPC maturation**
Blue: Early eye field transcription factors
Red: Photoreceptor
Purple: Ganglion cell
Brown: amacrine/ horizontal/Muller cell
Yellow: Bipolar cell
Green: RPE genes
Figure S3. The co-cultures were metabolically active. WA09-derived RPC, cultured on GHC-521, were cultured with hfRPE cultures that were matured and adapted to SFM. Cultures were incubated with Alamar Blue and normalized to parallel monocultures of hfRPE on D35.
Table S1: List of primers. Related to Quantitative Real Time RT-PCR (qRT2-PCR).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell type</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>OTX2</td>
<td>Early eye field</td>
<td>5'-GCA GAG GTC CTA TCC CAT GA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-CTG GGT GGA AAG AGA GAA GC TG-5'</td>
</tr>
<tr>
<td>NeuroD1</td>
<td></td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>Housekeeping gene</td>
<td>5'-TCA CCA GGG CTG CTT TTA AC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-GCA AAG CTT CCC GTT CTC AG-5'</td>
</tr>
<tr>
<td>LHX2</td>
<td>LIM Homeobox Protein 2 for neural cells</td>
<td>5'-TAG CAT CTA CTG CAA GGA AGA C-3'</td>
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<tr>
<td></td>
<td></td>
<td>3'-GTG ATA AAC CAA GTC CCG AG-5'</td>
</tr>
<tr>
<td>NANOG</td>
<td>ES cell proliferation, renewal, and pluripotency</td>
<td>5'-CAA AGG CAA ACA ACC CAC TT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-TCT GCT GGA GGC TGA GGT AT-5'</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Stem cells pluripotency marker</td>
<td>5'-CGA GCA ATT TGC CAA GCT CCT GAA-3'</td>
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<tr>
<td></td>
<td></td>
<td>3'-TTC GGG CAC TGC AGG AAC AAA TTC-5'</td>
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<tr>
<td>PAX6</td>
<td>Neural retinal development</td>
<td>5'-TCT AAT CGA AGG GCC AAA TG-3'</td>
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<td>RAX</td>
<td>Retina and Anterior Neural Fold Homeobox</td>
<td>3'-TGT GAG GGC TGT GTC TG-5'</td>
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<tr>
<td>SIX3</td>
<td>Neural progenitor cells</td>
<td>5'-GGA ATG TGA TGT ATG ATA GCC-3'</td>
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<td></td>
<td></td>
<td>3'-TGA TTT CGG TTT GTT CTG G-5'</td>
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### Table S2: List of Primary Antibodies. Related to Immunocytochemistry

<table>
<thead>
<tr>
<th>Target</th>
<th>Antigen</th>
<th>Host(^a)</th>
<th>Dilution(^b)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior forebrain</td>
<td>Sox1</td>
<td>RP</td>
<td>IF 1:400</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
|                                 | PAX6      | RM         | IF 1:100
|                                 |           |            | IB 1:5000             | Abgent              |
|                                 | RAX       | RP         | IF 1:200              | Novus Biologicals   |
| Early Eye Field                 | LHX2      | GP         | IF 1:300
|                                 |           |            | IB 1:5000             | Santa Cruz Bio      |
|                                 | CHX10 (VSX2) | SP   | IF 1:300              | EMD Millipore       |
|                                 | OTX2      | MM         | IF 1:200
|                                 |           |            | IB 1:3000             | Novus Biologicals   |
|                                 | Recoverin | RP         | IF 1:400
|                                 |           |            | IB 1:5000             | EMD Millipore       |
| Photoreceptor Rods/Cones        | CRX       | GP         | IF 1:300              | Fisher Scientific   |
|                                 | Rhodopsin | MP         | IF 1:300
|                                 |           |            | IB 1:3000             | Cell Signaling Technology |
| Neural retinal cells precursor  | Prox1     | RM         | IF 1:500              | Fisher Scientific   |
|                                 | β-tubulin III | MP     | IF 1:200              | Fisher Scientific   |
| Normalization                   | Actin     | Actin      | IB 1:5000             | Sigma               |

\(^a\)RP, Rabbit Polyclonal; RM, Rabbit Monoclonal; MM, Mouse Monoclonal; MP, Mouse Polyclonal; SP, Sheep Polyclonal; GP, Goat Polyclonal

\(^b\)IF, Immunofluorescence; IB, Immunoblot