Retinal stem cells: expansion and progeny characterization

by

Tahani Baakdhah

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Abstract

Growing retinal stem cell (RSC) clonal spheres in traditional static culture is associated with low expansion rate and poor survival attributed to loss of cells during the enzymatic dissociation steps. Another problematic issue is the difficulty to mimic *in vivo* stem cell niche conditions. Using our traditional lab protocol, only 0.2% of the pigmented ciliary epithelium cells (CE) give rise to floating RSC clonal spheres in serum free conditions. These issues have hindered our ability to study the signaling pathways governing RSC proliferation and expansion *in vitro*.

To overcome these limitations, I used microcarriers (MCs) in a suspension stirring bioreactor (SSB) to help achieve sufficient numbers suitable for differentiation and transplantation. Using this new protocol, I achieved a significant (10-fold) enrichment of RSC yield compared to conventional static culture techniques using a combination of FACTIII MCs and relative hypoxia (5%) inside the bioreactor. My work showed that hypoxia (5% O_2) was associated with better RSC expansion across all platforms which was attributed to hypoxia-induced boosting survival and/or symmetric division of stem cells.

RSC spheres were thought to be unvaried and were randomly picked and placed on laminin extracellular matrix (ECM) in static plates. In my work, I found noticeable variance in the pigment distribution between RSC spheres which led me to categorize them into three different groups according to their pigmentation, I also noted that this variance in pigment level and distribution was associated with contrasting differentiation potentials. RSCs were classified into three morphological groups: heavily pigmented (HP), lightly pigmented (LP) and

centrally pigmented (CP) spheres. Unlike the other two sphere types, CP spheres were capable of producing highly proliferative progenitors (producing large number of cobblestone-like cell lawns) in adherent culture that differentiate into retinal pigment epithelium (RPE) cells. I found that the individual stem cells that (clonally) formed the three sphere types appear homogeneous, but it is their downstream progenitors that are different. I showed that CP spheres contain a population of early RPE progenitors that respond to proliferative signals from the surrounding non-pigmented neural retinal cells while The HP and LP spheres do not respond to these signals.

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"Everything is theoretically impossible, until it is done." – Robert A. Heinlein.

Contributions

Chapter 2

Tahani Baakdhah and Derek van der Kooy designed the experiments. Tahani Baakdhah preformed the experiments and analyzed the data. Tahani Baakdhah wrote the manuscript and Derek van der Kooy revised the manuscript.

Chapter 3

Tahani Baakdhah and Derek van der Kooy designed the experiments. Tahnai Baakdhah. performed the experiments. Brenda Coles assisted in the dissection for the data presented in Figure 3.4, A-12, A-13,A-14 and A-15. Tahani Baakdhah analyzed the data and wrote the manuscript. Derek van der Kooy and van der Kooy's lab members revised the manuscript.

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List of Abbreviations

AIF Apoptosis-inducing factor
BDNF Brain-derived neurotrophic factor
BMP Bone morphogenetic protein
BrdU Bromo-deoxyuridine
Casz1 castor
CB Ciliary body
CE Ciliary epithelium
CER1 Cerberus 1
CGZ Circumferential germinal zone
Chx10 Ceh-10 homeodomain containing homolog, also known as Vsx2
CKIs Cyclin kinase inhibitors
CMZ Ciliary marginal zone
CNS Central nervous system
CNTF Ciliary neurotrophic factor
CP Centrally pigmented
CRABP Retinaldehyde-binding protein
CSF Cerebrospinal fluid
Crx Cone-rod homeobox
DMEM Dulbecco's modified eagle medium
E Embryonic
EB Embryoid body
EGF Epidermal growth factor
EM Electron microscopy
ERG Electoretinography
ESC Embryonic stem cell
eGFP Enhanced green fluorescent protein
eYFP Enhanced yellow fluorescent protein
FACS Fluorescence-activated cell sorting
FBS Fetal bovine serum
FGF Fibroblast growth factor
FITC Fluorescein isothiocyanate
GCL Ganglion cell layer
GFP Green fluorescent protein
GMEM Glasgow minimum essential medium
H Heparin
Hes Hairy and enhancer of split
HIF Hypoxia-inducible factor
hMSCs Human müller glia stem cells
HP Heavily pigmented
HSC Hematopoietic stem cell
IGF Insulin-like growth factor
Ikzf1 Ikaros
INL Inner nuclear layer
IPL Inner plexiform layer
iPSC Induced pluripotent stem cell
IRBP Interphotoreceptor retinoid binding protein
LEDGF Lens epithelium-derived growth factor
LEF Lymphoid enhancer factor
Lhx2 LIM Homeobox 2
LP Lightly pigmented

