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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Generation and clonal isolation of retinal stem cells from human embryonic stem cells

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Abstract

Retinal stem cells (RSCs) are present within the pigmented ciliary epithelium (CE) of the adult human eye and produce progeny that differentiate *in vitro* into all neural retinal subtypes and retinal pigmented epithelium (RPE). We hypothesized that a RSC population, similar to the adult CE-derived RSC, is contained within pigmented colonies that arise in long-term cultures of human embryonic stem cells (hESCs) suggested to recapitulate retinal development *in vitro*. Single pigmented hESC-derived cells were isolated and plated in serum-free media containing growth factors and, after 2 weeks, clonal sphere colonies containing both pigmented and non-pigmented cells were observed. These colonies expressed the early retinal transcription factors Rx, Chx10 and Pax6, and could be dissociated and replated as single cells to form secondary clonal colonies. When allowed to differentiate, expression of markers for both RPE and neurons was observed. Rhodopsin expression was detected after explant co-culture and transplantation into the developing mouse eye as well as following treatment with soluble factors *in vitro*. We show that RSCs emerge in an *in vitro* model of retinal development and are a potential source of human photoreceptors for use in transplantation.

Introduction

Rare, pigmented cells present in the ciliary epithelium (CE) of the adult human and mouse eye compose a population of retinal stem cells (RSCs; Tropepe *et al.*, 2000; Ahmad *et al.*, 2000; Coles *et al.*, 2004). Though quiescent *in vivo*, isolated single stem cells proliferate to form sphere colonies that consist of both heavily pigmented and lightly or non-pigmented cells. These clonal spheres have the capacity for long-term self-renewal and multilineage retinal differentiation *in vitro*, and can contribute to the retinal pigmented epithelium (RPE) and neural retina after transplantation (Ballios *et al.*, 2010; Inoue *et al.*, 2010).

The mammalian eye develops from an evagination of anterior neural tissue termed the optic vesicle. Progenitors competent to form both RPE and NR express a set of eye field transcription factors including Pax6, Otx2, Rx and Six3 (Grindley *et al.*, 1995; Oliver *et al.*, 1995; Furukawa *et al.*, 1997; Zuber *et al.*, 2003). The optic vesicle is then rapidly segregated into the inner presumptive neural retina and the outer presumptive RPE, which later becomes pigmented (Kaufman, 1992; Graw, 2010). The outer, pigmented layer of the CE where RSCs are found is continuous with the RPE. RSCs express genes essential for retinal development, such as the neural retina transcription factor Chx10 and Pax6, which is necessary for RSC proliferation (Tropepe *et al.*, 2000; Xu *et al.*, 2007).

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Pigmented cells have been reported to arise from primate and human embryonic stem cells (hESCs) that were allowed to differentiate for several weeks on stromal or fibroblast feeder layers (Kawasaki *et al.*, 2002; Klimanskaya *et al.*, 2004). These cells can be isolated and expanded to give rise to monolayers of RPE cells that express appropriate markers, have capacity to phagocytose and can rescue photoreceptor loss in RCS rats (Lund *et al.*, 2006). We asked whether pigmented cells spontaneously arising in differentiated hESCs contained a subpopulation of RSCs – single pigmented cells with the capacity for both self-renewal and multilineage retinal differentiation. Such a population would potentially be useful as a retinal-specific donor cell population for cell-based therapies to treat degenerative diseases of the eye, particularly as protocols to differentiate hESCs to pigmented cells appear to be easily translated to induced pluripotent stem cells (iPSCs; Hirami *et al.*, 2009; Meyer *et al.*, 2009).

Materials and methods

hESC culture

H9 cells were maintained in knockout Dulbecco's modified essential medium (DMEM) with 20% knockout serum replacement, 1% Glutamax^{TM}, 1% beta-mercaptoethanol, 1% non-essential amino acids and 4 ng/mL fibroblast growth factor (FGF)2 on inactivated mouse embryonic fibroblasts (MEFs). Cells were passaged using collagenase type IV onto fresh, irradiated MEFs at a ratio of 1 : 6 every 5 days or

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at 80% confluency (whichever came first). Pluripotency marker testing (SSEA3 and Tra-1-60) through FACS was performed every fourth passage; cells were discarded below 70/65, respectively.

hESC differentiation

H9 hESCs were differentiated into pigmented cells as described (Kawasaki *et al.*, 2002; Klimanskaya *et al.*, 2004). Briefly, H9 hESCs were either overgrown on MEFs and then switched to a media containing 13% KnockOut™ serum replacement in KnockOut™-DMEM (Gibco), or were grown on PA6 stromal cells in Glasgow minimal essential medium (GMEM; Sigma-Aldrich) containing 10% KnockOut™ serum replacement. Media were changed every 1–2 days. Pigmented colonies arose after 3–5 weeks. CA1 hESCs also gave rise to pigmented colonies under the same conditions.

Sphere-forming assay

Pigmented patches were picked using a pipette and dissociated into single cells using trypsin-EDTA. Cell viability was assessed immediately before plating using Trypan blue exclusion and cells were plated at 5 cells/μL (1389/cm^2) in serum-free media containing 10 ng/mL FGF2, 2 μg/mL heparin (both from Sigma) and B27 (Gibco) in 24-well plates (Nunclon™). The media were carefully refreshed once after approximately 10 days. Colonies were counted after 14–21 days. When indicated, the Rho kinase (ROCK) inhibitor Y-27632 (Calbiochem) was added to the media at a final concentration of 10 μM (Watanabe *et al.*, 2007).

Flow cytometry

Differentiated hESC monolayers were dissociated into single cells using trypsin-EDTA and plated at 1–2 million cells/75 cm^2 flask (Nunclon™) in serum-free media with FGF, heparin and B27, and allowed to proliferate for 5–7 days. Small sphere colonies were then dissociated a second time using 20 U/mL papain in trypsin-EDTA and analysed on a FACS Aria (BD Biosciences). A combination of side and forward scatter was used to delineate the fraction containing pigmented cells. Dissociated, non-pigmented neural colonies obtained from hESCs were used as a negative control.

