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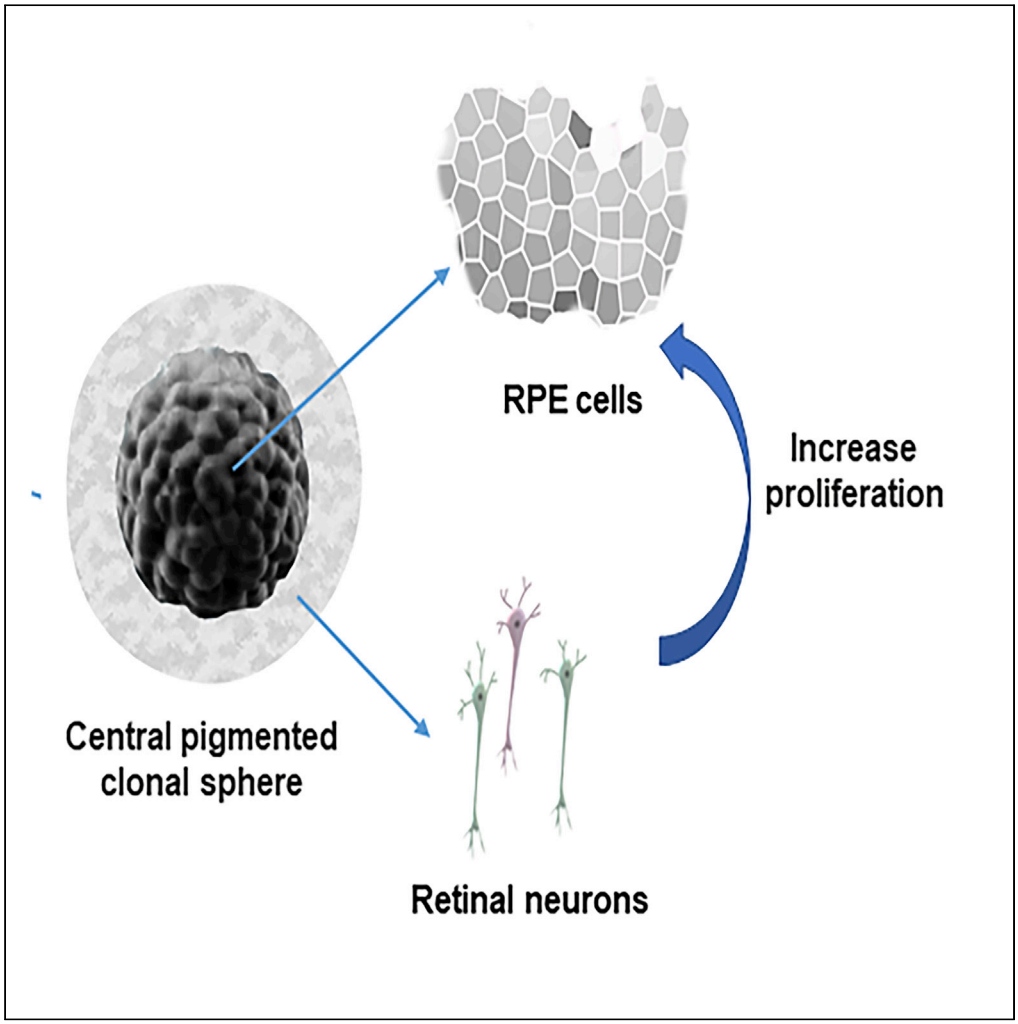

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Highlights

Three types of clonal retinal stem cell spheres form from the same single stem cell

Centrally pigmented spheres contain populations of early RPE progenitors

Heavily and lightly pigmented spheres contain populations of late RPE progenitors

Downstream RPE progenitors are different due to extrinsic and intrinsic factors

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A defined subset of clonal retinal stem cell spheres is biased to RPE differentiation

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SUMMARY

Retinal stem cells (RSCs) are rare pigmented cells found in the pigmented ciliary layer of the mammalian retina. Studies show that RSCs can replicate to maintain the stem cell pool and produce retinal progenitors that differentiate into all retinal cell types. We classified RSCs based on their level and distribution of pigment into heavily pigmented (HP), lightly pigmented (LP), and centrally pigmented (CP) spheres. We report that CP spheres are capable of generating large cobblestone lawns of retinal pigment epithelial (RPE) cells. The other clonal sphere types (HP and LP) primarily produce cells with neural morphology and fewer RPE cells. The RSCs are homogeneous, but their downstream progenitors are different. We found that CP spheres contain highly proliferative populations of early RPE progenitors that respond to proliferative signals from the surrounding non-pigmented cells. HP and LP spheres contain late RPE progenitors which are not affected by proliferative signals.

INTRODUCTION

Understanding retinal stem cell and progenitor heterogeneity is important in exploring the mechanisms by which the retina is built during development. During early stages of optic vesicle development, the retinal stem cell (RSC) population expands and starts producing retinal progenitors, and these progenitors will go through different stages of competence to differentiate into early and late retinal cells which will form the lining of the developing optic cup (Bassett and Wallace, 2012; Cepko, 2014; Dyer and Cepko, 2001; Prada et al., 1991; Wong and Rapaport, 2009; Young, 1985a, 1985b). This process happens in a conserved pattern across vertebrates. However, the size and the composition of cells within the stem cell colonies were widely variable (Cayouette et al., 2003; Cepko, 2014; Fekete et al., 1994; Harris, 1997; He et al., 2012; Trimarchi et al., 2008; Turner and Cepko, 1988; Wetts and Fraser, 1988). This process was found to be governed by both intrinsic and extrinsic signals *in vivo* (Rompani and Cepko, 2008; Trimarchi et al., 2008). *In vitro*, embryonic or adult RSC clones were grown and isolated from a specific proliferative population that resides in the pigmented ciliary epithelium (CE) at the periphery of the eye (Ahmad et al., 2000; Tropepe et al., 2000). This population can be distinguished from the surrounding pigmented CE by their size (large) and the level of pigmentation (heavily pigmented [HP]) (Ballios, Clarke, Coles, Shoichet and van der Kooy, 2012). *In vitro*, an adult RSC colony arises from the proliferation of a single pigmented ciliary epithelial cell. A previous study showed that 0.2% (1:500) of pigmented CE cells, either as single cells per well in 96-well plates or at low cell densities (less than 20 cells/μl in 24-well plates), produced clonal spheres after 7 days containing both pigmented and non-pigmented cells (Tropepe et al., 2000). The same paper showed that mixing green fluorescent protein (GFP+) with wild-type CE cells (at a total cell density of 20 cells/μl) resulted in the formation of spheres that contain GFP+ only cells or wild-type only cells and never clones with mixed populations, indicating that all the cells were derived clonally from single parent stem cells. The same study showed that many non-pigmented cells within the RSC colony express *Chx10* and nestin before differentiation (Tropepe et al., 2000). *Chx10* is a specific marker for neural retinal (NR) progenitors while nestin is a marker of undifferentiated cells. Small numbers of pigmented RSCs from the dissociated clonal spheres have the ability to self-renew and differentiate into all retinal cell types (Ahmad et al., 2000; Ballios et al., 2012; Tropepe et al., 2000). By adding the exogenous factors retinoic acid/taurine or coco, researchers were able to direct RSCs to differentiate into rods (Ballios et al., 2012) or cones (Khalili et al., 2018), respectively. Larger numbers of retinal pigment epithelial (RPE) cells could be produced from adult RSCs by adjusting culture density and media conditions (De Marzo et al., 2010). The division of RSCs is heterogeneous *in vivo*; they undergo a variable number of divisions producing clones with different sizes and cell type compositions. The apparent randomness of clonal size and cell fate distribution suggested a strong element of

