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Three-dimensional early retinal progenitor 3D tissue constructs derived from human embryonic stem cells

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ABSTRACT

Purpose: To develop three-dimensional (3D) constructs of retinal pigment epithelium (RPE) and early retina progenitor cells from human embryonic stem cells (hESCs).
Methods: 3D tissue constructs were developed by culturing hESC-derived neural retinal progenitors in a matrix on top of hESC-derived RPE cells in a cell culture insert. An osmolarity gradient maintained the nutrition of the 3D cell constructs. Cross-sections through hESC-derived tissue constructs were characterized by immunohistochemistry for various transcription factors and cell markers.
Results: hESC-derived tissue constructs expressed transcription factors characteristic of retinal development, such as pax6, Otx2, Chx10, retinal RAX; Brn3b (necessary for differentiation of retinal ganglion cells); and crx and nrl (role in photoreceptor development). Many cells expressed neuronal markers including nestin, beta-tubulin and microtubule-associated proteins.
Conclusions: This study shows for the first time that 3D early retinal progenitor tissue constructs can be derived from hESCs.

1. Introduction

Over 12 million Americans suffer from retinal diseases, with macular degeneration being the leading cause of vision loss. Micronutrient supplements (Berson et al., 2004) and gene therapy to introduce trophic factors (McGee Sanftner et al., 2001) or to correct mutated genes (Bainbridge et al., 2008; Maguire et al., 2008) may be helpful in the early stages, but once photoreceptors and RPE cells are lost, only techniques to reconstruct or bypass the damaged retina will restore vision (for review, see Cronin et al., 2007).

Since 1986 a unique model has been in development (Aramant and Seiler, 2004; Seiler and Aramant, 2005; Seiler et al., 2008) to replace both lost photoreceptors and RPE, by transplanting sheets of human fetal retinal progenitor cells together with its RPE to the subretinal space; this is currently the only approach to have demonstrated efficacy in both animals and humans (Radtke et al., 2008). Previous studies in different models of retinal degeneration have shown that transplanted layers of fetal retina, with or without its RPE, can restore visual responses in a specific area of the superior colliculus corresponding to the placement of the transplant in the retina (Arai et al., 2004; Sagdullaev et al., 2003; Thomas et al., 2004; Woch et al., 2001). However, there are both ethical and supply issues with the use of fetal tissue. Most other approaches are restricted to the rescue of endogenous retinal cells by a 'nursing' role of the implanted cells, an approach which does not restore lost function and is limited to the early stages of disease.

Human embryonic stem cells (hESCs) derived from human blastocysts maintain pluripotency, proliferative potential and karyotypic stability for prolonged periods (Carpenter et al., 2001). Clinical interest arises from their ability to provide an apparently unlimited cell supply for transplantation, and from the hope that they can be directed to desirable phenotypes in high purity (Coutts and Keirstead, 2008). As the supply of fetal derived neuroblastic tissue is limited, induction of hESCs to three-dimensional tissue constructs of retinal progenitors and RPE cells would provide a useful supply of human retinal tissue for developmental studies and clinical applications. This study shows that hESCs can be directed towards early retinal differentiation and stably united with co-cultured RPE to form 3D retinal tissue constructs, using a cell culture insert system with an osmolarity gradient. To our knowledge, this is the first 3D organized tissue to be generated solely from hESCs.

Abbreviations: hESC, human embryonic stem cells; RPE, retinal pigment epithelium; MAP, microtubule-associated protein; NF, neurofilament; Dkk, Dickkopf 1.

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2. Materials and methods

2.1. Cell lines

hESCs derived from the H7 line (NIH code WA07) or from the parthenogenetic stem cell line P6 (International Stem Cell Corporation, Oceanside, CA) were expanded on Matrigel (BD Biosciences, San Jose, CA) using media conditioned on a mitotically inactivated feeder layer of mouse fibroblasts, containing 10 ng/ml FGF. The cells were passaged every 5–7 days using 1 mg/ml collagenase IV (Invitrogen, Carlsbad, CA) with a splitting ratio of 1:4 to 1:6.