Ficoll gradient

A 25% stock solution of ficoll (Sigma) was prepared in Hank's balanced salt solution (HBSS) and autoclaved, then diluted in HBSS; 16 mL of 15%, 8 mL of 10% and 8 mL of 5% ficoll were layered in a 50-mL Falcon tube and cells were separated by centrifugation for 20 min at 650 × g (Heller & Jones, 1980).

Sphere passaging

Single spheres were selected and dissociated using trypsin-EDTA and 20 units/mL papain (Worthington) for 5 min at 37 °C followed by brief manual trituration. Dissociation to single cells was verified after plating in individual wells of 48-well plates in serum-free media with B27, FGF and heparin. ROCK inhibitor was added where noted.

Sphere differentiation

Spheres were selected onto individual wells of laminin-coated 24-well plates and allowed to attach (laminin from Sigma). Differentiation media either consisted of serum-free media with B27 and 1% foetal bovine serum (FBS) with FGF2 and heparin alone or with the addition of 100 μM taurine and 500 nM RA (both from Sigma).

Explant cultures

Retinal explants from adult male and female C57Bl/6 mice were cultured as described (Lamba *et al.*, 2006). The intact retina excluding the RPE was removed in artificial cerebrospinal fluid. Small incisions in the periphery were made to flatten the retinas, which were then placed photoreceptor side down on CM 0.4-μm cell culture inserts (Millipore) in DMEM/F12 containing 10% FBS and N2 supplement just covering the tissue. Media were changed every second day.

Immunocytochemistry

Immunostaining was performed as previously described (Coles *et al.*, 2004). Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed in phosphate-buffered saline (PBS) and permeabilized in 0.3% Triton X-100 (Sigma) before being blocked for 1 h in 10% normal goat serum and incubated overnight in primary antibody at 4 °C. The following primary antibodies were used – mouse anti-rhodopsin (RetP1 1 : 250 and Rho1D4 1 : 500; Millipore), mouse anti-Pax6 (1 : 300; Developmental Studies Hybridoma Bank), rabbit anti-Chx10 (a gift from Dr R. McInnes), mouse anti-bestrophin (1 : 500), mouse anti-calbindin (1 : 500), anti-Cralbp (1 : 500; Abcam) and anti-GFP (1 : 500; Invitrogen). All antibodies were tested on sections of mouse neural retina/RPE to confirm cell type-specific staining (see also Coles *et al.*, 2004; Ballios *et al.*, 2012). Cells were then rinsed in PBS and incubated in Alexa-tagged secondary antibodies (Molecular Probes, Invitrogen) at 1 : 400 for 1 h at 37 °C, rinsed and counterstained with Hoechst 33342 (Invitrogen). Cells were visualized in PBS at room temperature using a Zeiss Axiovert inverted fluorescence microscope, and images were acquired with AxioVision v4.6 imaging software and AxioCam MRm camera with monochrome CCD sensor.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted using a Qiagen RNeasy extraction kit with DNase to remove genomic DNA contamination. RNA was quantified using Nanodrop, and a specified amount of cDNA was reverse-transcribed using SuperscriptIII (Invitrogen). PCR was carried out using standardized Taqman Gene Expression Assays in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Quantification was performed using the ΔC_t method with 18S or Gapdh as an endogenous control template.

Transplantation

Experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals, and approved by the Animal Care Committee at the University of Toronto. Pigmented cells were isolated by FACS and infected with eGFP lentivirus (a gift from Dr John Dick) 6–12 h after plating. Spheres were then grown for 3–4 weeks before being dissociated into a single cell suspension using 20 U/mL papain in trypsin-EDTA. NOD/SCID pups aged 0–2 days were anaesthetized using isoflurane, and 5–10 000 cells were transplanted into the vitreous fluid using a glass pipette. Confocal images

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of transplanted eyes were acquired with an OLYMPUS fluoview 1000 and FV10-ASW 2.0 imaging software.

Statistics

Student's *t*-tests were used for comparison of two groups, and ANOVA with multiple comparisons and *post hoc* Bonferroni tests were used for multiple comparison and were performed using PRIZM statistical software with an overall significance level of 0.05.

Results

Rare pigmented cells from differentiated hESCs are proliferative retinal progenitors

Pigmented colonies arose within differentiating hESCs (H9 and CA1) after approximately 4 weeks in culture on PA6 stromal cells or 5 weeks in culture on fibroblast feeders. These colonies were found adjacent to Tuj1-positive cells as well as Pax6-positive neural or retinal precursors and Chx10-positive neural retinal precursors (Fig. 1A). mRNAs for Rx, Pax6 and Mitf, genes expressed during eye field specification, were upregulated before the appearance of pigmentation, indicating that unspecified retinal progenitor cells are present before the appearance of RPE and RPE precursor cells (Fig. 1B). By immunocytochemistry, we found many non-pigmented, Chx10- and Pax6-positive cells representing proliferating precursors. Pigmented colonies derived in these culture conditions have been presumed to contain only RPE-lineage cells (Kawasaki *et al.*, 2002; Klimanskaya *et al.*, 2004). Pax6 is expressed in RPE progenitors (Quinn *et al.*, 1996; Bäumer *et al.*, 2003), and expression was seen in pigmented cells, though pigmentation often masked immunofluorescence particularly in multilayered colonies (Fig. 1C). In areas where pigmented cells were well spread, we noticed a small subset of pigmented cells that were double positive for Pax6 and Chx10. This rare subset may represent a RSC population.