LP Lens placode
LRAT Lecithin retinol transferase
m Mouse
Mab21 Male-abnormal 21
MCs Microcarriers
MerTK MER proto-oncogene tyrosine kinase
Mitf Microphthalmia-associated transcription factor
NR Neural retina
Nrl Neural retina-specific leucine zipper
OC Optic cup
OFL Optic fiber layer
ONL Outer nuclear layer
OPL Outer plexiform layer
OS Optic stalk
Otx2 Orthodenticle Homeobox 2
OV Optic vesicle
P Postnatal day
PAR Polarity protein
Pax6 Paired box gene 6
Pax2 Paired box gene 2
PBS Phosphate-buffered saline
PCM Pigmented ciliary margin
PDGF Platelet-derived growth factor
PC Primordial germ cell
PEDF Pigment epithelium-derived factor
PRs Photoreceptors
RA Retinoic acid
RAR Retinoic acid receptor
Raldh Retinaldehyde dehydrogenase
Rb Retinoblastoma
RDH Retinol dehydrogenase
RNA Ribonucleic acid
RPCs Retinal progenitor cells
RPE Retinal pigmented epithelium
RSC Retinal stem cell
RT-PCR Reverse transcription polymerase chain reaction
RXR Retinoid X receptor
Rx/Rax Retina and anterior neural fold homeobox
SEM Standard error of the mean
sFRP Frizzled-related protein
Shh Sonic hedgehog
Six3 SIX Homeobox 3
Sox SRY-box containing gene
SSB Stirring suspension bioreactor
TCF T-cell factor
TGF Transforming growth factor
TIM Tissue inhibitor of matrix metalloprotease
TNF Tumor necrosis factor
VEGF Vascular endothelial growth factor
Wnt Wingless-related MMTV integration site
YFP Yellow fluorescent protein
ZO Zonula occludens

Chapter 1: General introduction

1.1 Eye development

The vertebrate eye is formed through coordinated interactions between neuroepithelium, surface ectoderm and extraocular mesenchyme which originates from two sources: neural crest and mesoderm (Cvekl & Tamm, 2004; Gage, Rhoades, Prucka, & Hjalt, 2005; Sevel, 1981). After the establishment of the three germ layers during gastrulation, the notochord induces the formation of neural tube within the overlying ectoderm. The neuroepithelium undergoes massive nonuniform cell proliferation, which establishes the three primary brain vesicles, the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). In the forebrain, eye development begins as a single eye field located in the anterior neural plate which then splits into two lateral fields through the activation of the sonic hedgehog gene (*Shh*) by the prechordal plate and the ventral midline of the diencephalon which, in turn, down regulate *Pax6* (The paired box gene) expression in the midline and the single eye field splits into two separate eye fields, located on either side of the diencephalon (Adelmann', 1929; Chow & Lang, 2001; Loosli, Köster, Carl, Krone, & Wittbrodt, 1998; Sinn & Wittbrodt, 2013). As the lateral walls of the diencephalon begin to bulge out during neurulation, the eye primordium becomes grossly identifiable as the optic pit around E8.5 in mouse (day 22 of gestation in human). By E9 (day 24 of gestation in human), the optic pits will enlarge to form the optic vesicles (OV) which is connected to the brain by a thinner optic stalk (OS). The distal end of the optic vesicle will finally induce the non-neural ectodermal surface to form the lens and cornea (Chow & Lang, 2001; Grainger, 1992; Henry et al., 2002). This early induction signal to the non-neural ectoderm operates to induce the lens placode (LP), which in turn reciprocally induces the OV to invaginate and transform into optic cup (OC) as well as to form the lens vesicle starting at E10 (30 days' gestation in human). The outer layer of the bi-layered OC will form the retinal pigmented epithelium (RPE), the inner layer forms the neural retina (NR) (Hirashima, Kobayashi, Uchikawa, Kondoh, & Araki, 2008; Kagiyama et al., 2005). The transitional area between the two regions forms the ciliary body (CB) and iris (Gilbert, 2011; Graw, 2010; Kaufman, 1992) (Figure 1.1). However, this is not always the case, as was shown by a study using three-dimensional culture of mouse embryonic stem cell aggregates to derive hollowed spheres of neuroepithelium containing Rax-positive domains. Many of these regions spontaneously invaginated to form the OC in the absence of a lens or ectodermal tissue (Eiraku et al., 2011). Conversely, the apposition of the LP and the OV appears to be important for placode invagination and lens formation (Chauhan et al., 2009; Graw, 2010; Streit, 2007).