2.2. Derivation of neural progenitor cells and RPE

Cells were differentiated using a modification of the protocol of Osakada et al. (2008), with the difference that Osakada et al. used non-adherent cultures, and our protocol used adherent cultures from day 14 after induction of differentiation. After obtaining confluence of 75–100%, hESC cultures were induced to differentiate by exposing the cells to a serum free "induction media" formulated of DMEM/F12 high glucose, B27 supplement, Insulin–Selenite–Transferrin (IST), triiodothyronine (T3), Taurine 2.5 g/l; Hyaluronic Acid (HA) 250 mg/l; Dickkopf-1 (Dkk) 25 ng/ml, LeftyA (TGF-beta ligand and antagonist of Nodal signaling) 50 ng/ml, FGF 5 ng/ml (Invitrogen, Carlsbad, CA). From day 10–13, 10 μM retinoic acid (RA) was used for neural induction, from a stock solution of 20 mM prepared in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO). (In initial experiments, RA was applied on day 1–3 which resulted in too early neuronal differentiation and low yields of RPE.) At day 7, the cultures were dissociated using collagenase and transferred to ultra-low adherent flasks. During the following 7 days (feeding every second day), formation of spherical cell agglomerates was observed. At day 14, the cell suspension was transferred to an adherent substrate of collagen type 1 and laminin in "maintenance media" (DMEM/F12 high glucose, B27, IST, T3, Taurine 2.5 g/l; Hyaluronic Acid 250 mg/l, FGF 5 ng/ml). The cell conglomerates attached overnight and individual cells migrated immediately, forming a monolayer of epithelial shaped cells. At day 42, pigmented RPE colonies were hand picked and plated in collagen/laminin coated inserts or, alternatively, on collagen/laminin coated dishes for expansion and later plated in collagen/laminin coated inserts. RPE cells derived from hESCs were maintained in maintenance media with 10% FBS, without FGF.

At day 50–60, the neural cultures were replated on RPE seeded tissue culture inserts (Corning Transwell PET or PTFE, 3 μm pore) at high density.

2.3. Preparation of culture inserts

A larger Petri dish was used as an external reservoir for the inserts. The bottom of the dish was used either smooth (as manufactured) or deeply scratched with a sterile 18G needle in a dense cross-pattern. Inserts with various materials and pore sizes ranging from 0.2 μm to 3 μm and diameters ranging from 5 mm to 3 cm (Corning, Lowell, MA) were tested. The inserts were coated with poly-L-lysine and laminin, or collagen type 1 and laminin by overnight exposure at room temperature. Matrigel was used as matrix in initial experiments.

2.4. Media formulation

The media used to feed the cells in the insert's "top media" was designed to have an osmolarity of 320–340 mmol/kg. A higher osmotic pressure was provided by a hyaluronic acid (0.4%)–albumin (0.4%) complex and taurine 0.5% supplement added to maintenance media.

The media surrounding the inserts was designed to have a 270 mmol/kg osmolarity formulated using Knockout DMEM:F12 (Invitrogen, Carlsbad, CA), Insulin–Selenite–Transferrin 1% (Invitrogen, Carlsbad, CA) and supplemented with either 10% fetal bovine serum or B27 supplement. The serum containing or the serum free media was used alternately for 1 week each for the first 28 days after assembling the system. From that point, only the serum free formulation was used.

Table 1
Overview of antibodies used.

Antibody specificity	Species	Specific for	Dilution	Supplier
Chx10/Vsx10	Goat	Homeodomain transcription factor (Rowan and Cepko, 2004)	1:200	Santa Cruz Biotechnology Inc., Santa Cruz, CA
Otx2	Rabbit	Orthodenticle homeobox 2 gene (necessary for eye development) (Martinez-Morales et al., 2003; Nishida et al., 2003)	1:1000	Chemicon, Temecula CA
mitf	Mouse	Microphthalmia associated transcription factor, regulates RPE development	1:50	Santa Cruz Biotechnology Inc.
Retinal Rax	Rabbit	Homeobox gene necessary for eye development (Mathers and Jamrich, 2000)	1:200–1:400	Santa Cruz Biotechnology Inc.
Crx	Rabbit	Cone-rod homeobox gene	1:400	Santa Cruz Biotechnology Inc.
Nrl	Rabbit	Neural retina leucine zipper transcription factor, marker for photoreceptor precursors	1:50	Santa Cruz Biotechnology Inc.
Beta-tubulin	Mouse	Marker for early neurons	1:50	ICN (now MP Biomedicals), Irvine CA
Pax6	Rabbit	"master control" gene for development of eyes and sensory organs,	1:500	Chemicon
Pax6	Mouse	neural and epidermal tissues (Mathers and Jamrich, 2000)	1:50–1:100	
NeuN	Mouse	Neuronal nuclei	1:200	Chemicon
Vimentin	Mouse	Intermediate filament; marker for early embryonic and mesenchymal cells	1:500	ICN
Brn3b	Goat	Brain-specific homeobox/POU domain protein 3b; regulates ganglion cell development	1:100	Santa Cruz Biotechnology Inc.
NeuroD	Goat	Neurogenic bHLH transcription factor	1:100	Santa Cruz Biotechnology Inc.
MAP2	Mouse	Microtubule-associated protein 2 (neuronal marker)	1:200	Chemicon
MAP2	Rabbit		1:500	Chemicon
CRALBP	Rabbit	Cellular retinaldehyde binding protein, marker for RPE and retinal Muller glial cells	1:1000	Saari, Univ. of Washington, Seattle WA (Saari et al., 1984)
Glutamine synthetase	Mouse	Glial cell marker (retinal Muller cells)	1:1000	Transduction Labs, Lexington KY
Recoverin	Mouse	Photoreceptors and cone bipolar cells	1:500	McGinnis (McGinnis et al., 1997); Univ. of Oklahoma