Chx10 is only expressed in the early neural retina and in RSCs (Liu *et al.*, 1994). This suggested the presence of pigmented RSCs in hESC cultures. To investigate this possibility, we employed a clonal, sphere formation assay. Pigmented cell patches were removed using a pipette and dissociated into a single cell suspension and then plated at 5 cells/μL in serum-free media containing FGF. We found that in 2–3 weeks, similar to the time required to form clonal brain neural colonies from hESCs (Chaddah *et al.*, 2012), colonies composed of both pigmented and non-pigmented cells were present. These were

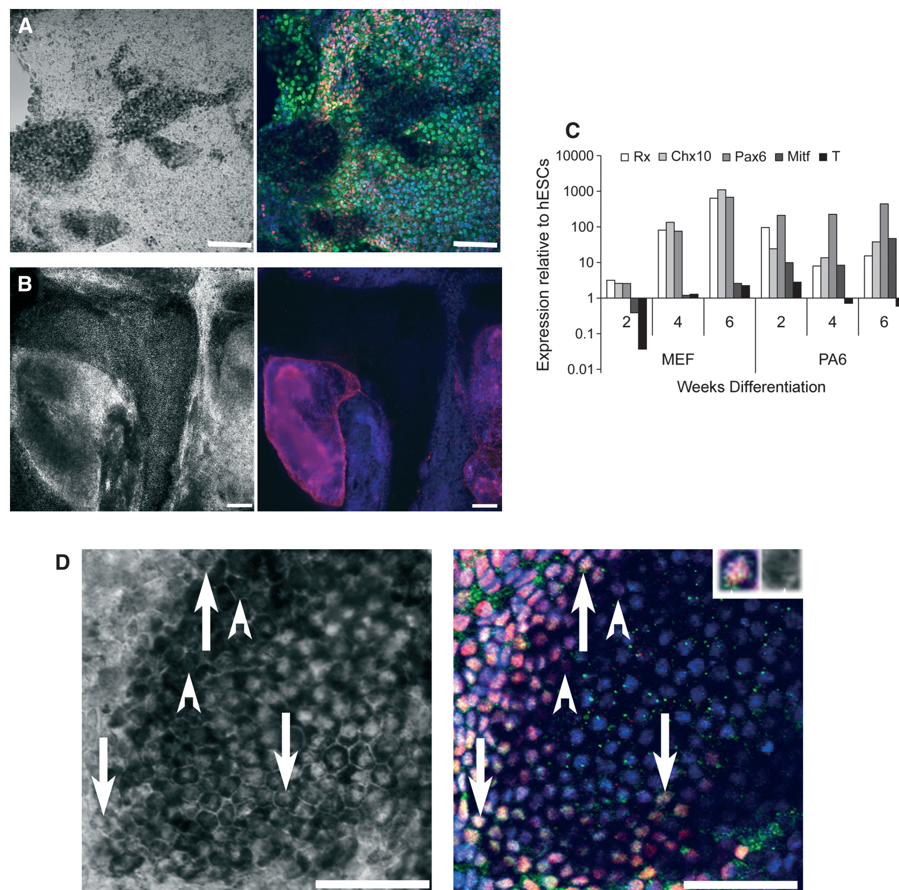

FIG. 1. Human embryonic stem cells (hESCs) differentiate into retinal cells in the absence of exogenous factors. (A) Pigmented patches (grey scale, left) in 12-week differentiated hESCs on mouse embryonic fibroblasts (MEFs) were found along with Pax6-positive (red, right panel) neural/retinal and Chx10-positive (green) neural retinal progenitors/neurons. Scale bar: 100 μm. (B) Clusters of neuron-specific progenitors were also seen (Tuj1-positive, red). Scale bar: 200 μm. (C) hESCs differentiating on either MEFs or PA6 stromal cells upregulated early retinal transcription factors by Q-PCR, but not the mesodermal marker brachyury (T) or the pluripotency marker Oct4. Levels were normalized to starting hESCs. (D) Confocal images show that rare pigmented cells co-expressed nuclear Pax6 (red) and Chx10 (green), indicating that some pigmented cells are undifferentiated retinal progenitor cells (white arrows, right panel inset shows an enlargement of a single Pax6- Chx10-positive pigmented cell). Pigmented cells often expressed Pax6 alone (white arrowheads). Scale bar: 50 μm. Nuclei are counterstained with Hoechst (blue).

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similar in appearance to colonies derived from human CE (Coles *et al.*, 2004), and formed at a low frequency (approximately 1 in 500 pigmented cells from MEF cultures, 1 in 1000 from PA6 cultures, $n = 2$). The relative rarity of colony-forming cells suggested that only a subset of pigmented cells are proliferative RSCs and that other cells are likely committed RPE cells.

Single pigmented colony-forming precursor cells can be isolated using FACS

Hand-picking colonies gave a high purity of pigmented cells; however, it did not allow for efficient isolation of large numbers of pigmented cells. We determined whether pigmented cells could be isolated using FACS based on side scatter. Entire differentiated hESC monolayers containing pigmented patches were first dissociated into single cells and allowed to proliferate in serum-free media for 5–7 days, then dissociated a second time and analysed. For comparison, hESCs in maintenance conditions were cultured under the same conditions. We found that in cells from the differentiated cultures containing pigmented colonies, a population of cells with high side scatter was visible (Fig. 2A). By visual inspection, the starting population contained approximately 8.7 ± 2.7% ($n = 9$) pigmented cells, and the high side scatter fraction contained < 95% pigmented cells. This high-purity pigmented population gave rise to clonal colonies mottled with pigmentation, suggesting proliferation of the starting pigmented cell to give rise to separate pigmented or non-pigmented cells (Figs 2B and C). A second method of purifying pigmented cells was also tested. A 15%/10%/5% ficoll density gradient was used, and similarly resulted in a pigmented population containing rare, colony-forming cells (Fig. 2C). The recovery rates using ficoll (4.9 ± 2.4%) and those using FACS (5.6 ± 1.6%) were not significantly different ($F_{1,11} = 0.59$, $P > 0.5$), nor was there an effect of adding the survival factor ROCK inhibitor ($F_{1,11} = 0.54$, $P > 0.5$), known to