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stochasticity (Cayouette et al., 2003; Cepko, 2014; Fekete et al., 1994; Harris, 1997; He et al., 2012; Trimarchi et al., 2008; Turner and Cepko, 1988; Wetts and Fraser, 1988). Downstream retinal progenitor cells pass through a series of competence states to produce all retinal cell types. There are, however, conserved patterns in cell birth with ganglion cells born first and Muller glial cells born last (Bassett and Wallace, 2012; Cepko, 2014; Dyer and Cepko, 2001; Prada et al., 1991; Wong and Rapaport, 2009; Young, 1985a, 1985b). Others have observed heterogeneity even among cells isolated from the same developmental time point. Some clones were found to have multiple types of retinal neurons and some were restricted to one type of retinal neurons (Alexiades and Cepko, 1997; Buenaventura et al., 2018; Schick et al., 2019; Trimarchi et al., 2008).

In this paper, we studied the behavior of RSCs and retinal progenitor cells on the clonal sphere level *in vitro*. We identified three morphologically distinct groups of RSC clonal spheres: HP, lightly pigmented (LP), and centrally pigmented (CP) spheres, one of which (CP spheres) was biased to produce huge lawns of RPE cells. Indeed, two populations of RPE progenitors with divergent gene expression and different response to proliferative signals were found: early RPE progenitors in CP spheres and late RPE progenitors in HP and LP spheres.

The RPE is a monolayer of pigmented cells situated between the neuroretina and the choroids. They are important in maintaining healthy and functioning photoreceptors through ensuring (1) proper transport of nutrients, ions, and water (2) absorption of light and protection against photooxidation, (3) reisomerization of all-*trans*-retinal into 11-*cis*-retinal, which is a key element of the visual cycle, (4) phagocytosis of shed photoreceptor membranes, and (5) secretion of various essential factors that support the structural integrity of the retina (Simó et al., 2010). Loss of RPE cells will consequently result in loss of photoreceptors and subsequently blindness (Hollyfield and Witkovsky, 1974; Miller et al., 2017; Mitrousis et al., 2020). Replacement of dead or dysfunctional RPE with stem cell-derived RPE along with photoreceptors is a promising alternative to treat age related macular degeneration (AMD) and some forms of retinitis pigmentosa (RP) (Surendran et al., 2021). We suggest that the highly proliferative early RPE progenitor population in the CP spheres will provide an enriched and safe source to replace lost RPE in retinal degenerative diseases.

RESULTS

Three types of clonal RSC spheres were isolated from the pigmented ciliary epithelium

In vitro, adult RSC colonies arise from the proliferation of single pigmented ciliary epithelial cells in low density cultures (Tropepe et al., 2000). RSC colonies were thought to be homogeneous in terms of their pigmentation level, proliferation potential, and types of differentiating progenitors they produce (Ahmad et al., 2004; Ballios et al., 2012; Coles et al., 2004; Das et al., 2005; Tropepe et al., 2000). However, we now classify adult mouse and human clonal RSC spheres based on the distributions and levels of pigmentation observed into HP, LP, and CP spheres (Figures 1A and 1B). The same three clonal retinal sphere types also were grown from embryonic day 14 (E14) mouse presumptive CE with differentiation morphologies like those observed with adult clonal spheres (Figure 1C). Further, quantitative analyses of cryostat sections through the centers of each sphere type revealed significantly greater percentage of pigmented surface areas in the centers of CP spheres compared to the other two sphere types (Figures 1D and 1E). CP spheres (27 $\pm$ 3% of all clonal spheres) produced highly proliferative progenitors in adherent cultures that differentiated into large sheets of confluent cells that had a cobblestone-like appearance and much fewer neurite process bearing NR cells (Figure 2A). In comparison, HP (18 $\pm$ 3% of all clonal spheres) and LP (54 $\pm$ 6% of all clonal spheres) colonies produced more NR cells and only scattered pigmented cells (Figures 2B and 2C). Clonal CP spheres from cadaveric adult humans also gave rise to sheets of cobblestone cells when differentiated on laminin in pan-retinal conditions, and again no cobblestone morphologies were observed in human HP and LP sphere cultures (Figure 2D). The average sphere diameters from the three types were similar after 7 days, but significantly larger diameters of the CP spheres compared to the other two sphere types were observed by day 14 (Figures 3A and 3B). This difference may be due to increased proliferation and/or better cell survival inside the CP spheres. Based on these proliferation and differentiation potentials, we hypothesize that CP spheres contain populations of highly proliferative early RPE progenitors, while HP and LP contain populations of late RPE progenitors that lack the proliferative capabilities of early RPE progenitors.

All RSC clonal sphere types are derived from the same parent stem cell

To test the hypothesis that mouse HP, LP, and CP spheres are all derived from the same RSC type, spheres of each type (HP, LP, and CP) were bulk passaged separately (10 spheres/passage). We found that every

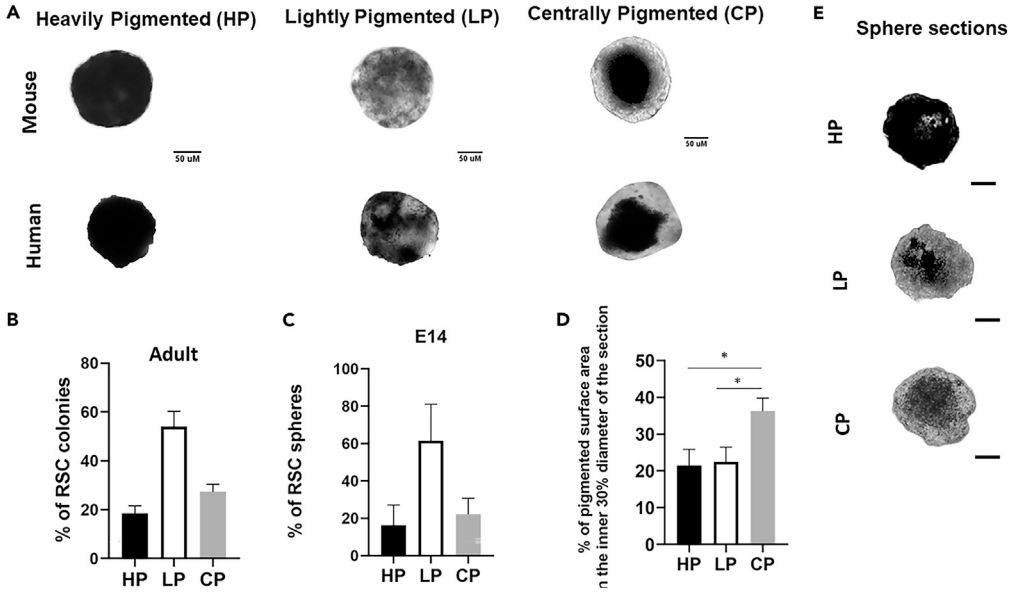

Figure 1. Three types of clonal RSC spheres can be isolated from adult and E14 pigmented ciliary epithelium

(A) Types of retinal stem cell clonal spheres based on their pigmentation levels and distributions in mouse and human. HP, heavily pigmented; LP, lightly pigmented; and CP, centrally pigmented.