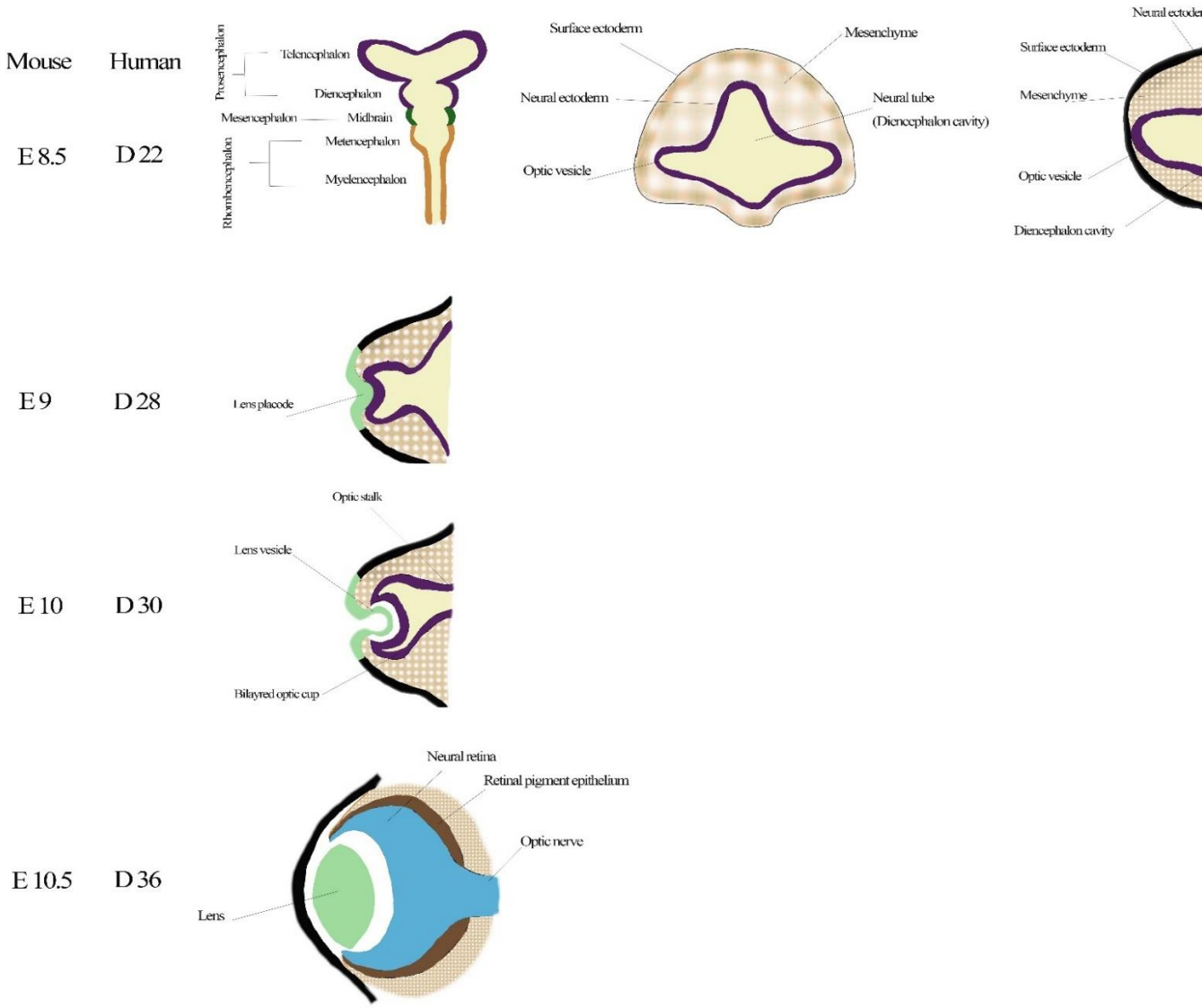

Figure 1.1: Embryonic eye development.

Normal eye development proceeds through several steps: at E 8.5 the diencephalon evaginates to form the optic vesicle. The optic vesicle then contacts the lens placode (E 9). The lens vesicle begins to invaginate at E 10 concomitantly with the distal tip of the optic vesicle. Later the optic vesicle will invaginate, forming the optic cup (E 10.5).

A set of transcription factors has been first identified in Xenopus that defines the eye field within the anterior neural plate (Zuber, Gestri, Viczian, Barsacchi, & Harris, 2003) but these factors were found to be highly conserved throughout vertebrates (Achberger, Haderspeck, Kleger, & Liebau, 2018; Lee et al., 2006; Mellough et al., 2019; K. B. Moore, Mood, Daar, & Moody, 2004; Zaghloul, Yan, & Moody, 2005) (Figure 1.2). These factors include: Otx2, Sox2, Rax, Lhx2, Pax6 and Six3.

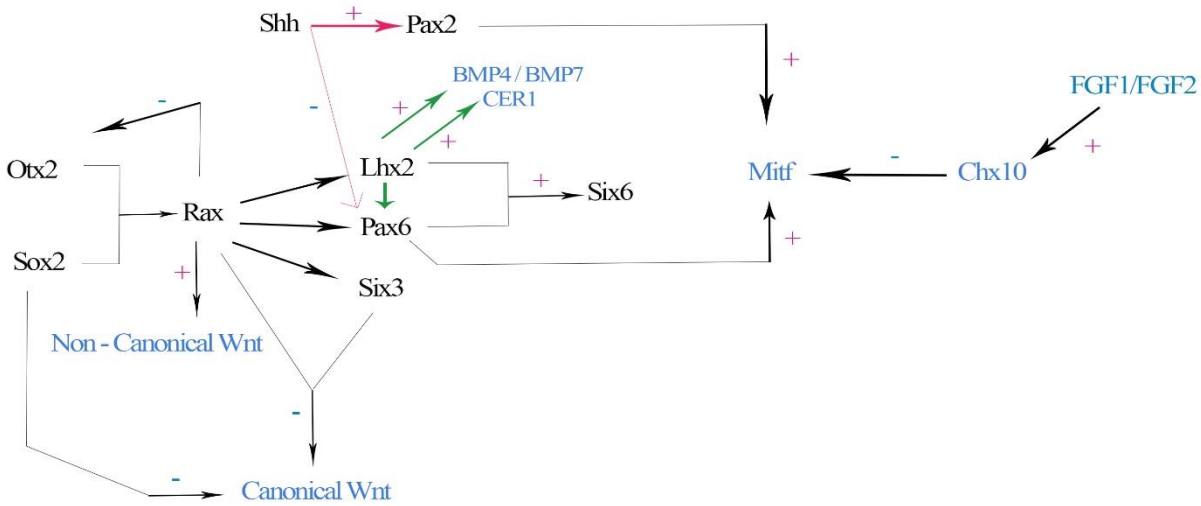

Figure 1.2: Network of transcription factors that establish the eye field.
The neural ectoderm transcription factors Sox2 and Otx2 activate Rax expression in the prospective eye field, which is located in the ventral forebrain. Rax is required for the up regulation of the EFTFs Lhx2, Pax6, and Six3 in the eye field. Lhx2 coregulate the expression of Rax, Pax6, and Six3 in the eye field. The EFTFs then coordinate the cell's intrinsic and extrinsic signaling pathways that regionalize the optic vesicle along its axes.