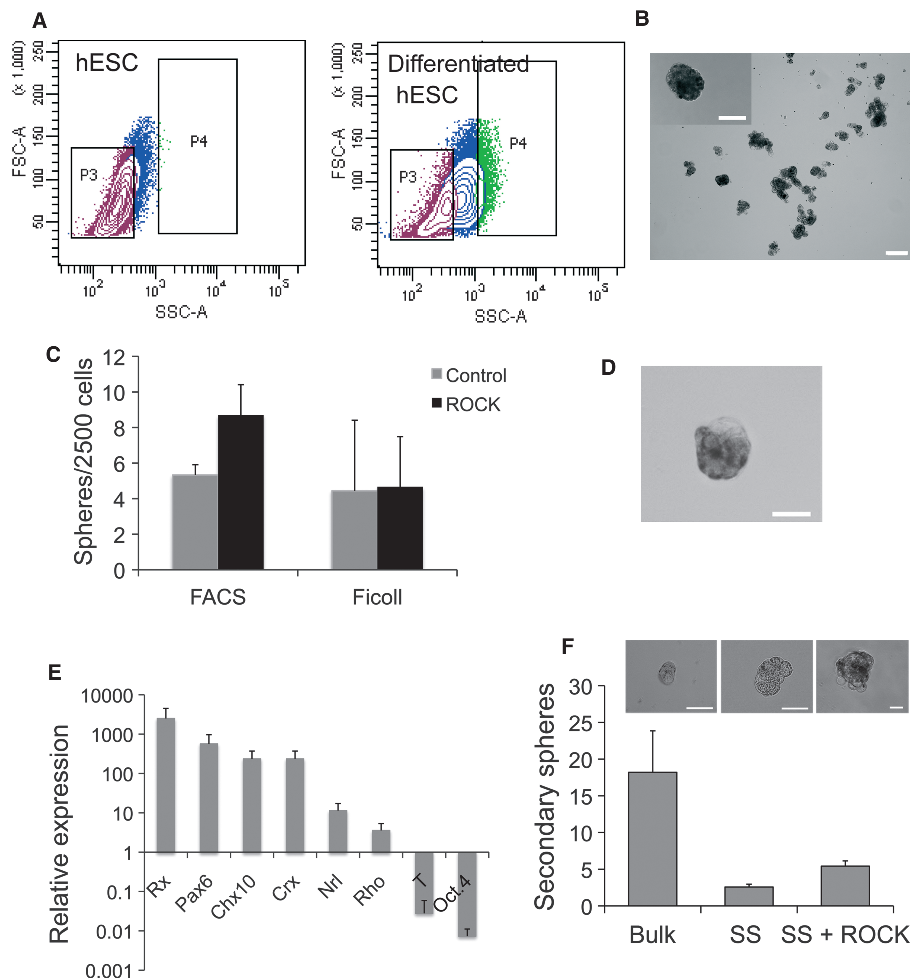

FIG. 2. Single pigmented cells give rise to self-renewing colonies. Pigmented cells could be isolated using FACS. A high side scatter (SSC) population was not present in cells from undifferentiated human embryonic stem cells (hESCs; A, left), but was seen in differentiated hESC colonies (A, right) and contained pigmented cells. Rare single pigmented cells plated in serum-free media with FGF gave rise to colonies containing pigmented and non-pigmented cells. (B) Sphere colonies collected together in the centre of a field after counting. Scale bar: 200 μm; 100 μm (inset). (C) Pigmented cells could also be isolated by centrifugation using a ficoll gradient. There was no significant difference in sphere frequency between FACS and ficoll ($F_{1,11} = 0.59$, $P > 0.5$) or after the addition of ROCK inhibitor ($F_{1,11} = 0.54$, $P > 0.5$). (D) Example of a colony derived from a well with a single cell pigmented cell. Scale bar: 50 μm. (E) Q-PCR revealed expression of early retinal transcription factors in sphere colonies, as well as low levels of more differentiated markers, Crx, Nrl and rhodopsin (Rho), but not the mesoderm marker brachyury (T) or the pluripotency marker Oct4. Levels are normalized to hESCs. (F) Colonies could be passaged in bulk (spheres/2500 cells) and as single spheres. Passaged spheres also formed from single pigmented cells (inset, representative images of single cells on Days 3, 10 and 21 after passaging). Single sphere passaging was significantly improved in the presence of ROCK inhibitor ($t_{98} = 3.60$, $P < 0.5$). Scale bars: 50 μm. All data are shown + SEM.

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promote survival of hESCs after dissociation (Watanabe *et al.*, 2007). In ficoll-purified cultures, however, we observed rare, non-pigmented colonies, likely neural stem cell-derived neurospheres. Colony-forming neural stem cells are present in differentiating hESC colonies (R. Chaddah, M. Arntfield, D. van der Kooy, submitted), and would be expected in our culture conditions as cells first adopted a neural identity and then a subset became specified to retinal fate. We used FACS to obtain all subsequent colonies.

Using a FACS purification strategy also allowed us to sort single cells into individual wells of 96-well plates. The presence of only a single pigmented cell (97.8% of well) or single non-pigmented cell (2.2% of wells) was visually confirmed for each individual well. The frequency of colony formation from single pigmented cells was very similar to bulk cultures, indicating that spheres are clonally derived. Three colonies were derived from 1614 single cells from MEF cultures (0.19% colony formation), compared with 4.1 per 2500 cells in control bulk culture (0.16% colony formation; Fig. 2D). We also observed eight colonies per 2823 cells from PA6 cultures in a separate experiment (0.28% colony formation). No spheres arose from non-pigmented single cells. In addition, when the non-pigmented (low side scatter) fraction was plated after FACS, no pigmented spheres formed ($n = 4$). Thus, single pigmented cells can be FACS isolated, and rare pigmented cells give rise to clonal sphere colonies consisting of pigmented and non-pigmented cells.