(B)The percentages of the three types of adult mouse RSC colonies.

(C) RSC clonal sphere percentages from E14 ciliary epithelium.

(D) The percentages of pigmented surface areas in the inner 30% of the sphere diameter from the sections through the centers of spheres for the three sphere types. CP sphere sections have more areas of pigment within their inner 30% diameter areas than the other two sphere types (CP compared to LP spheres ($t = 2.456$, $p = 0.036$) and HP spheres ($t = 2.56$, $p = 0.03$) ($n = 4–7$) (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

(E) Cross section of HP, LP, and CP spheres. Data represent means ± S.E.Ms.

sphere type could produce new clonal spheres of each of the three sphere types (Figure 3C), suggesting that all three sphere types share the same clonal origin (equivalent parent RSCs). However, we noticed that HP spheres gave rise to significantly more HP spheres than LP or CP spheres (Figure 3C). This bias may be due to either an intrinsic stem cell difference or extrinsic influences on downstream progenitors. However, the data above suggest that the original stem cell in all sphere types may be the same, but interactions between downstream progenitors may influence progenitor fate decisions. To distinguish between these possibilities, one dissociated clonal HP sphere was passaged alone, and cells were all plated in a clonal sphere assay. Indeed, HP sphere stem cells (free from the excess of HP progenitor cells) were able to produce all sphere types in percentages similar to those observed after primary culture (compare Figures 1B and 3D), indicating that the stem cell itself is not biased toward producing more HP spheres. The bias we observed (HP spheres giving rise to more HP spheres) was limited to bulk passaged HP spheres (passaging 10 HP spheres together), suggesting that this is not an intrinsic attribute of the stem cell, but rather the result of extrinsic signals received from the large number of surrounding HP progenitors in the culture. In conclusion, all three sphere types are the product of the same (parent) stem cell, but the downstream progenitors in the sphere types are different.

Cobblestone morphologies are observed only with differentiated CP spheres

Individual primary RSC spheres were differentiated in the presence of 1% fetal bovine serum (FBS), fibroblast growth factor (FGF), and heparin for 21 days on laminin (pan-retinal conditions). CP spheres produce large number of cobblestone cells while HP and LP spheres do not. In order to determine if large lawns of cobblestone cells can be generated from other sphere types, HP spheres were differentiated at 6 spheres/well (rather than 1 sphere/well) on laminin for 21 days. No cobblestone morphologies were observed in these higher density cultures (Figure S1). To check if the early RPE progenitors from CP spheres can influence late RPE progenitors or NR progenitors in HP spheres to change fate and differentiate into larger numbers of cobblestone cells, one HP YFP+ sphere was co-cultured with one CP sphere derived from a

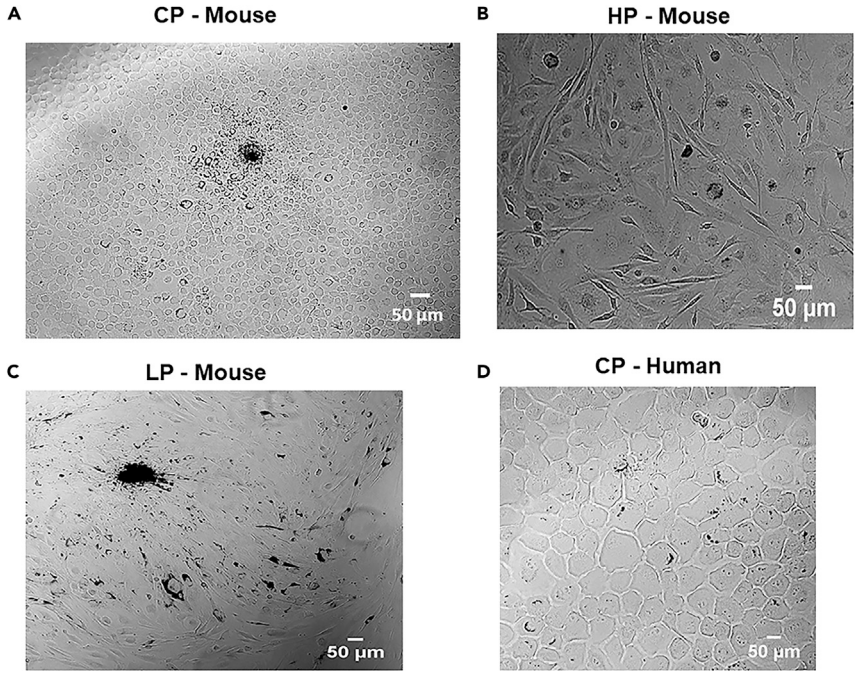

Figure 2. Centrally pigmented spheres produce large numbers of cobblestone cells resembling RPE morphologies

(A) Mouse CP spheres after pan-retinal differentiation.

(B and C) Mouse HP and LP spheres after pan-retinal differentiation.

(D) Human CP spheres after pan-retinal differentiation. Spheres were plated on laminin (1 sphere/well) for 21 days.

C57BL/6 wild-type mouse during differentiation on laminin for 21 days. All YFP+ HP cells maintained their primarily NR identity and did not display the cobblestone differentiation pattern (Figure S2). In conclusion, late RPE progenitors from the HP spheres did not produce cobblestone morphologies when differentiated with other HP sphere cells or when differentiated with a CP sphere, indicating that the cobblestone differentiation pattern is exclusive to early RPE progenitors in the CP spheres.

Cobblestone cells express RPE markers

The differentiation data demonstrated that the CP spheres gave rise to more cells when plated on laminin for 21 days compared to LP and HP (2586 $\pm$ 566, 446 $\pm$ 218, and 430 $\pm$ 140, respectively) (Figure 3E). We also found that non-pigmented cobblestone cells from CP spheres expressed the early RPE marker microphthalmia-associated transcription factor (MITF) (28 $\pm$ 5%) more than cells from LP spheres (4 $\pm$ 3%) and HP spheres (0.388% $\pm$ 0.280). Some differentiated CP sphere cells showed more RPE65 expression compared to cells from LP and HP spheres (Figures 3F and S3A). Rhodopsin expression was also observed in all sphere types both with immunostaining (Figure S3A) and quantitative PCR (qPCR) analyses (data not shown). The tight junction protein ZO-1 (zonula occludens-1), which is important for accelerating claudin polymerization/assembly, binding to F-actin and initiating cell signaling (McNeil et al., 2006; Rizzolo, 2007; Shin and Margolis, 2006), was expressed in CP sphere cells but not in the HP sphere cells (Figures S3A and S3B), indicating that ZO-1 expression may be essential for the formation of the cobblestone morphology. As in mouse, the human CP spheres also had more cells compared to HP and LP cultures, and more human cells from the CP spheres expressed RPE markers such as bestrophin (Figures S4A and S4B). Thus, CP spheres make larger clones compared to HP and LP spheres. CP cells also express more RPE markers than the other two sphere types suggesting that the CP spheres may contain a highly proliferative population, specifically early RPE progenitors that maintain their proliferative potential during sphere formation and differentiation. Our culture duration in mouse (21 days) and human (60 days) is not enough to promote full maturation of RPE cells. During the culture period, most of the pigmented RPE cells extrude their pigment and become less pigmented or non-pigmented. In CP sphere culture, these non-pigmented cells maintain their cobblestone morphology, sometimes proliferate and express early RPE