The homeodomain transcription factor Otx2 is required for the specification of the forebrain, and allows the neural plate to become competent for eye development by coordinating the expression of eye field initiators *Six3*, *Rax*, and *Pax6* (Chow & Lang, 2001; Chuang & Raymond, 2002; Danno et al., 2008; Loosli et al., 1998; Satou et al., 2018; Sinn & Wittbrodt, 2013; Zagozewski, Zhang, & Eisenstat, 2014; Zuber et al., 2003). Together with *Sox2* they activate the *Rax* gene during early retinal development (Danno et al., 2008). Afterward, *Rax* will downregulate *Otx2* expression in the developing optic vesicle (Furukawa, Kozak, & Cepko, 1997; Muranishi et al., 2011). *Otx2* will then regulate RPE specification (Martinez-Morales, Signore, Acampora, Simeone, & Bovolenta, 2001) and photoreceptor and bipolar cell (BP) differentiation and maturation (Koike et al., 2007; Nishida et al., 2003; Sato et al., 2007). After birth, *Otx2* expression in photoreceptor cells is significantly downregulated when retinal BP cells begin expressing *Otx2* in the postnatal retina (Muranishi et al., 2011), *Otx2* expression will be maintained in RPE and BP cells throughout life (Fossat et al., 2007). Mutations of this gene in humans have been linked to ocular malformation including retinal dystrophy and photoreceptor degeneration, pituitary defects and mental retardation (Abdalla-Elsayed, Schatz, Neuhaus, & Khan, 2017; Béby et al., 2010).

Sox2 - a member of the SoxB1 family of transcription factors is initially expressed in the anterior neural plate and optic vesicle. Its expression in the OV is eventually restricted to the progenitor cells of the neural retina and then to amacrine cells and Müller glial cells (Lin, Ouchi, Satoh, & Watanabe, 2009). During early eye field specification phase, *Sox2* works synergistically with *Otx2* to activate the *Rax* gene (Danno et al., 2008; Pevny & Placzek, 2005; Van Raay et al., 2005). Next, *Sox2* antagonizes canonical WNT signaling to maintain neurogenic fate in the mouse OC where its expression will be restricted to the presumptive NR (Heavner, Andoniadou, & Pevny, 2014). Ablation of *Sox2* in OC progenitor cells resulted in loss of neural competence and cell fate conversion of the NR to CE primordium accompanied by an increase in WNT signaling but no retinal dystrophy phenotype observed (Matsushima, Heavner, & Pevny, 2011). S*ox2* mutations are associated with anophthalmia (absent eye) and account for 10 to 20% of cases of severe bilateral ocular malformation, including microphthalmia (small eye) (Fantes et al., 2003; Ragge et al., 2005).

The retinal homeodomain transcription factor Rx/Rax is a master key regulator in eye field development that is conserved among all vertebrates (Andreazzoli, Gestri, Angeloni, Menna, & Barsacchi, 1999; Kennedy et al., 2004; Mathers, Grinberg, Mahon, & Jamrich, 1997a). Once activated by *Sox2* and *Otx2* (Danno et al., 2008), *Rax* will promote the expression of other key regulators of early eye formation such as *Lhx2, Pax6, Mab21l2*, and *Six3* to control retinal progenitor cells (RPCs) specification in the optic vesicle. *Rax* participates in suppressing the canonical Wnt pathway with the help of *Six3* to prevent the induction of posterior fates of the anterior neural plate in-order to activate *Shh* signalling in the ventral midline and facilitate midline division and the formation of bilateral visual field (Fuhrmann, 2008a). It also promotes noncanonical Wnt signaling that controls morphogenetic movements of ocular cells and works in different pathways to promote the formation and/or maintenance of the eye field (Martínez-Morales et al., 2003; Maurus, Héligon, Bürger-Schwärzler, Brändli, & Kühl, 2005). Null or hypomorphic Rx alleles display anophthalmia in humans (RAX), mouse (Rax), frog (Rx1), zebrafish (Rx3/chokh), and medaka (eyeless), indicating that *Rax* genes are essential for early eye development (Harding & Moosajee, 2019; Loosli et al., 2003; Tucker et al., 2001; Voronina et al., 2004).

Lhx2 (LIM homeobox transcription factor) is required in the early optic vesicle development for specification into both neural retina and RPE and to regulate optic cup formation (Porter et al., 1997; Roy et al., 2013; Zuber et al., 2003). Lhx2 has many roles including: 1) regulating OV regionalization, patterning, and lens formation (Sanghee Yun et al., 2009). 2) it is important for the expression of certain EFTFs (such as, *Rax, Pax6*, and *Six3*) at the anterior