Sphere colonies are self-renewing and multipotential

Sphere colonies were characterized for expression of genes involved in retinal development. Using quantitative RT-PCR we found that Rx and Pax6 were highly upregulated in hESC-derived spheres compared with hESCs, while the pluripotency gene Oct4 had highly reduced expression (Fig. 2E), and that spheres also expressed low levels of transcription factors involved in photoreceptor differentiation, Crx and Nrl as well as rhodopsin. Sectioned sphere colonies were immunostained for rhodopsin, but sufficient protein was not detected in single cells.

To test whether colonies exhibited self-renewal, we passaged colonies using enzymatic dissociation to a single cell suspension both in bulk and as single spheres. A small number of clonal secondary spheres formed in both conditions (18.2 ± 5.6 spheres/2500 bulk cells, 2.6 ± 0.4 spheres/single sphere; Fig. 2F). This number was increased twofold by the addition of ROCK inhibitor (5.4 ± 0.7 spheres/single sphere, $t_{98} = 3.60$, $P < 0.5$). Single pigmented cells were observed to give rise to secondary spheres, and all secondary spheres contained some pigmented cells. We also found that cells could be expanded as monolayers in 10% FBS, with a single cell-derived sphere giving rise to 3.2 ± 1.7 million cells after three passages ($n = 6$). This is an underestimation, as trypsinization to a single cell suspension was associated with cell loss.

When differentiated on laminin-coated plates in the presence of 1% FBS, hESC-derived colonies produced bestrophin-positive RPE cells as well as neurons and glia after 4 weeks (Fig. 3). Rhodopsin-positive cells were only rarely observed (< 1% of cells), though the low levels of Crx, Nrl and rhodopsin mRNA in sphere colonies suggested that hESC-derived colonies have the potential to differentiate into rod photoreceptors. This was tested by co-culturing cells with retinal explants. Primary spheres were labeled with GFP-expressing lentivirus by infecting single pigmented cells 24 h after FACS. Spheres were then dissociated into small clumps and co-cultured with adult or embryonic neural retinal explants. Approximately 50% of surviving cells were found in the outer nuclear layer. All of cells in the outer nuclear layer either expressed rhodopsin or remained pigmented and undifferentiated (Fig. 4A), while cells residing in other retinal layers were all rhodopsin-negative. No cells developed outer segments, though some extended short processes. This indicates that the progeny of RSCs derived from hESC colonies can upregulate photoreceptor markers given the appropriate environment.

hESC-derived colonies can integrate into the RPE and neural retina after transplantation into the neonatal mouse eye

To determine whether hESC-derived RSCs could produce multilineage retinal progeny *in vivo*, we transplanted dissociated clonal sphere colonies that had been labelled with GFP-expressing lentivirus into the developing, early postnatal mouse retina; 1–5% of transplanted cells survived after 3–4 weeks, which is similar to findings after transplantation of human eye-derived RSCs and mouse retinal progenitors (Coles *et al.*, 2004; MacLaren *et al.*, 2006; Inoue *et al.*, 2010). More than 90% of the surviving cells were integrated into the RPE or were found between the RPE and photoreceptor nuclei (Figs 4C–F). All cells in the RPE were pigmented and expressed bestrophin (Figs 4C and D), while cells adjacent to the outer nuclear layer expressed rhodopsin (Fig. 4E). More rarely (< 10% of cells), nuclei were observed integrated into the outer nuclear layer or into other retinal layers (Fig. 4F). These results are similar to what is seen after transplantation of mouse CE-derived RSC colonies, multilineage differentiation with preferential integration into the RPE and presence in the subretinal space (Ballios *et al.*, 2010).

hESC-derived RSCs can be directed to a photoreceptor fate in vitro by addition of exogenous factors

Retinoic acid (RA) and taurine have been shown to drive mouse and human RSC progeny to a photoreceptor fate (Ballios *et al.*, 2012). These factors were added to our differentiation media to determine if a similar effect was found in RSCs derived from hESCs. The differentiation period employed for mouse RSCs was 44 days, and we expected a more protracted course with human cells. Serum was maintained in the hESC-derived colony differentiation media, as survival beyond 4–6 weeks was extremely low in serum-free differentiation media. Colonies were allowed a 5–10-day expansion period in FBS with FGF before RA and taurine were added to the media. Under these conditions, most cells remained undifferentiated at 8 weeks, staining positive for the progenitor marker Pax6 (Fig. 4G). By Q-PCR, the rod-specific marker Nrl marker was upregulated at 4 weeks, a 20.9 ± 12.2-fold increase over hESC levels, and remained high at 8 weeks, 14.3 ± 4.8-fold higher than hESCs and 77-fold higher than at Day 0 of RA and taurine ($n = 5$ each). No rhodopsin mRNA was detected at Day 0 of differentiation conditions ($n = 2$), but low levels started to be expressed at 8 weeks, 0.56 ± 0.7-fold of the levels in sphere colonies (not detected in hESCs, $n = 5$). Rhodopsin-positive cells by immunocytochemistry were very rare (< 1% of cells) at 8 weeks of differentiation; however, by 16 weeks, 20.6 ± 3.2% of cells were rhodopsin-positive (Figs 4G–J). In addition, 16-week differentiated cells cultured for an additional 7 days on early postnatal retinal explants were 47% rhodopsin-positive, and a subset of these cells displayed outer segment-like morphology (Fig. 4K). Thus, hESC-derived RSCs can be directed toward a rod photoreceptor fate.