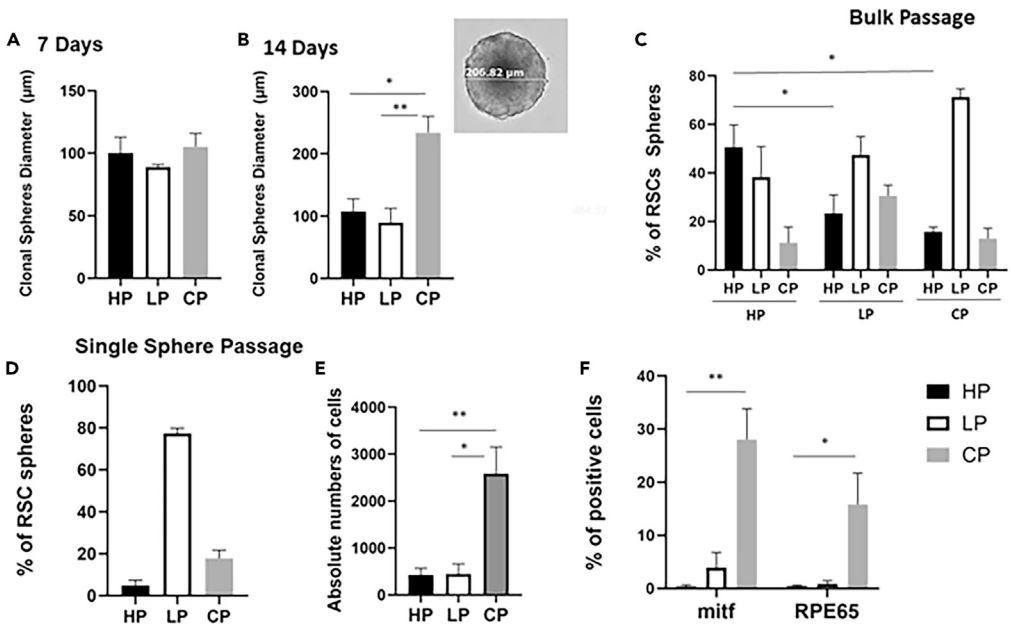

Figure 3. CP spheres express RPE markers and are more proliferative than HP and LP spheres

(A) and (B) Average sphere diameter comparisons of HP, LP, and CP sphere colonies revealed no significant difference after 7 days, but the diameters of CP spheres were significantly larger than those of HP ($n = 3$, $t = 3.85$, $p = 0.0183$) and LP spheres ($n = 3$, $t = 4.185$, $p = 0.0139$) after 14 days of culture. A two-way analysis of variance (ANOVA) revealed a significant interaction of sphere types with 7 versus 14 days of culture ($F(2,12) = 8.287$, $p = 0.005$). The diameters of the CP spheres are significantly greater after 14 than 7 days of culture ($n = 3$, $t = 4.59$, $p = 0.01$). Spheres were imaged, and their diameters were measured using Ziess microscopy software (Axio Observer D1; Carl Zeiss) with AxioVision 4.8 software (Carl Zeiss).

(C) Bulk passaging 10 spheres of each of the three sphere types (HP, LP, and CP) showed that each type was capable of producing all three types of spheres. HP spheres gave rise to significantly more HP spheres at passaging, when compared to HP spheres from CP spheres ($n = 3$, $t = 2.45$, $p = 0.035$) or to HP spheres from LP spheres ($n = 3$, $t = 2.276$, $p = 0.031$).

(D) Single HP spheres were dissociated and passaged. Clonal spheres were counted after 7 days, and the percentages of each sphere type were calculated. We found that the stem cells in the HP sphere were able to produce all three sphere types when passaged as single spheres rather than bulk passaged, with the percentages of HP spheres decreasing significantly when passaged as one sphere compared to bulk passaging ($n = 3$, $t = 4.127$, $p = 0.009$).

(E) CP spheres have more differentiated cells when compared to HP ($n = 3$, $t = 3.173$, $p = 0.005$) and LP spheres ($n = 3$, $t = 2.467$, $p = 0.027$). Cells were fixed after 21 days of differentiation, counter stained with Hoechst, and then cells were counted using the ImageJ program.

(F) Percentages of MITF and RPE65 expression in CP, HP, and LP sphere differentiation cultures. Compared to HP spheres, CP spheres expressed more MITF ($t = 4.75$, $p = 0.0089$) and RPE65 ($t = 3.11$, $p = 0.026$) ($n = 3$–5) (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Data represent means ± S.E.Ms.

genes like *Mitf* and *Otx2*; however, many genes associated with RPE function and polarity are not highly expressed. We suggest that extended culture duration is needed, along with supplementing the culture with factors that are known to accelerate RPE maturation.

This difference may be due to (1) intrinsic differences in the CP early RPE progenitors or (2) extrinsic factors such as the outer non-pigmented cells in the CP spheres secreting proliferative factors favoring the production of more RPE cells. Alternatively, perhaps, factors inhibitory to RPE proliferation may not diffuse well to the sphere center, which again may favor RPE proliferation (Leschey et al., 1990; Schönfeld, 2000; Spraul et al., 2004).

To investigate potential intrinsic differences between HP, LP, and CP RPE progenitors, qPCR expression analyses were done on pigmented cells sorted from spheres of the 3 types. Higher expression levels of Otx2 were observed in the pigmented cells from CP spheres in comparison to pigmented cells from the other two sphere types (Figures 4A and 4B). Otx2 is a homeodomain-containing transcription factor known for its essential role in forebrain formation (Martínez-Morales et al., 2003; Martinez-Morales et al., 2001). In vertebrate eyes, Otx2 is

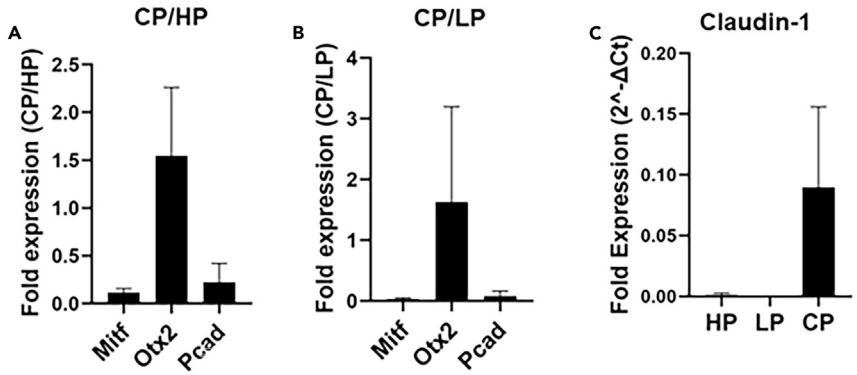

Figure 4. Pigmented cells in CP spheres express early markers of retinal differentiation

(A) Fold changes of Mitf, Otx2, and Pcad in CP pigmented cells compared to those of pigmented cells from HP spheres using delta delta ct method (n = 2).