2.5. Co-culture—assembling the system

After 3 weeks of hESC neural differentiation and RPE differentiation (in separate cultures), the neural progenitors were plated at a concentration of 200,000 cells/cm^2 on top of the RPE cultures in the inserts. At this point, the system consisted of a layer of porous membrane (polycarbonate) with various pore sizes, a layer of extracellular matrix (collagen and laminin; or poly-L-lysine and laminin), a layer of RPE cells and a dense layer of neural progenitors. Each insert received, according to the insert size, 5 mm of "Top" media above the cells (equivalent to 4 ml for the large inserts). The inserts were placed in the larger Petri dish (150 mm), scored with a 18G sterile syringe needle in a crisscross pattern. The media in the inserts was changed every second day and supplemented with 5 ng/ml FGF. The large Petri dish containing 4–6 inserts was filled with 60 ml of the low osmolarity media which was replaced once a week. A diagram of the culture setup is shown in Fig. 1A. Fig. 1B shows a diagram of the differentiation timetable.

2.6. Histology

Three-dimensional tissue sheets were fixed with 4% paraformaldehyde, infiltrated with 30% sucrose, and frozen in OCT. Ten μm cryostat sections were stained with hematoxylin-eosin or used for immunocytochemistry.

2.7. Immunocytochemistry

In most experiments, sections were pretreated with an antigen retrieval solution (HistoVTOne, Nakalai USA Inc., San Diego, CA) for 20 min at 70 °C prior to blocking. Sections were incubated in blocking serum (10% goat serum for mouse and rabbit antibodies; 10% donkey serum for goat antibodies in PBS/1% BSA) for 1–3 h at room temperature, then incubated with various primary antibodies overnight at 4 °C (see Table 1), washed 3 times with PBS and incubated with fluorescent secondary antibodies, conjugated with Alexa Fluor 488 or Rhodamine Red X, respectively (1:200, Molecular Probes, Eugene, OR) for 1 h. Nuclei were labeled blue with 4,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlingame, CA). Fluorescent images (2–6 microscopic fields per section) were scanned in a Zeiss LSM510 or LSM710 Confocal microscope (Carl Zeiss, Germany) at 40× magnification. Five to 10 images of one field at several focus depths were combined into one image.

3. Results

3.1. Extracellular matrix

Initial experiments which used Matrigel for creating a three-dimensional matrix all resulted in overgrowth of the cultures