Discussion

ESCs acquire a neural identity in the absence of exogenous factors (Tropepe *et al.*, 2001; Smukler *et al.*, 2006), and neural progenitors specifically will adopt a rostral identity (Gaspard *et al.*, 2008; Wataya

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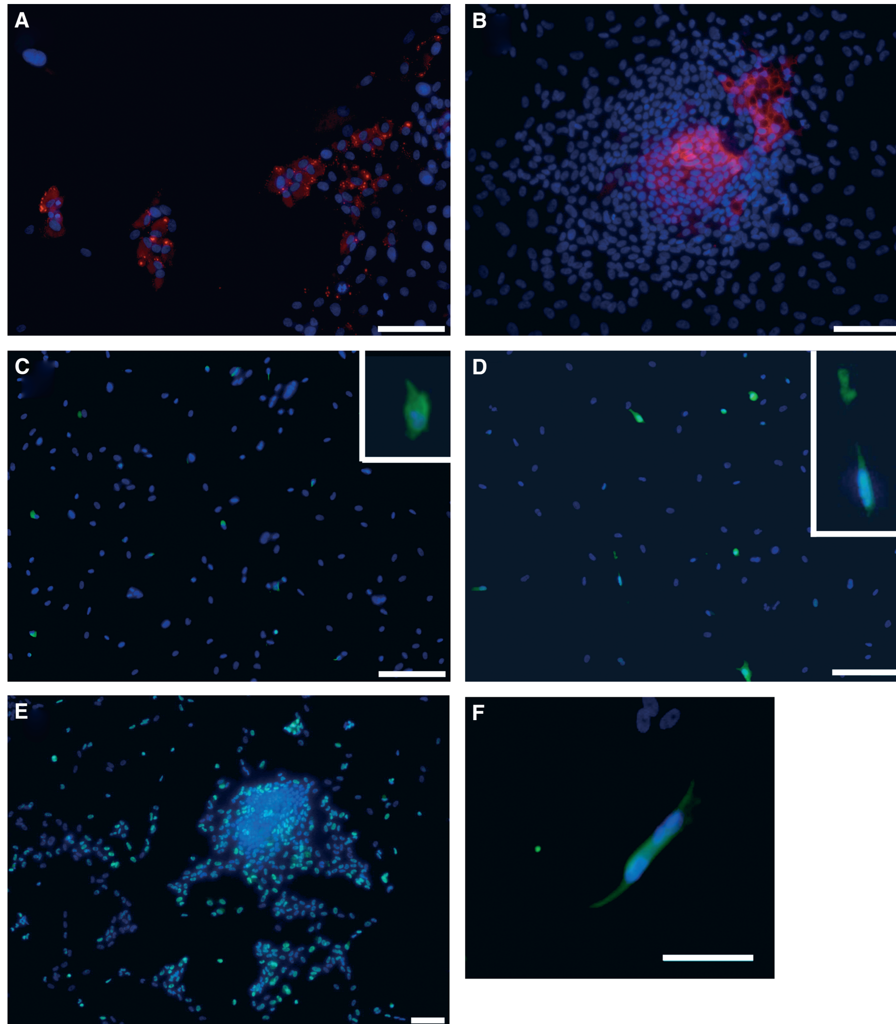

FIG. 3. hESC-derived colonies differentiate into RPE cells, neurons and glia in 1% FBS. RPE cells expressing bestrophin (A), Cralbp-positive Muller glia (B), and Tuj1 (C) and calbindin-positive neurons (D) were detected after 4 weeks. Many undifferentiated cells were present, marked by nuclear Pax6 staining (E). Scale bars: 200 μm. Rhodopsin-positive photoreceptors were rare (F, a few or none in each differentiated clonal colony). Scale bar: 50 μm. Nuclei are counterstained with Hoechst (blue).

et al., 2008). The eye is derived from the embryonic forebrain at the telencephalon/diencephalon border, and ESCs allowed to differentiate in minimal media on MEFs for long periods will subsequently give rise to retinal progenitors (Fig. 1; Klimanskaya *et al.*, 2004; Meyer *et al.*, 2009). Retinal cells also arise from primate ESCs exposed to stromal cell-derived inducing activity, which previously was demonstrated to be neuralizing (Kawasaki *et al.*, 2002). These data suggest that retinal specification may be a default fate for a subset of cells following neural induction (Eiraku *et al.*, 2011).

The first studies focusing on these differentiation protocols characterized the pigmented cells that arose as specific RPE progenitors, which could be expanded to give rise to large sheets of RPE (Kawasaki *et al.*, 2002; Klimanskaya *et al.*, 2004). These conditions also give rise to neural retinal cells (Fig. 1; Meyer *et al.*, 2009); however, the clonal derivation of pigmented and non-pigmented cells from a single RSC-like cell has not been demonstrated previously. Other studies using small molecules to derive neural retinal precursors reported the concomitant emergence of pigmented cells (Ikeda *et al.*, 2005; Osakada *et al.*, 2008), suggesting that bipotential (RPE and neural retinal), optic vesicle-like progenitors are generated, and that embryonic retinal development is recapitulated *in vitro* (Meyer *et al.*, 2009).

RSCs are pigmented cells found in the CE of the adult mammalian eye that also have the capacity to give rise to both RPE and retinal neurons and glia. We find that RSCs are contained within the pigmented cells that arise spontaneously in differentiating hESCs. These cells may be the origin of both neural retina and RPE colonies, or may represent a stem cell compartment that only emerges later within the RPE compartment. Pigment itself is not an early RSC marker, as pigmentation only appears *in vivo* after morphogenesis of the optic cup (Buse & de Groot, 1991). It also does not appear to have a functional role, as RSCs can easily be isolated from albino animals (Tropepe *et al.*, 2000). Muller glia also have been suggested to act as RSCs; however, clonal analyses have not been performed to assay for multipotentiality and self-renewal. Moreover, Muller cells have not been demonstrated to give rise to pigmented epithelial cells and make only a very limited contribution to retina regeneration even after a combination of retinal injury and growth factors or genetic manipulation (Ooto *et al.*, 2004; Osakada *et al.*, 2007; Wan *et al.*, 2007).