(B) The fold change of Mitf, Otx2, and Pcad in CP pigmented cells compared to LP spheres (n = 2).

(C) Pigmented cells in CP spheres express higher level of Claudin-1 when compared to pigmented cells in LP and HP sphere (n = 2). Data represent means ± S.E.Ms.

initially expressed in the entire embryonic optic vesicle, but its expression soon becomes restricted to the presumptive RPE, where it is maintained throughout adulthood (Lane and Lister, 2012; Martinez-Morales et al., 2001; Schmitt et al., 2009; Zuber et al., 2003). Early RPE progenitors in CP spheres concentrate more in the centers of the spheres. Although this may happen stochastically, a perhaps more likely explanation is higher expression of tight and/or adherence junction genes. Epithelial cells, including RPE cells, are known to be connected to each other as well as to their extracellular matrix by a subset of molecules forming tight junctions, adherence junctions, and/or gap junctions (Rizzolo, 2007). Some of these molecules include but are not limited to E-cadherin, occludin, integrin, Claudin (Rahner et al., 2004; Rizzolo, 2007; Rizzolo et al., 2011). CP pigmented cells express more claudin-1 (tight junction gene) than pigmented progenitors in HP and LP spheres (Figure 4C), but knocking down claudin-1 did not result in a decrease in the types, numbers, or sizes of the spheres compared to controls (Figure 5). qPCR expression analysis confirmed that claudin-1 expression was 98% lower after our siRNA treatment compared to controls (Figure 5). In early RPE progenitors (the pigmented cells from CP spheres), claudin-1 might work with other genes that regulate strong adhesion among pigmented cells at the center of the sphere. In the claudin-1 knockdown experiment, the stem cell could produce all three sphere types indicating that suppressing claudin-1 did not affect stem cell self-renewal or progenitor proliferation potentials. Blocking claudin-1 in LP spheres showed no significant difference in the total number or size of passaged spheres compared to control (Figure S5). To conclude, the posited early RPE progenitors in CP spheres express higher levels of Otx2 and claudin-1 compared to the posited late RPE progenitors from HP and LP spheres. Blocking claudin-1 did not disrupt the central concentration of the pigmented RPE cells in CP spheres nor the stem cell survival and proliferation capabilities.

Early RPE progenitors proliferate in response to extrinsic signals received from NR cells

To test whether early RPE pigmented progenitors in the CP spheres are proliferating in response to extrinsic signals from the surrounding non-pigmented NR progenitors, pigmented YFP+ cells from CP, HP, and LP spheres were cultured alone or in combination with CP, LP, or HP YFP- non-pigmented cells on laminin for 12 days in 1% FBS + FH. YFP+ cells were counted in each condition every 4 days before the cells were fed with fresh media Figure S6. Co-culturing 5 pigmented cells with 20 non-pigmented cells (all from CP spheres) increased the number of pigmented YFP+ cells 3-fold compared to culturing 5 CP sphere pigmented cells alone (Figure 6A). Similar increases in expansion were observed when CP sphere pigmented cells were co-cultured with non-pigmented cells from the other adult sphere types (Figure 6B). Further, these expansions of CP sphere pigmented cells were due to proliferation of the cells, as these cells were labeled with the proliferation marker Ki67 (Figure 6C). To summarize, early RPE progenitors in CP spheres are responsive to signals received from non-pigmented cells derived from all sphere types.

Non-pigmented NR progenitors isolated from adult RSC (HP, LP, and CP) spheres were proliferative when cultured at a 20 cells/well density. We found that culturing 5 non-pigmented cells/well from CP spheres

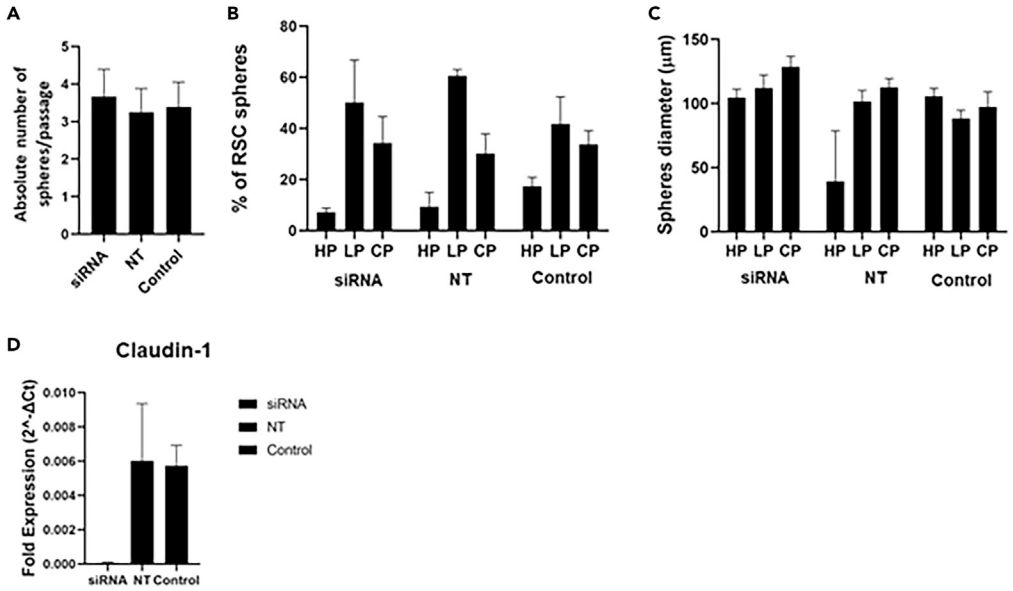

Figure 5. Knockdown of claudin-1 in CP spheres did not affect sphere diameters nor passaging

(A) siRNA depletion of claudin-1 did not affect the numbers of passaged spheres compared to non-targetting (NT) siRNA and no siRNA treatment (control) (n = 3, one-way ANOVA, F (2,6) = 0.0989, p = 0.907).

(B) The percentages of RSC spheres of each of the three types of RSC clonal spheres that were similar across conditions. No significant interaction was observed between sphere types and siRNA conditions (n = 3, two-way ANOVA, F (4,17) = 0.718, p = 0.591) and only a significant main effect of sphere type was seen (F (2,17) = 14.67, p = 0.0002, Tukey-Kramer post-hoc, $p < 0.05$), indicating that knocking down claudin-1 did not disrupt the central concentration of the pigmented RPE cells in CP spheres nor stem cell survival or proliferation capabilities.

(C) No significant differences in the sphere diameters were observed (no interaction of siRNA and control conditions with sphere type (n = 3, two-way ANOVA, F (4,18) = 2.39, p = 0.088) nor any main effect of sphere type (F (2,18) = 2.878, p = 0.082).