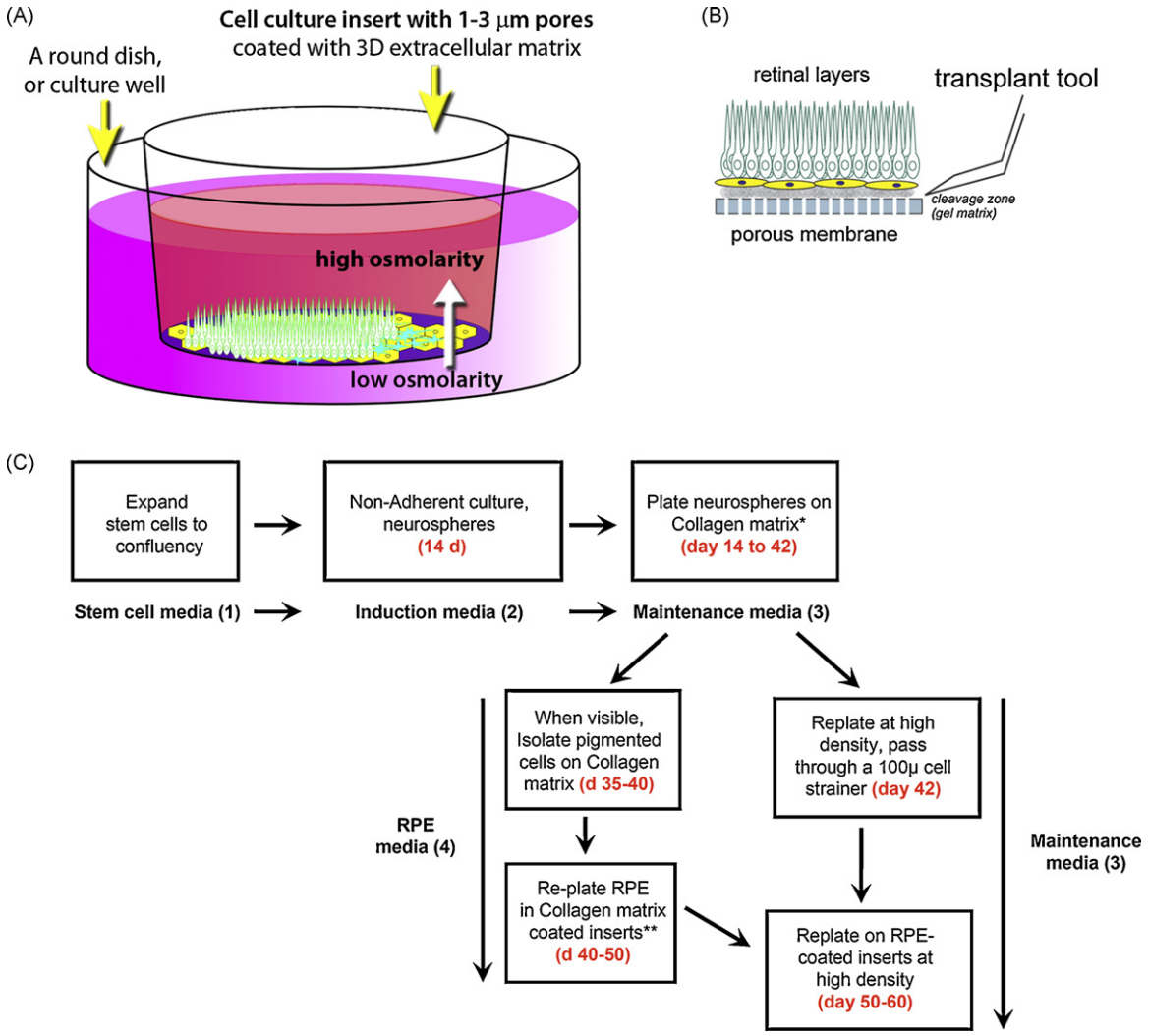

Fig. 1. Experimental setup. (A) Setup of insert culture. (B) Separation of layers from membrane insert. (C) Time schedule for differentiation. *Collagen Type 1 matrix: Cell Gel (California Stem Cell). **Inserts: Corning Transwell PET or PTFE, 3 μm pore (the polycarbonate or Anapore inserts do not work).