neural plate stage and the expression of regional retinal markers *like Mitf, Chx10* and *Pax2* (S. Yun et al., 2009; Zuber et al., 2003). 3) initiating expression of *Bmp4* and *Bmp7* in the optic vesicle (Hägglund, Dahl, & Carlsson, 2011). *Bmp4* is expressed in the distal OV, whereas *Bmp7* is enriched in surface ectoderm and the dorsal OV (Dudley & Robertson, 1997; Furuta & Hogan, 1998; Müller, Rohrer, & Vogel-Höpker, 2007). Distal OV-enriched *Bmp4* is especially crucial for lens induction, and *Bmp7* is essential in facilitating OV-LP interaction for eye morphogenesis. *Bmp4* expression requires maintained *Lhx2* expression in the eye committed progenitor cells whereas *Bmp7* expression appears to not be dependent on maintained *Lhx2* expression (Hägglund et al., 2011). 4) promoting the expression of Wnt and Bmp signaling antagonist Cerberus 1 (CER1) in the optic cup to regulate the neural differentiation (Hou et al., 2013; S. Yun et al., 2009). 5) promoting maintenance of stem cells and progenitors and preventing premature differentiation (Gordon et al., 2013; Hägglund et al., 2011). 6) *Lhx2* is required for *Six6* expression initiation in the optic vesicle. *Lhx2* and *Pax6* are genetically required for *Six6* expression and can cooperate to trans-activate the *Six6* gene synergistically (Tétreault, Champagne, & Bernier, 2009). During embryonic development *Lhx2* was found to be expressed in most, if not all, RPCs within the neuroblast layer of the retina, as well as in the retinal pigment epithelium. Postnatally, expression was maintained in müller glia and a subset of amacrine cells (de Melo et al., 2018, 2016). In *Lhx2* mouse mutants, expression of other eye field transcription factors initiates normally, but eye development arrests at the optic vesicle stage, and the lens fails to form (Porter et al., 1997; Tétreault et al., 2009; S. Yun et al., 2009), a more detailed analysis revealed that expression of optic vesicle regional patterning markers is severely disturbed, for example, the expression of *Mitf, Chx10* is never initiated, while expression of *Pax2* and *Rax* is initiated but not maintained (S. Yun et al., 2009).

The progression from the optic vesicle to the optic cup requires the expression of *Lhx2* and the action of retinoic acid. Retinoic acid (RA) signaling is required for the optic cup morphogenesis and ventral invagination of the optic cup. This step is controlled by Retinaldehyde dehydrogenases (Raldh1, 2, 3) where both mediate the final step of retinoic acid synthesis. *Raldh2* is present in the mesenchyme and *Raldh3* is expressed in the RPE (Duester, 2008; Molotkov, Molotkova, & Duester, 2006). At later stages, *Raldh1* expressed in dorsal neural retina and *Raldh3* expressed in ventral neural retina (Molotkov et al., 2006).
In the developing OC the relationship between Chx10 and Mitf defines the boundaries between NR and RPE (Horsford, 2004; Nguyen & Arnheiter, 2000) while the relation between Pax2

and Pax6 establish the boundary between the optic stalk and the NR (Heavner & Pevny, 2012; Morcillo et al., 2006).

NR/RPE margin is established through the initial expression of *Mitf* throughout the dorsal OV, but *Mitf* becomes down-regulated distally upon the expression of *Chx10* (Nguyen & Arnheiter, 2000). The function of Mitf in boundary formation is supported by the observation that mice with loss-of-function mutations in *Mitf* show a conversion from RPE to NR (Bumsted & Barnstable, 2000; Nguyen & Arnheiter, 2000). *Mitf* is then down regulated by *Chx10* (the earliest marker of neural retina). It becomes first expressed in the inner layer of the optic cup (I. S. Liu et al., 1994). *Chx10* homozygous mutation produces the ocular retardation mouse mutant, which shows greatly decreased progenitor proliferation resulting in a hypocellular neural retina (Burmeister et al., 1996). Double Chx10/Mitf mutants have a less severe phenotype than each individual mutant, it is thought that Chx10 represses Mitf to specify the neural retina (Horsford, 2004; Rowan, Chen, Young, Fisher, & Cepko, 2004).
OC differentiation is also controlled by signals from the surrounding tissue. The inner layer of the optic cup is in close contact with extraocular mesenchyme, which secretes TGFß signaling factors to maintain RPE identity (Fuhrmann, 2010a). The outer layer of the optic cup will undergo extensive proliferation to produce the neurons and glia of the neural retina. FGF signaling from the overlying surface ectoderm directs the acquisition of a neural retinal fate (Pittack, Grunwald, & Reh, 1997; Shulei Zhao, Thornquist, & Barnstable, 1995). Removal of extraocular mesenchyme in chick embryos causes upregulation of neural retinal markers and downregulation of RPE markers in the outer prospective RPE. Removal of surface ectoderm causes an analogous, inappropriate gene expression pattern (Fuhrmann, 2010a; Hyer, Mima, & Mikawa, 1998).

The exogenous influences are not absolutely required for mouse eye development. Eiraku et al. in 2011 demonstrated the formation of optic cups and well-organized neural retinae containing all mature retinal cell types *in vitro* from mESCs in the absence of exogenous influences (Eiraku et al., 2011).

OV dorsal-ventral and proximal-distal patterning is mainly controlled by *Shh* gene which is secreted from the ventral forebrain (X. M. Zhang & Yang, 2001). *Shh* is essential for the bilateral separation and proximal-distal patterning of the OV (Amato, Boy, & Perron, 2004; W. K. Jin & Lemke, 2006) as well as for specification of the ventral and proximal optic vesicle structure and for the inhibition of dorsal-distal fate (i.e., NR and RPE). *Shh* promotes expression of *Pax2* (optic stalk marker) and represses expression of Pax6 (retinal marker) (Amato, Boy, et al., 2004; Ekker et al., 1995; Macdonald et al., 1995). *Pax2* and *Pax6*

transcriptionally repress each other, forming a precise boundary between the retina and the optic stalk (Heavner et al., 2014; Morcillo et al., 2006; Schwarz et al., 2000). The optic vesicles receive two antagonistic signals: *Shh* from the ventral midline and *Bmp4* from the dorsal part of the optic vesicle. These molecules act in coordinated manner to pattern the eye along the D–V axis, repressing each other (Ohkubo, Chiang, & Rubenstein, 2002).