(D) A 98% depletion of claudin-1 was observed after siRNA treatment compared to the no treatment and non-targetting siRNA controls. Data represent means ± S.E.Ms.

alone negatively affected their survival (from 5 cells/well to 1–2 cells/well after 12 days of culture), but culturing them at the higher density of 20 non-pigmented cells/well increased their survival and proliferation (Figure 6D), as reflected by higher cell expansion and positive Ki67 staining (Figures S67A and S6B). No changes in the Ki67 labeling of non-pigmented cells were observed when non-pigmented cells from each sphere were co-cultured with pigmented cells from each sphere type including CP (Figure S7C), HP (Figure S8A), and LP (Figure S9A), indicating that proliferation of non-pigmented cells is not dependent on culturing with pigmented cells but rather on proliferative factors secreted by other non-pigmented cells from any sphere type. Thus, non-pigmented NR progenitor cells from all sphere types proliferate in response to signals received from other non-pigmented cells but not from pigmented (RPE) cells. Culturing HP and LP pigmented cells in combination with non-pigmented cells from either HP, LP, or CP spheres did not increase the proliferation of HP and LP pigmented cells relative to controls (Figures S8B, S8C, S9B, and S9C). The increased expansion and proliferation are exclusive to the pigmented early RPE progenitor cells derived from CP spheres and are not observed in cells derived from the late RPE progenitors from the two other sphere types. In conclusion, pigmented cells in CP spheres do not show increased proliferation on their own but depend upon proliferative signals received from the surrounding non-pigmented cells. Conversely, late RPE progenitors in HP and LP do not proliferate in response to these same signals from NR progenitors. Proliferation of adult RSC derived non-pigmented progenitors is independent of the pigmented RPE progenitors, and non-pigmented NR progenitors appear to proliferate in response to factors secreted from other non-pigmented NR cells in the cultures.

DISCUSSION

We studied RSC and downstream retinal progenitor cell heterogeneity on a clonal level. The data showed that there are three clonal sphere types arising from the same parent retinal stem cell in the CE. These

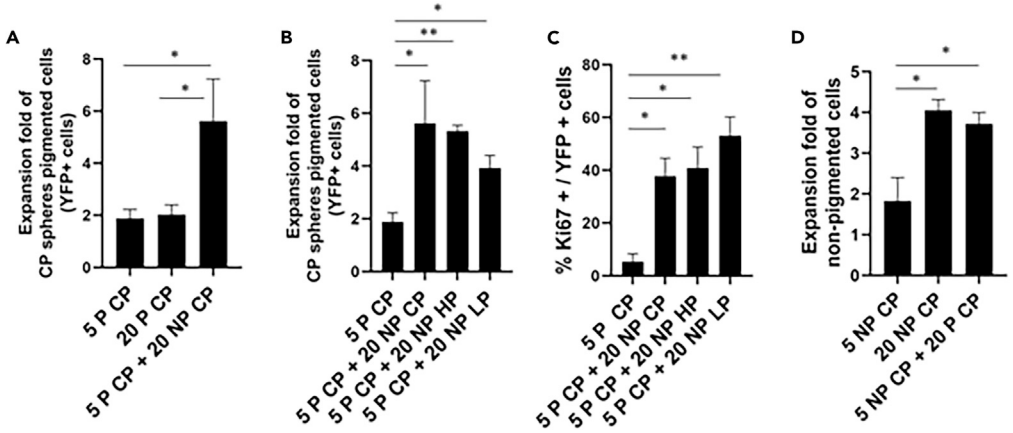

Figure 6. Early RPE progenitors in CP spheres proliferate in response to extrinsic signals received from the surrounding non-pigmented NR cells

(A) Expansion fold of CP pigmented YFP+ cells achieved after 12 days of culture. Pigmented cells from YFP+ CP spheres were cultured alone at 5 or 20 cells/well or co-cultured at 5 cells/well with 20 CP non-pigmented C57BL/6 cells/well. A significant fold increase of YFP+ cells was observed in the co-culture experiment compared to the experiments with 5 pigmented cells alone ($t = 2.26$, $p = 0.044$) or to 20 pigmented cells alone ($t = 2.16$, $p = 0.048$) ($n = 3$).

(B) The effect of combining non-pigmented cells from different sphere types on the expansion of pigmented cells from CP spheres. Significant fold increases were found when pigmented CP cells were co-cultured with NP CP ($t = 2.26$, $p = 0.044$), NP HP cells ($t = 7.171$, $p = 0.0028$), and NP LP cells ($t = 3.608$, $p = 0.0183$) compared to the 5 cells/well pigmented cells alone control ($n = 2$–3 biological replicates, 4–12 technical replicates per biological replicate).

(C) Percentages of YFP+ cells double labeled with the Ki67 proliferation marker. Five pigmented CP cells co-cultured with C57BL/6 non-pigmented cells from CP, HP, or LP spheres showed significant increases in Ki67 double labeling compared to 5 pigmented cells cultured alone (one-way ANOVA $F(3,8) = 9.951$, $p = 0.0045$, Tukey-Kramer post-hoc tests, $p < 0.05$).

(D) Expansion fold of YFP+ non-pigmented cells after 12 days of culture. Non-pigmented cells from YFP+ CP spheres were cultured either alone at 5 and 20 cells/well or co-cultured at 5 cells/well with 20 cells/well of CP pigmented cells. Significant increases were found when comparing the co-culture experiment to 5 non-pigmented cells alone but not to 20 non-pigmented cells alone ($n = 3$, one-way ANOVA, $F(2,6) = 9.033$, $p = 0.0155$, Tukey-Kramer post-hoc tests, $p < 0.05$) (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Data represent the means ± S.E.Ms. P = pigmented, NP = non-pigmented.

colonies were classified based on the distributions and levels of pigmentation into HP, LP, and CP spheres. To explain these observed differences, we suggest that the stem cells forming each of the sphere types are identical, but the downstream progenitors are different. The stem cells within each clonal colony were able to self-renew and continued to give rise to all three sphere types after repeated passaging *in vitro* and were capable of differentiating into NR and RPE cells, indicating their multipotentiality. The data demonstrated that the progenitors downstream of the homogeneous RSCs were different due to intrinsic differences in gene expression in addition to extrinsic proliferative factors secreted by the surrounding NR cells. CP spheres contain a population of highly proliferative RPE progenitors that produce a large number of cobblestone cells resembling RPE *in vivo*. On the other hand, HP and LP spheres did not differentiate into lawns of cobblestone RPE cells, indicating that the cobblestone differentiation pattern is exclusive to early RPE progenitors in the CP spheres. .Differentiated CP sphere cells express Mitf, RPE65, and ZO-1 more than LP and HP spheres. ZO-1 was not expressed in the HP sphere cells indicating that ZO-1 may be important for the formation of the cobblestone morphology. CP spheres also make larger clones compared to HP and LP spheres, suggesting that the CP spheres may contain a highly proliferative population, specifically early RPE progenitors that maintain their proliferative potential during sphere formation and differentiation. Sorting pigmented (RPE) and non-pigmented cells allowed us to study the proliferation potential of each cell type alone or in combination with each other. Our experiments revealed that the pigmented cells from CP spheres were not proliferative on their own and only unleash their intrinsic proliferation potential upon reception of signals from the surrounding non-pigmented NR cells. Unlike CP spheres, pigmented cells from the HP and LP spheres did not respond to these proliferative signals from NR cells. Based on gene expression and response to external signals, we classified the pigmented cells in RSCs clonal spheres into early (CP spheres) and late (HP and LP spheres) RPE progenitors. Indeed, the gene expression of RPE genes and adhesion was different among HP, LP, and CP pigmented cells. The pigmented cells of CP spheres expressed early RPE genes (MITF

and Otx2) in addition to the claudin-1 gene (a tight junction gene). These genes are expressed in presumptive RPE during early development suggesting that the RPE progenitors in the CP spheres are of early origin. In a claudin-1 knockdown experiment, the RSCs could produce clonally all three sphere types, indicating that suppressing claudin-1 did not affect stem cell self-renewal or progenitor proliferation potentials. Blocking claudin-1 did not disrupt the aggregation of pigmented cells at the centers of the CP spheres, hinting that other adhesion genes might work separately and/or in conjugation with claudin-1 to regulate adhesion in the core of CP spheres.