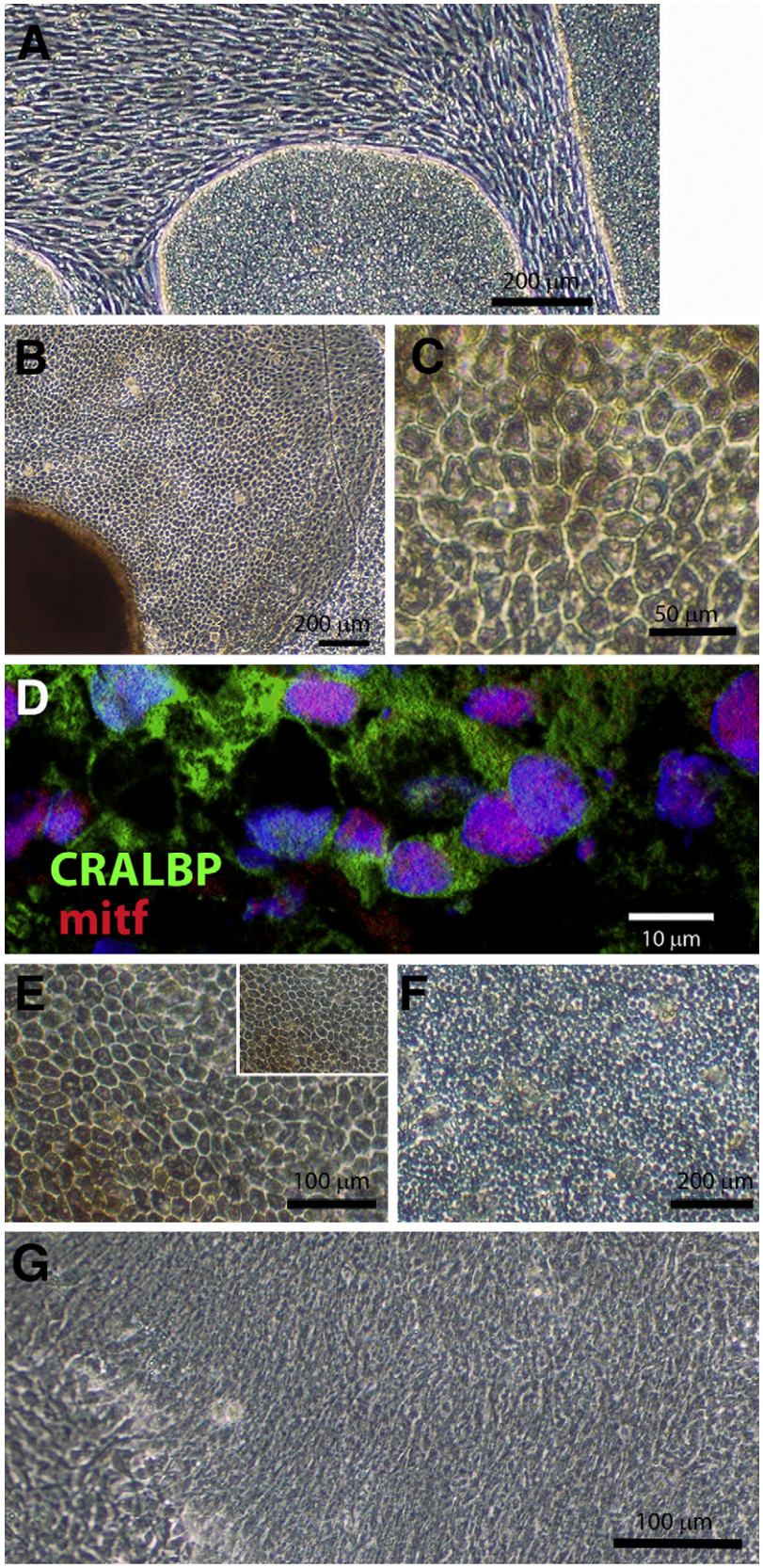

Fig. 2. Phase-contrast images of cultures in vitro. (A) Undifferentiated hESC culture on Matrigel in phase contrast. (B and C) Island of a developing RPE layer in a mixed culture at an early stage of differentiation (30 days). (D) Stain of RPE layer cross-sections for the RPE-specific transcription factor mitf (red) and for cellular retinaldehyde binding protein (CRALBP). Nuclei are counterstained with DAPI (day 76 of differentiation). (E) RPE cells on collagen/laminin before co-culture (day 47 since induction with Dkk and LeftyA). Insert: same magnification as picture in (F). (F) Neural progenitor cells before co-culture (day 47 since induction of differentiation). (G) Co-culture of neural progenitors on top of RPE layer, 30 days after plating neural progenitors. Magnification bars = 200 μm for (A, B, F); 50 μm for (C), 100 μm for (E), 10 μm for (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with mesodermal components (fibroblasts, osteoblasts and chondrocytes) in long-term cultures (data not shown). Therefore, we switched to a mixture of collagen type 1 and laminin.

3.2. Cell growth

Co-cultures of hESC-derived neural progenitors and rat RPE cells in a cell culture insert (Fig. 1) were maintained for 60–120 days. Fig. 2 shows phase-contrast images of the hESCs (Fig. 2A), RPE cells and neural progenitors, before starting the RPE and neural progenitor co-culture. RPE cells formed a cuboidal epithelium, but lost their pigment with time in culture (Fig. 2B, C and E). RPE cells expressed cellular retinaldehyde binding protein (CRALBP) and microphthalmia inducing transcription factor (mitf) early on (Fig. 2D). RPE cells had larger cell sizes than neural progenitor cells (compare Fig. 2E insert and F). Multi-layering of the neural cells could be observed after 90 days, but because of increased density, phase-contrast observation became impractical (Fig. 2G). The multi-layered sheets could be collected using a custom-made spatula and/or a fine Dumont forceps and placed in a separate Petri dish containing media, cut into pieces, then transplanted or fixed for histology.

3.3. Influence of pore size

Cell culture insert pores with a small diameter (0.2–1.0 μm) did not allow for sufficient media exchange, and the bottom layer soon become necrotic with areas of detachments from the substrate. When neural cells were added to RPEs seeded on cell culture inserts consisting of small diameter pore sizes, sheets lifted from the substrate within 5–7 days, and formed clumps resembling neurospheres that were interspersed with a multitude of floating debris, indicative of dead cells. Media within these cultures turned yellow relatively quickly, indicative of accumulation of metabolic byproducts resulting in a drop of culture pH.

Larger pores (3 μm) allowed for nutrient exchange evidenced by healthy 3D tissue constructs with no evidence of necrosis or sheet detachment. When neural cells were added to RPEs seeded on cell culture inserts consisting of large diameter pore sizes, sheets remained attached to the cell culture inserts and homogeneously distributed across the inserts without clumping. Media within these cultures remained orange-red, indicative of the lack of accumulation of metabolic byproducts and a generally healthy cellular metabolism. Media within these cultures contained little or no floating debris.

3.4. Influence of the insert/container size

Small (5 mm) cell culture inserts did not allow for sufficient media supply, and the cultures became necrotic and detached from the substrate. Larger (up to 3 cm) cell culture inserts placed in the 200 mm Petri dishes resulted in tissue survival without signs of necrosis and detachment beyond 90 days (similar to Fig. 2G). When the cell culture inserts were in intimate contact with the smooth bottom of the Petri dish, media exchange was suboptimal, evidenced by cell necrosis and detachment of the sheet. Addition of deep scratches to the dish bottom allowed for sufficient media exchange, resulting in tissue survival without signs of necrosis and detachment beyond 90 days (similar to Fig. 2G).