Pigmented Chx10 and Pax6-double positive cells have not been described in the developing eye. Pax6 is expressed throughout the optic cup and in early RPE and neural retinal progenitors, but Chx10 is

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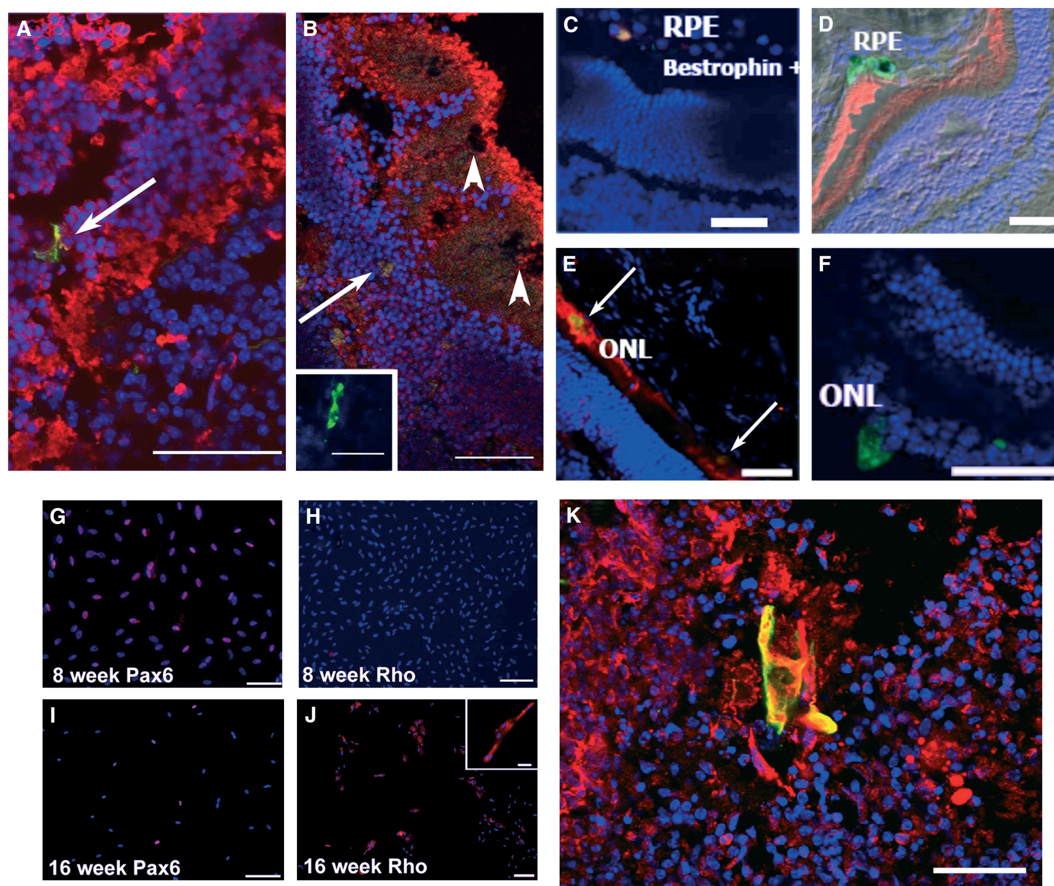

FIG. 4. Cells from hESC-derived colonies can differentiate into rhodopsin-positive cells. Fifty percent of surviving GFP-labelled cells settled in the outer nuclear layer and expressed rhodopsin when co-cultured with adult neural retina (A, B, arrows, rhodopsin-red). They often extended short processes, but did not show fully mature photoreceptor morphology (A). The remaining 50% of the cells either retained pigment and stayed undifferentiated (B, arrowheads) or were found in other retinal layers and were rhodopsin-negative (B, inset – inner nuclear layer). Scale bars: 50 μm; 25 μm (B, inset). Dissociated GFP-marked primary sphere cells also integrated into the developing eye after transplantation into the vitreous fluid of neonatal NOD/SCID mice. Many of the surviving cells integrated into the retinal pigmented epithelium (RPE) and expressed the marker bestrophin (B, C, red-human specific antibody) and were pigmented (C, D). Cells expressing rhodopsin (E) were also found between the RPE and outer nuclear layer (ONL) (red-rhodopsin in D, E marking rod photoreceptors). Some nuclei were seen integrated into neural retinal layers (F). Scale bars: 40 μm (C–F). Rod photoreceptor differentiation could be induced in culture using RA and taurine. At 8 weeks of differentiation in RA and taurine most cells remained undifferentiated Pax6-positive progenitors (G, red), and rhodopsin-positive cells were rare (H, red). By 16 weeks, few cells were Pax6-positive (I), and the number positive for rhodopsin had greatly increased (J). Scale bars: 200 μm (G–J); 50 μm (J, inset). (K) Sixteen-week differentiated rhodopsin-positive cells cultured on perinatal retinal explants for an additional 7 days extended outer segment-like processes. Scale bar: 40 μm. Nuclei are counterstained with Hoechst (blue).

a specific marker of the inner optic cup that will give rise to the neural retina (Liu *et al.*, 1994). Single Chx10-positive cells in our cultures represent restricted neural retinal progenitors with limited or no self-renewal (Tropepe *et al.*, 2000). Most pigmented cells were positive only for Pax6 or negative for both markers, indicating that the majority of pigmented cells in our cultures are RPE progenitors or mature RPE. Rare, double positive cells likely represent RSCs, and we predict they also would be present in the developing eye at a low frequency. Many double positive cells appeared at the edges of pigmented patches. This may reflect a capacity to give rise to both pigmented and non-pigmented retinal cells, or may simply be a consequence of immunostaining of more centrally located pigmented cells being masked by surrounding, heavily pigmented cells. FACS showed that approximately 1 in 500 pigmented cells proliferated to form colonies, similar to the frequency of RSCs from the human CE (Coles *et al.*, 2004).