Similar EFTF expression pattern has been demonstrated in human induced pluripotent stem cells (iPSCs) derived retinal organoid (Akhtar et al., 2019a; Fligor et al., 2018a; Quinn, Buck, Ohonin, Mikkers, & Wijnholds, 2018; Manuela Völkner et al., 2016).

1.2 The retina

The retina is a highly conserved structure in vertebrates, having almost identical anatomical and physiological characteristics in multiple taxa. All vertebrates have a three-layered retina consisting of a photoreceptor layer containing rod and cone photoreceptors, an inner nuclear layer containing the cell bodies of processing neurons, and a ganglion cell layer containing the cell bodies of ganglion cells (Stenkamp, 2007).

The different cell types in the retina are organized in distinct laminae that are divided into nuclear and plexiform layers. The cell bodies are located in the nuclear layers and the axons and dendrites reside in the plexiform layers. The layers are termed the outer nuclear layer, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer and the optic fibre layer (Figure 1.3). Together they form an elaborate neuronal network that accomplishes the tasks of image detection, processing, and transmission in a similar way to other parts of the central nervous system.

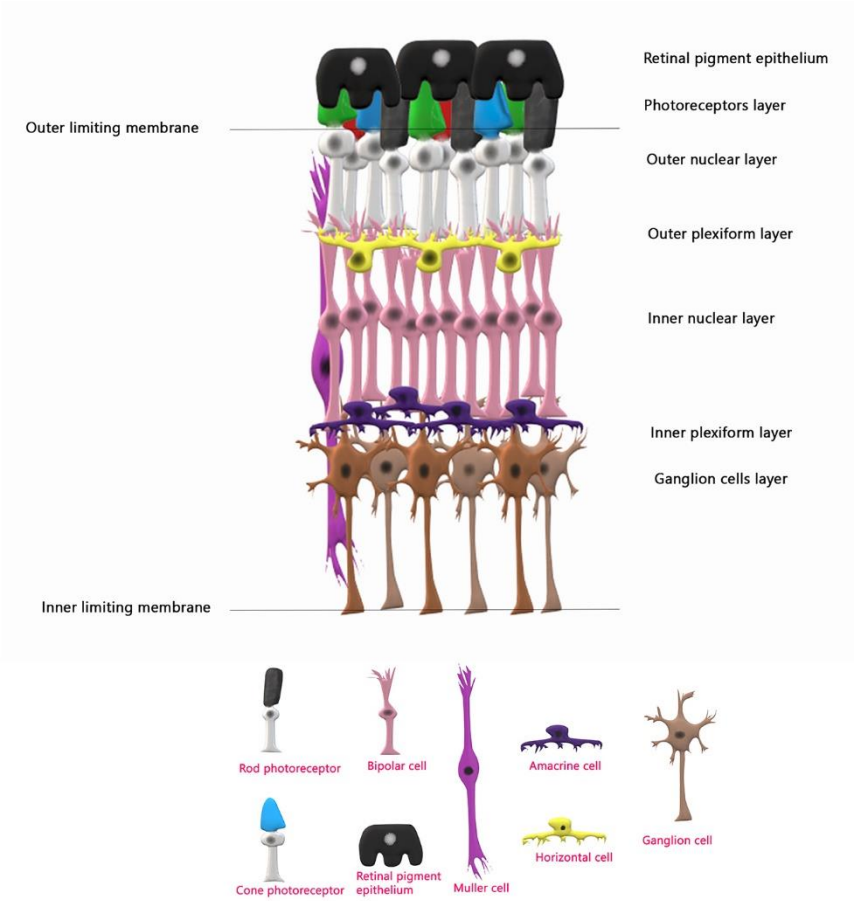

Figure 1.3: Retinal cells distribution throughout the retina.

The outer nuclear layer contains the rod and cone photoreceptors, which are responsible for converting light into electrical signals. Cone are active in bright light conditions and are responsible for high-resolution perception that discriminates colors and visual details. There are three types of cone photoreceptors in the retina. Each cone has a specific photo pigment that can absorb light of a specific wavelength, which in turn makes the cone able to detect colors. Rod photoreceptors, on the other hand, are extremely sensitive to light and are important for dim light vision. The Inner nuclear layer contains the cell bodies of müller cells, horizontal cells, bipolar cells and amacrine cells. Horizontal cells are a class of interneurons that are involved in enhancing contrast between light and dark regions. They act by integrating and modulating the output from PRs to BPs by means of inhibitory synapses. Bipolar cells act as relay stations for visual signals, which means that they relay information from photoreceptors to the ganglion cells. Amacrine cells integrate and modulate the signals from bipolar cells to Ganglion cells through both excitatory and inhibitory synapses. Mular glia are the primary glial cells of the retina, they support the retinal structure and regulate retinal homeostasis. The Ganglionic cell layer contains ganglion cells. Ganglion cells are the last

neurons in the retina to receive the visual signals and their function is to convey the information to the brain.