3.5. Immunohistochemistry for transcription factors and cell markers

After 90 days in culture, up to 8 cell layers typically developed on the cell culture inserts. The layers could be peeled off the cell culture inserts by careful dissection and cut into pieces. Cross-sections were cut after embedding in OCT and processed for immunohistochemistry. Fig. 3 shows transcription factor and marker expression in neural layers after induction and before co-culture with RPE (10 weeks after starting the differentiation process with Dkk and LeftyA). Fig. 4 shown marker expression after 1–2 weeks of co-culture. Strong staining of cell processes for CRALBP was observed 10 weeks after induction (Fig. 3A). The retina-specific transcription factor RAX was found in large neurons immunoreactive for MAP2 (Figs. 3B and 4D). Staining for the transcription factor OTX2 was found throughout the cultures, with some areas more

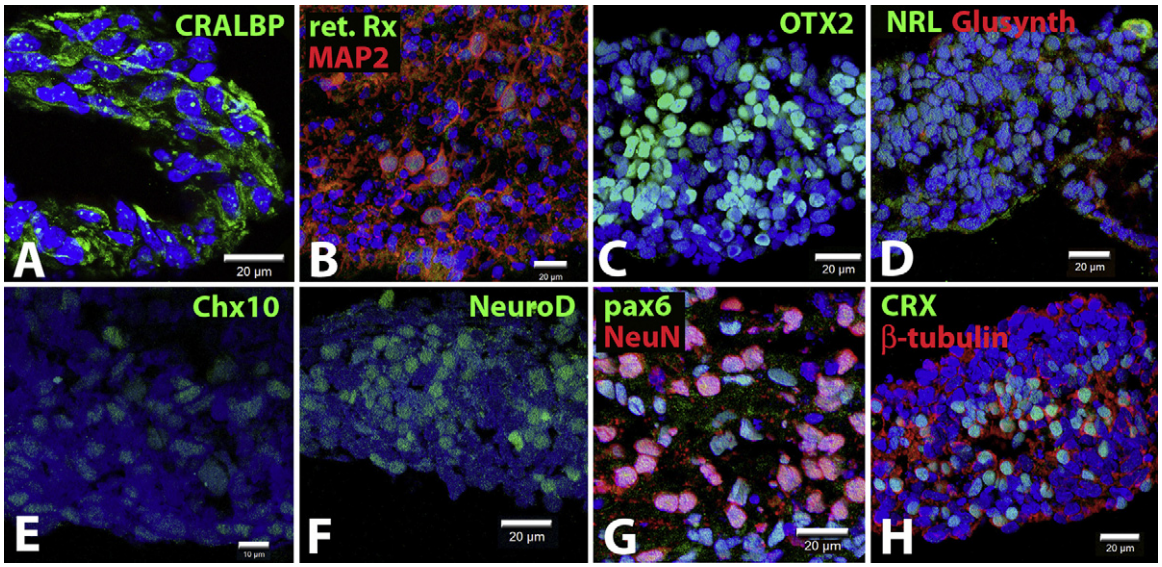

Fig. 3. Expression of transcription factors and neuronal/glial markers within layers before co-culture. Nuclei are counterstained with DAPI (blue). (A) Strong CRALBP expression in the cytoplasm of many cells. (B) Strong staining for MAP2 (red), clear staining for transcription factor retinal RAX (green) in many large neurons. (C) Many cells express the transcription factor OTX2. (D) Many cells faintly stained and one cell strongly stained for photoreceptor-progenitor transcription factor NRL (green), almost no staining for glial marker glutamine synthetase (red). (E) Chx10/Vsx10 staining is distributed throughout layers (sample with bleach DAPI counterstain). (F) Strong expression of transcription factor NeuroD in some areas. (G) NeuN (red)/pax6 (green) staining shows partial overlap. (H) Beta-tubulin/CRX: Beta-tubulin is distributed throughout the sheet whereas CRX appears in some areas. Magnification bars = 20 μm (A–D, F–H); 10 μm (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