Limitation of the study

Due to short culture period, many genes associated with RPE function and polarity are not highly expressed (RPE65, ZO-1, and Best-1). We suggest that extended culture duration is needed along with supplementing the culture with factors that are known to accelerate RPE maturation and maintain their fate.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102574.

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AUTHOR CONTRIBUTIONS

D.v.d.K. and T.W.B. designed the experiments. T.W.B. and B.C performed the experiments; T.W.B analyzed the data. T.W.B. wrote the manuscript and D.v.d.K. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-MITF	Thermo Fisher Scientific	Cat#MA5-14146, RRID:AB_10979903
anti-RPE65	Millipore	Cat#MAB5428, RRID:AB_571111
anti-Best1	Thermo Fisher Scientific	Cat#MA1-16739, RRID:AB_568460
anti-ZO-1	Thermo Fisher Scientific	Cat#61-7300, RRID:AB_138452
anti-Rhodopsin	Millipore	Cat# MAB5316, RRID:AB_2156055
Alexa fluor 568	Thermo Fisher Scientific	Cat # A-11004, RRID:AB_2534072
Alexa fluor 488	Thermo Fisher Scientific	Cat # A-11001, RRID:AB_2534069
Ki67 conjugated monoclonal antibody, FITC	eBioscience	Cat#11-5698-82, RRID:AB_11151330
Ki67 conjugated monoclonal antibody, PE	eBioscience	Cat#12-5698-82, RRID:AB_11150954
Biological samples		
Human eyes	Eye Bank of Canada	https://www.kensingtonhealth.org/eye-bank
Chemicals, peptides, and recombinant proteins		
FGF2	Sigma-Aldrich	Cat# F0291
Heparin	Sigma-Aldrich	Cat# H3393-10KU
Trypsin inhibitor	Roche	Cat# 10109886001
Propidium iodide	Thermo Fisher Scientific	Cat# P1304MP
Elastase	Sigma-Aldrich	Cat#E1250-10MG
Laminin	Sigma-Aldrich	Cat# L4544-100UL
Heat-inactivated fetal bovine serum	Thermo Fisher Scientific	Cat# 10082139
DNase	Qiagen	Cat# 79254
Experimental models: Organisms/strains		
C57BL/6J mice	The Jackson Laboratory	http://www.jaxmice.jax.org
EYFP mice	The Jackson Laboratory	Tg (ACTB-EYFP) 7AC5Nagy
Oligonucleotides		
Claudin-1	Thermo Fisher Scientific	Cat#Mm00516701_m1
Occludin	Thermo Fisher Scientific	Cat# Mm00500912_m1
Integrin 5 alpha	Thermo Fisher Scientific	Cat#Mm00439797_m1
Cdh1	Thermo Fisher Scientific	Cat#E- cadherin: Mm01247357_m1
Mitf	Thermo Fisher Scientific	Cat# Mm00434954-m1
Cdh3	Thermo Fisher Scientific	Cat#Mm249209-m1
Otx2	Thermo Fisher Scientific	Cat#Mm00446859-m1
Claudin-1 pooled siRNAs	Horizon Discovery	Cat # E-059246-00-0010
Software and algorithms		
Adobe Photoshop Version 20.0.0	Adobe	http://adobe.com
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
BD FACS Diva Software V6.1.2	FacsAria II (BD)	https://www.bd.com/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AxioVision 4.8 software	Carl Zeiss	https://www.zeiss.com/
QuantStudio 6 Fast Real-Time PCR System	Applied Biosystems	https://www.thermofisher.com/ca/en/home/life-science/pcr
GraphPad Prism 8	GraphPad Software Inc	https://www.graphpad.com/
Other		
RNeasy Mini Kit	Qiagen	Cat# 74104
iScript cDNA Synthesis Kit	Bio-RAD	Cat#170-8890

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tahani Baakdhah (tahani.baakdhah@mail.utoronto.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse eyes source

RSCs were derived from the ciliary epithelium of adult male and female (7 – 8 week-old) C57BL/6J mice or of (7 – 16 week-old) Actin promotor driven ubiquitous enhanced yellow fluorescent protein expressing mice (EYFP), Tg (ACTB-EYFP) 7AC5Nagy (Jackson Laboratories, Bar Harbor, ME, http://www.jaxmice.jax.org), on a F129S1/SvImJ background. All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Toronto.

Human eyes source

Human eyes of adult male and female were procured from the Eye Bank of Canada (Toronto, ON) within 24h postmortem and their use approved of by the UofT Human Research Ethics Board. The ciliary epithelium was dissected and cells were cultured under clonal density (RSC clonal spheres forming assay).

METHOD DETAILS

Clonal RSC spheres assay

We dissected the ciliary epithelium from adult C57BL/6 or YFP+ mice (using a previously published protocol (Tropepe et al., 2000)). Briefly, we dissected the pigmented ciliary epithelium and then transferred the strips into a 35-mm dish containing 2 mL of Dispase (Corning Inc., Massachusetts, United States) and incubated in a 37° C incubator for 10 minute. Cells were then moved into a dish containing 2 mL of filtered Trypsin, Hyaluronidase, and Kynurenic Acid (Sigma-Aldrich, Oakville, ON). and placed at 37 °C incubator for 10 minutes. Under the dissecting microscope, the pigmented ciliary epithelial cells were scraped away from the sclera and the sclera was discarded. Using a fire-polished cotton-plugged pipette, this solution was transferred into a 14-ml tube, triturated, centrifuged and the supernatant was discarded. Trypsin inhibitor (Roche) was added, centrifuged and the supernatant was discarded. Next, 1 mL of the plating medium was added and the cells were counted and on non-adherent tissue culture plates (Nunc; Thermo Fisher Scientific, Rochester, NY) at a density of 20 cell/μL in serum-free media (SFM) with FGF2 (10 ng/mL, human recombinant; Sigma-Aldrich) and heparin (2 ng/mL; Sigma-Aldrich) (FH) in humidified 5% CO_2 incubator with temperature of 37 ºC. One week later, clonal spheres were counted and discriminated based on their pigmentation levels and pigmentation distributions.