In amphibians and teleost fish, the growth of the retina is coordinated with the overall growth of the eye. They continuously add new neurons and glia to the retina throughout life. Undifferentiated precursor cells are located in the most peripheral part of the retina, termed the ciliary marginal zone (CMZ), a transitional region between the neural retina and the iris/ciliary epithelium (Perron & Harris, 2000; Reh & Levine, 1998), while more centrally are situated committed proliferative neuroblasts, followed by differentiating precursors and finally postmitotic neurons (Figure 1.4) (Hollyfield, 1968; Johns, 1977) and as the retina grows in size, new cells are added in concentric rings with cells situated more peripherally being younger than central ones (Hollyfield, 1971; Straznicky & Gaze, 1971). Progenitors in the CMZ maintain expression of early eye field transcription factors (Perron, Kanekar, Vetter, & Harris, 1998). By comparison, most of the retina in birds is generated in ovo during early stages of development. In the chicken, for example, at least 90% of the retina is produced more than one week before hatching, by the time of hatching, chicks will have fully functional retinae capable of detecting images and conveying visual information to higher visual centers in the brain (A. J. Fischer, 2005).

In fish, the retina increases in size throughout adulthood by three mechanisms (Johns, 1977). The first mechanism is one of balloon-like expansion of existing retina and enlargement of existing retinal cells. The second mechanism is the addition of new retinal tissue at the retinal periphery, generated by a circumferential germinal zone (CGZ). The third mechanism is the insertion of additional rod photoreceptors into the existing sheet of photoreceptors in the outer nuclear layer. In contrast to the amphibian retina, in which retinal precursors generate all retinal cell types, fish rod photoreceptors are generated during embryogenesis in a secondary wave of neurogenesis by specialized rod precursors situated in the ONL. Similarly, in the post-embryonic fish, rods are not produced together with the other retinal cell types at the ciliary margin (Otteson & Hitchcock, 2003). Consequently, the newly generated ONL that is close to the ciliary margin contains only cone photoreceptors. More centrally, the photoreceptor layer contains cones, rods and dividing rod precursors. It has been shown that rod precursors derive from a mitotic population of fusiform cells in the underlying INL that migrate to the ONL (Johns & Fernald, 1981; Julian, Ennis, & Korenbrot, 1998). These elongated progenitors have a restricted lineage as they only generate rods. They probably derive from a slow dividing population of spherical cells that have been found in the inner part of the INL (Otteson,

D'Costa, & Hitchcock, 2001). These cells have some stem cell characteristics, i.e. they self renew and divide asymmetrically. Regeneration studies have demonstrated that the INL stem cell population consists of multipotent cells. Indeed, removing part of the retina by surgical lesion triggers a regenerative response in the INL and the ONL with the formation of a regenerative blastema made of radially elongated cells that surround the lesioned area (Hitchcock, Myhr, Easter, Mangione-Smith, & Jones, 1992). This blastema behaves like a ciliary margin and fills the space generated by the lesion (Hitchcock et al., 1992; Otteson & Hitchcock, 2003). This experiment demonstrated that INL stem cells that normally generate only rod cells could, following injury, give rise to other retinal neurons.

In amphibians (Xenopus), CMZ has been divided into four principal zones. Gene expression in these different zones recapitulates the sequential expression occurring in retinoblasts during embryonic development (Dorsky, Rapaport, & Harris, 1995; Harris & Perron, 1998; Locker et al., 2006). Retinal stem cells reside in the first zone, while committed retinoblasts and differentiating neurons are found from the second zone onwards. These stem cells are able to self-renew and differentiate to all retinal cell types. Labeling the frog CMZ cells with a tracer and analyzing their lineages showed that retinal stem cells generate large clones containing all cell types including glial and pigmented cells (Amato, Arnault, & Perron, 2004a). Stem cells in the CMZ participate in the regeneration process after retinal injury (Henningfeld, Locker, & Perron, 2007; Keefe, 1973; Reh & Nagy, 1987; Yoshii, Ueda, Okamoto, & Araki, 2007). Yoshii et al., 2007 showed that NR and lens regenerate following the surgical removal of these tissues in the anuran amphibian, *Xenopus laevis*, even in the mature animal. The NR is regenerated from both, the RPE cells (by transdifferentiation) as well as the stem cells in the CMZ (by differentiation) (Yoshii et al., 2007).

In the bird, mitotic CMZ precursors persist only for the first few weeks after hatching but Fischer and Reh have demonstrated that a subgroup of cells in the CMZ of adult birds (4.5 months of age) exists in a pattern similar to that seen in the eyes of younger chicks, less than 30 days of age. These precursors may not be multipotential, as only a subset of retinal neurons appears to be produced including bipolar and amacrine cells only (A. J. Fischer & Reh, 2000). In chicken postnatal retina, no retinal regeneration has been detected after acute injury and even those proliferated in response to exogenous factors did not show functional recovery (A. J. Fischer & Reh, 2003). Regeneration was only detected during early embryonic chick development from the transdifferentiation of the RPE cells (Araki, 2007; A. J. Fischer & Reh, 2003). In embryonic chicks (stage 22–24) such a transdifferentiation process can occur only if some neural retina is left behind. If all neural retina is removed, regeneration occurs only by

treatment with b-FGF (Guillemot & Cepko, 1992; Park & Hollenberg, 1989; Pittack et al., 1997). FGF, therefore, must be the most important factor in the induction of transdifferentiation and neural retina regeneration.