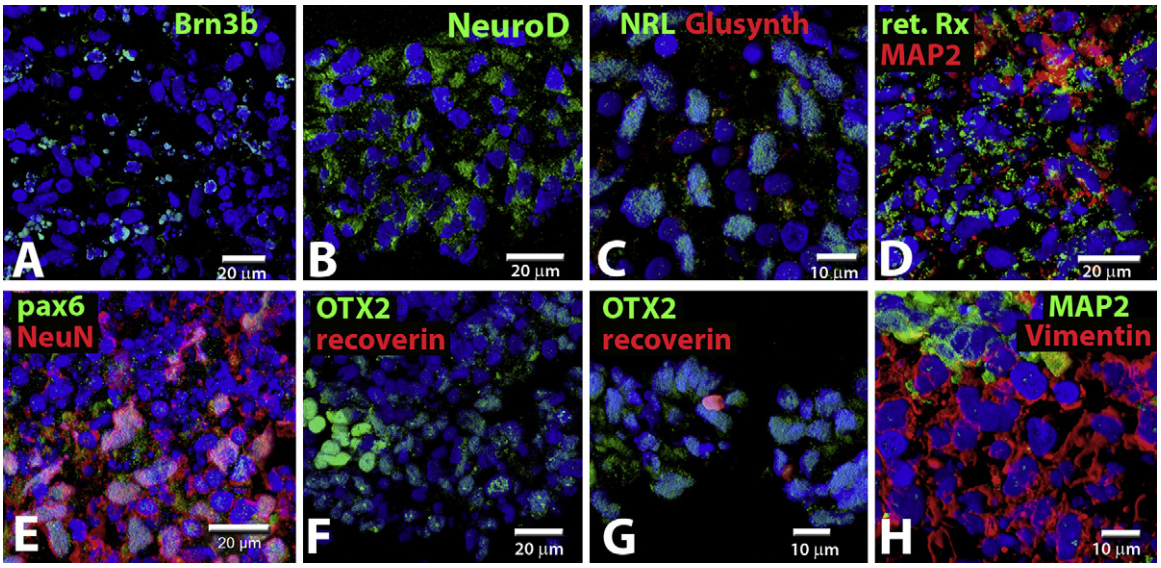

Fig. 4. Expression of transcription factors and neuronal/glial markers within layers during co-culture. Nuclei are counterstained with DAPI (blue). (A) Scattered cells stain for transcription factor Brn3b (green), 1 week after co-culture. (B) Cytoplasmic staining for transcription factor NeuroD (green), 2 weeks after co-culture. (C) Many cells are strongly stained for photoreceptor-progenitor transcription factor NRL (green), almost no staining for glial marker glutamine synthetase (red), 1 week after co-culture. (D) Scattered staining for transcription factor retinal RAX (green) and neuronal marker MAP2 (red), 1 week after co-culture. (E) NeuN (red)/pax6 (green) staining shows partial overlap, 1 week after co-culture. (F and G) Double staining for OTX2 (green) and recoverin (red). Note that there is almost no recoverin staining except for few cells in (G). (F) 1 week after co-culture. (G) 2 weeks after co-culture. (H) MAP2 (green)/Vimentin. Strong Vimentin stain throughout; MAP2 concentrated in one area, 1 week after co-culture. Magnification bars = 20 μm (A, B, D–F); 10 μm (C, G, H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

immunoreactive than others (Figs. 3C and 4F). Immunoreactivity for the photoreceptor-progenitor-specific transcription factors NRL and CRX could be demonstrated (Figs. 3D and H and 4C, F and G). The transcription factor NeuroD was found in nuclei before co-culture, after 10 weeks of differentiation (Fig. 3F), but in the cytoplasm of many cells after 2 weeks co-culture (Fig. 4B). Some nuclear staining for the transcription factor Brn3b (involved in ganglion cell differentiation) was observed before co-culture (data not shown) and 1–2 weeks after co-culture (Fig. 4A). Staining for the transcription factor pax6 was observed in many cells throughout the cultures, with partial overlap to the neuronal marker NeuN (Figs. 3G and 4E). Beta-tubulin, a marker for early neurons, was distributed throughout the cultures whereas CRX appeared in some areas (Fig. 3H). Cell sheets were strongly immunoreactive for the intermediate filament Vimentin (Fig. 4H). Staining for recoverin, a marker for photoreceptors and cone bipolar cells, was mostly absent (Fig. 4F and G). No staining for markers of mature photoreceptors such as rhodopsin and arrestin was detected (data not shown).

4. Discussion

This study shows that hESCs can be directed towards retinal differentiation by media and substrate manipulations, and co-culture with RPE cells. To our knowledge, this is the first 3D organized tissue to be generated solely from hESCs.

4.1. Comparison to other approaches of retina-specific differentiation of hESCs

Several laboratories have developed procedures for differentiating hESCs into RPE cells (Carr et al., 2009; Klimanskaya et al., 2004; Klimanskaya, 2006) and retinal progenitor cells which developed further to express photoreceptor markers (Meyer et al., 2009; Osakada et al., 2008). hESC-derived neural progenitors that are clearly differentiated towards a retinal fate develop photoreceptor markers after injection into a newborn retina, or a retina that contains a full photoreceptor layer (Lamba et al., 2009). However, they fail to develop photoreceptor outer segments after injection into a retina that has lost its photoreceptors but can induce responses to strong light (Lamba et al., 2009). Less differentiated cells fail to develop markers of mature photoreceptors although they can express markers of retinal progenitors. Co-culture of human retinal neurospheres with RPE did prolong and enhance growth of human prenatal retinal neurospheres, which went on to express numerous neurodevelopmental and eye specification genes and markers characteristic of neural and retinal progenitor cells (Gamm et al., 2008).