Embryonic rodent and chick RPE can be induced to produce neural retina through a transdifferentiation process (Guillemot & Cepko, 1992; Zhao *et al.*, 1995). In this case, the vast majority (> 80%) of pigmented cells become neural retinal cells and they are not thought to go through an undifferentiated stem/progenitor state. We find that a rare subset of pigmented cells derived from hESCs possesses the two cardinal stem cell properties of multipotentiality and self-renewal. RSC colonies derived clonally from hESCs showed self-renewal in bulk passages, as monolayers and at the level of single clonal colonies. Colonies differentiated into both RPE and multiple neural retinal cell types *in vitro* and integrated into the developing eye after transplantation. Spheres also could be derived from wells containing single pigmented cells, which then proliferated to produce both pigmented cells and non-pigmented neural retinal progenitor cells. In addition, RSC spheres expressed both the neural retinal-specific progenitor marker Chx10 as well as Pax6 by Q-PCR. These data are inconsistent with a model of aggregated RPE cells simply transdifferentiating to produce neural retinal cells.

hESC-derived RSCs gave rise to a small number of rhodopsin-positive photoreceptors when differentiated for 4 weeks in 1% FBS. Outer segments were not observed, as mature photoreceptor morphology is never seen with *in vitro*-derived photoreceptors, and is even lost

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when young photoreceptors are cultured (Ballios *et al.*, 2012). RA and taurine promote rod photoreceptor differentiation in cultured mouse RSCs and also promoted rod differentiation in our hESC-derived RSCs, though cells required a longer differentiation period than the 3–6 weeks used for mouse RSCs (Tropepe *et al.*, 2000; Ballios *et al.*, 2012). Human eye development takes place over a much longer time course than in mouse. Nrl precedes rhodopsin expression by approximately 7 days in mouse (Akimoto *et al.*, 2006), but in humans this difference is about 5 weeks (Hendrickson *et al.*, 2008). We observed a similar delay between Nrl and rhodopsin expression in our cultures, suggesting that a normal developmental timeline is followed in hESC-derived RSC rod differentiation. Other studies have reported similar prolonged time courses to observe photoreceptor differentiation from hESCs. Very low numbers of rhodopsin-positive cells have been detected after 2–3 weeks of differentiation (Lamba *et al.*, 2006). Meyer *et al.* (2009) first reported opsin at Day 70, and one group reported no opsin expression after 130 days of differentiation and > 10% rhodopsin-positive cells even at Day 200 (Osakada *et al.*, 2008).

The formation of elongated outer segments was not observed after transplantation of RSCs derived from hESCs into the developing eye. This lack of full morphological photoreceptor differentiation also has been reported after transplantation of mouse Muller glial or embryonic retinal progenitors (Yang *et al.*, 2002; Qiu *et al.*, 2005; Tomita *et al.*, 2006; Singhal *et al.*, 2008; Wan *et al.*, 2008). A single demonstration of outer segment formation after transplantation of retinal progenitors exists in a study using cells derived from hESCs (Lamba *et al.*, 2009); however, cell fusion was not ruled out as a potential origin of labelled photoreceptors. When this group used the same protocol to differentiate human iPSCs but then specifically selected for committed photoreceptors, no outer segments formed after transplantation (Lamba *et al.*, 2010). While this could represent an important difference between hESCs and iPSCs, it has been previously shown using mouse neural retinal cell transplants that there is a critical window in the timing of photoreceptor development that results in successful integration and morphological differentiation after transplantation (MacLaren *et al.*, 2006). It is possible that similar transplantation of partially differentiated cells would result in mature rod photoreceptor differentiation; however, we have not yet determined the appropriate timing for hESC-derived RSC progeny differentiation.

A recent study reported the *in vitro* derivation of complete optic cups followed by production of mature neural retinal cell types arranged in appropriate layers from mouse ESCs (Eiraku *et al.*, 2011). While exciting, this finding will need to be replicated in human cells. In addition, the use of ESC-derived cells for therapeutic purposes requires the elimination of residual pluripotent cells due to their potential tumorgenicity. We describe a protocol for clonal isolation of pigmented cells that selects for specified RSCs. Degenerative diseases of the eye often affect only one retinal cell type, in many cases, photoreceptors. RSC colonies can produce multiple retinal cell types. Moreover, they can be specifically directed toward a rod photoreceptor fate and, in principle, other desired retinal cells.

Human retinal stem cells (hRSCs) are potentially a useful cell population in cellular therapy to replace photoreceptors and other cells lost in retinal degenerative disease; however, obtaining autologous tissue is technically difficult and cadaveric donors are limited. hESC-derived RSCs are a potentially unlimited source of retinal cells that can be directed to a photoreceptor fate, and if derived instead from iPSCs (Hirami *et al.*, 2009; Meyer *et al.*, 2009) would avoid immunological rejection. Clonal selection of pigmented cells would eliminate carryover of undifferentiated and potentially tumorigenic cells. We have also shown that bona fide hRSCs emerge in an *in vitro* model of retinal development. These cultures represent a useful and readily available system for investigating the mechanism of hRSC specification and their differentiation into specific retinal cells.

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Abbreviations

CE, ciliary epithelium; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; FGF, fibroblast growth factor; HBSS, Hank's balanced salt solution; hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; RA, retinoic acid; RPE, retinal pigmented epithelium; RSCs, retinal stem cells; RT-PCR, reverse transcriptase polymerase chain reaction.

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