Human eye dissection

The basement membrane and the sclera were removed, leaving minimal muscle tissue still attached to the pigmented epithelium. The tissue was then digested in dispase for 20 min followed by a digestion for 20 min in a solution containing 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid at 37°C. The pigmented and nonpigmented ciliary epithelial cells then were gently scraped off the basement membrane, and the nonepithelial tissue was removed from the dish. Cells were then transferred to separate tubes, mechanically triturated, and spun down. The supernatants were removed and replaced with 1 mg/ml trypsin inhibitor (Roche), and the tissues were mechanically dissociated into single cells with a small borehole pipette. Each of the solutions were passed through a 70-μ m cell strainer, rinsed with serum-free media (SFM), spun down again and resuspended in SFM. The cells were counted and plated at either 10 or 20 cells per μ l in SFM containing FGF2 plus heparin (Sigma-Aldrich). Cells proliferated to form floating sphere colonies in cell suspension, and these retinal spheres were counted after 7 days in culture.

RSC sphere type identification and characteristics

In this study, clonal spheres were classified into heavily pigmented (HP), lightly pigmented (LP) and centrally pigmented (CP) spheres. This classification was based on 1) Percentage of the pigmented surface area observed in each type under microscopy using imageJ program. HP sphere: the pigment covers more than 80% of the surface area. LP sphere: variable level of pigmentation (anywhere from 12% to 64%) with random distribution throughout a sphere's surface area. CP sphere: the pigment is heavily concentrated in the center of the sphere surrounded by a clear non-pigmented rim. The diameter of the central pigmentation ranges from 40 to 80 % of the sphere diameter while the surrounding non-pigmented rim accounts for the remainder of the sphere diameter. 2) We also measured the distribution pattern of pigment in the spheres by analyzing sections through the centers of each sphere type in Adobe Photoshop (Version 20.0.0).

Sphere sections

Spheres were collected into 1.5mL microcentrifuge tube in minimal volume (under 20μl), fixed by adding 200μL of fresh 4% PFA for 10 minutes then centrifuged and supernatant was removed. Spheres were kept in 500uL of 20-30% sucrose at 4°C overnight. On the next day sucrose was removed, and the spheres were transferred to plastic embedding molds. Tissuetek was added to each mold and put on a shaker for at least 1 hour at room temperature. Molds were kept in a -80°C Freezer spheres until ready for sectioning. Sphere sections (10 μm) were prepared on a cryostat (-20°C).

FACS sorting

After 4-5 days of clonal sphere assays, CP, HP and LP spheres derived from C57BL/6 or YFP mice were picked and dissociated into single cells using the following enzymes: trypsin 1.33 mg/mL, hyaluronidase 0.67 mg/mL, kynurenic acid 0.2 mg/mL, 0.5 mg/mL collagenase I, 0.5 mg/mL collagenase II, 0.1 mg/mL elastase (Sigma-Aldrich). Cells then were counterstained with propidium iodide (0.9 μg μl−1, Thermo Fisher Scientific) to assess viability and sorted based on their pigmentation level into pigmented and non-pigmented cells using a FacsAria II (BD) instrument and data were analyzed using BD FACS Diva Software V6.1.2. Pigmented cells were plated alone or in different combinations with non-pigmented cells in 96 well clear flat-bottom plates (Nunc; Thermo Fisher Scientific), coated with laminin (50 ng/mL, Sigma-Aldrich). Pigmented YFP+ cells were then cultured either alone at 5 cells or 20 cells per well, or in combination with 20 non-pigmented cells derived from C57BL/6 mice for 12 days in 1% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific , Burlington, ON) + FH. For every biological replicate experiment, we dissected both eyes from 30 mice (15 C57BL/6 and 15 YFP+).

RSC differentiation and immunostaining

Mouse RSC clonal spheres (CP, HP and LP) with average size 80-100 μm in diameter were picked after 7 days and plated individually on laminin in 24 well plates in humidified 5% CO_2 incubator and differentiated using pan-retinal differentiation conditions that involved 1% FBS + FH for 21 days. (Tropepe et al., 2000). Human spheres were cultured for 21 or 60 days in the same conditions. Media and growth factors were replaced every 4 days. Cells were then rinsed with phosphate buffered saline (PBS) and fixed using 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 10 minutes and pre-blocked with 2% normal goat serum and bovine serum albumin (BSA) for 1 hour at room temperature. Cells were then incubated overnight at 4°C in the following primary antibodies: anti-

MITF (MA5-14146, 1:100; Thermo Fisher Scientific), anti-RPE65 (MAB5428, 1:250; Millipore), anti-Best1 (MA1-16739, 1:100; Thermo Fisher Scientific), anti-ZO-1 (61-7300, 1:100; Thermo Fisher Scientific), and anti-Rhodopsin (MAB5316, RetP1, 1:250; Millipore). Next day cells were washed 3 times with PBS and incubated in the following secondary antibodies: Alexa fluor 568 or 488 (1:400; Thermo Fisher Scientific) for 1 hour at room temperature. Nuclei were stained with Hoechst dye (1:1000; Sigma-Aldrich). Staining was examined under fluorescence microscopy (Axio Observer D1; Carl Zeiss) with AxioVision 4.8 software (Carl Zeiss).

FACs treated cells were fixed after 12 days using 4% PFA then blocked in blocking solution containing 2% normal goat serum and BSA. Cells were then stained overnight in 4°C using Ki67 conjugated monoclonal antibody (SolA15), FITC (11-5698-82, 1:100; eBioscience) or Ki67 monoclonal antibody (SolA15), PE (12-5698-82, 1:200; eBioscience). Antibodies were washed off 3 times using PBS next day and counterstained with Hoechst dye.

RNA extraction and Q-PCR

RNA was extracted using RNeasy Mini Kit (Cat# 74104) with DNase to remove genomic DNA contamination (Qiagen, Cat# 79254). RNA was quantified using Nanodrop and a specified amount of cDNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-RAD, Cat#170-8890). PCR was carried out using standardized TaqMan Gene Expression Assays in a QuantStudio 6 Fast Real-Time PCR System (Applied Biosystems). Quantification was performed either using the delta delta Ct method or delta Ct methods with 18s rRNA and GAPDH as endogenous controls. For every biological replicate experiment, we dissected 15 mice C57BL/6 (30 eyes per experiment). The following TaqMan assays (ThermoFisher Scientific, US) were used: Claudin-1 (Mm00516701_m1), Occludin (Mm00500912_m1), Integrin 5 alpha (Mm00439797_m1), Cdh1 (E- cadherin: Mm01247357_m1), Mitf (Cat# Mm00434954-m1), Cdh3 (Cat#Mm249209-m1) and Otx2 (Cat#Mm00446859-m1).

Knockdown of claudin-1

Cells from primary RSC spheres were dissociated and cultured at clonal densities, and mixed with Claudin-1 pooled siRNAs (cat # E-059246-00-0010, Horizon Discovery, Dharmacon , United Kingdom) at a final concentration of 1 μM siRNA in 200 μl media with 4000 cells/well in a 48-well (Nunc plate), negative control cells were treated with pooled non-targeting control siRNA or as untreated controls. All cells were plated in Accell delivery media (cat # B-005000-500, Horizon Discovery, Dharmacon) containing FGF2 and heparin. RSC spheres were counted after 7 days.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Student's *t*-test through the program GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel. Data are presented as means ± SEMs. Significance was calculated using Student's t test to compare two groups, or using one-way or two-way ANOVAs, with Tukey-Kramer post-hoc analyses adjusted p-values to compare multiple groups to each other or to the control. The significance value was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).