However, no attempts were made to develop three-dimensional tissue constructs of retinal progenitor cells.

4.2. A unique procedure to develop layers

Few attempts to construct 3D layers in tissue culture have been reported (Duong et al., 2005; Hara et al., 2008; Lawrence et al., 2009; Srouji et al., 2008). In most cases, the three-dimensional substrate is provided by a gel (Dongari-Bagtzoglou and Kashleva, 2006; Lawrence et al., 2009) or electrospun nanofibers (Jinno et al., 2008; Pham et al., 2006; Srouji et al., 2008) in which cells are grown. Our use of an osmotic gradient across a neural and RPE multi-constituent structure is a unique approach that mirrors development and contributes to the individual cell orientation and layered architecture. The osmotic gradient establishes a unidirectional flow, causing the factors secreted by the bottom layers (RPE) to be forced towards the neural progenitor layers. Initially, Matrigel was employed as a matrix, as it contains a complex arrangement of growth factors and extracellular matrix proteins (Kleinman and Martin, 2005), and has been shown to promote differentiation of multiple cell types (Kleinman and Martin, 2005) and to support neural differentiation of hESCs (Ma et al., 2008; Prokhorova et al.,

2008). However, Matrigel consistently resulted in overgrowth of long-term cultures with mesodermal elements (fibroblasts, chondrocytes and osteoblasts). This was avoided using a collagen 1 and laminin-based matrix.

4.3. Importance of RPE co-culture

RPE cells play an important role during retinal development. Although the RPE cells used for this study maintained an epithelial morphology prior to the addition of neural progenitor cells, they had lost their pigment, an anticipated occurrence when subculturing RPE cells (Valtink et al., 1999). In other studies, co-culture of human retinal neurospheres with RPE prolonged and enhanced the growth of human prenatal retinal neurospheres, which went on to express numerous neurodevelopmental and eye specification genes and markers characteristic of neural and retinal progenitor cells (Gamm et al., 2008). Gong et al. cultured hESC-derived neural progenitors on the ARPE19 RPE cell line and also observed expression of the photoreceptor precursor markers Crx and Nrl (Gong et al., 2008). However, they never attempted to obtain 3D tissue constructs.

4.4. Expression of markers specific for eye development

Our differentiation protocol resulted in the expression of multiple markers of eye development within the hESC-derived layers, including Rax, Pax6, Chx10, Mitf, Otx2, Crx, Nrl, Brn3b, NeuroD, and CRALBP. The homeobox genes Rax and Pax6 are expressed early in the development of eye primordium, regulate cell proliferation, and are required for the initial determination to retinal and lens cell fate, respectively (Mathers and Jamrich, 2000). Pax6 appears to play additional roles in the formation of the retina and cornea (Mathers and Jamrich, 2000), and overexpression is associated with transdifferentiation of RPE to retina (Azuma et al., 2005). Chx10 is a homeobox-containing transcription factor that is required for retinal progenitor cell proliferation as well as bipolar cell determination in the developing retina (Rowan and Cepko, 2004). Bipolar cell specification is achieved in coordination with the bHLH transcription factors Mash1 and Mash3 (Hatakeyama et al., 2001). Inappropriate expression of Chx10 in the developing RPE causes downregulation of Mitf, Tfec, and associated pigment markers, leading to a nonpigmented RPE, linking Chx10 and Mitf to maintenance of the neural retina and RPE fates, respectively (Rowan et al., 2004). Otx2 is important both for RPE development (Martinez-Morales et al., 2003) and later for cell fate specification of photoreceptors (Nishida et al., 2003). The atonal homolog 5 (ATH5) basic helix-loop-helix (bHLH) transcription factor controls the expression of the beta3 subunit of the neuronal nicotinic receptor during specification of retinal ganglion cells (Skowronska-Krawczyk et al., 2005). Crx, an Otx-like homeodomain transcription factor, controls gene expression for development and maintenance of photoreceptors (Hennig et al., 2008). The rod-determining factor neural retina leucine zipper protein (Nrl) is expressed by post-mitotic photoreceptor precursors, and acts together with Crx to regulate rhodopsin transcription (Mears et al., 2001; Oh et al., 2008). Many cells expressed vimentin, indicating an early developmental stage. Vimentin is highly expressed in the part of the optic vesicle that develops into the retina (Iwatsuki et al., 1999).

5. Conclusions

These data provide strong evidence that our differentiation protocol directed hESCs towards a retinal cell fate. Induction of hESCs to layers of retinal progenitors and RPE cells will provide a useful and abundant source of human retinal tissue for developmental studies.

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