In mouse and other mammals, multipotent RSCs are present in the central part of the inner layer of the optic cups and have the ability to generate all retinal cell types (Marquardt & Gruss, 2002). In mammals RSCs were identified in the pigmented ciliary epithelium. Interestingly, the location of the pigmented ciliary epithelium is topographically analogous to the CMZ in the lower vertebrates (Amato, Arnault, & Perron, 2004b; Kubo & Nakagawa, 2008). When adult mouse RPE, NR and CE were dissociated and cultured as single cells, the only proliferative population was found in the CE (Tropepe et al., 2000). Similar cells have been isolated from this region in embryonic (P. Yang, Seiler, Aramant, & Whittemore, 2002) and adult human eye (Coles, Angenieux, et al., 2004). No stem cell proliferation can be detected within the mammalian retina postnatally *in vivo*, hence no new cells are being produced in healthy retina or following disease or injury (Ahmad, Tang, & Pham, 2000; Tropepe et al., 2000). These adult RSCs are localized in the pigmented ciliary margin and not in the central or peripheral RPE. This suggested that the cells might be homologous to the germinal zone of lower vertebrates which is part of a bigger analogy that exists between the pigmented epithelium of the CB and the CMZ of lower vertebrates, in which the adult germinal zone lies. While the ciliary CMZ consists of undifferentiated neuroepithelium, the pigmented epithelium of the CB is a mature, differentiated epithelial monolayer. It is physically distinct from the retina and lies anterior to the ora serrata (Ballios & van der Kooy, 2010).

RSC colonies were both self-renewing and multipotential, generating all retinal cell types and thus were named retinal stem cells (Ahmad et al., 2000; Tropepe et al., 2000). The generation of retinal cells is often divided into an “early phase” when ganglion cells, horizontal cells and cone photoreceptors are born, and a “late phase”, when bipolar cells, rod photoreceptors and müller cells are born. The amacrine cells are generated in between these phases (Prada, Puga, Pérez-Méndez, López, & Ramírez, 1991; Venters, Mikawa, & Hyer, 2015).

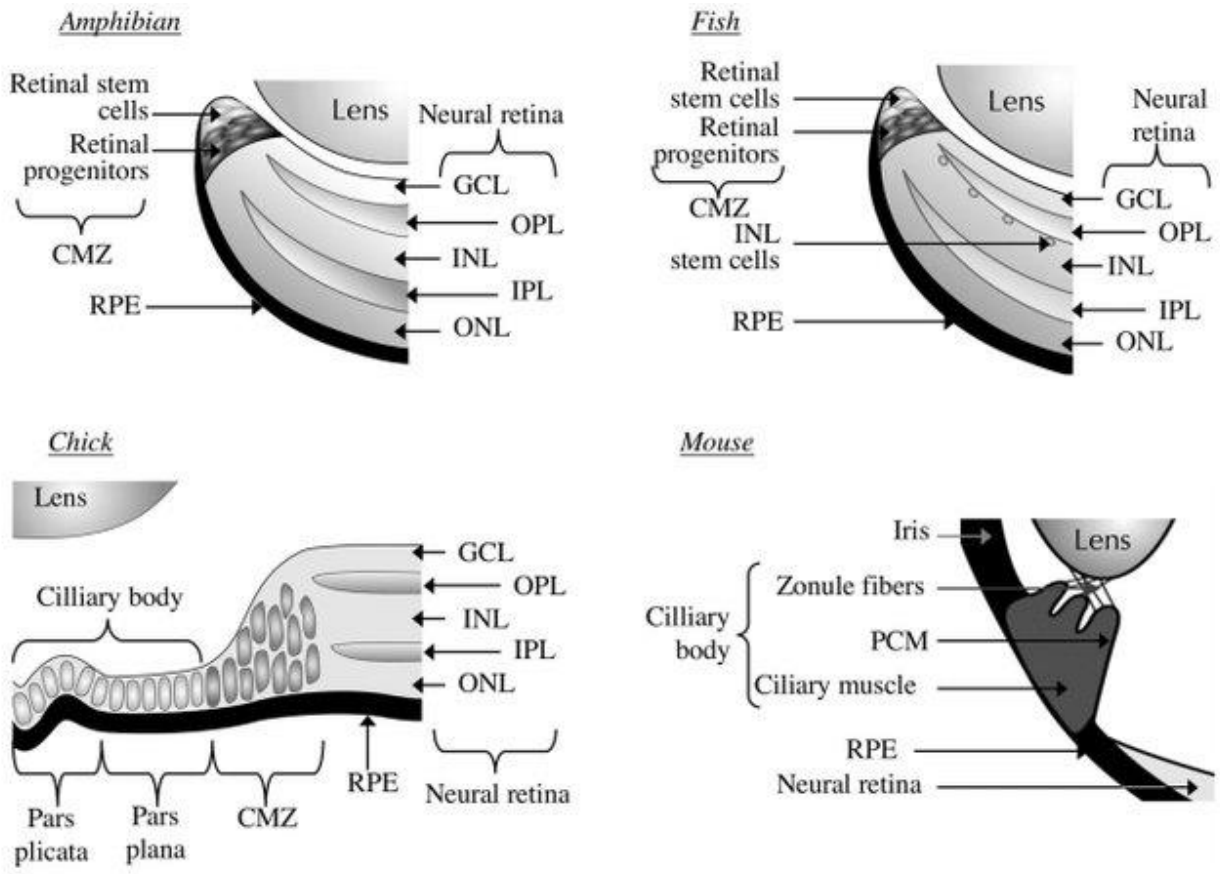

Figure 1.4: Localization of retinal stem cells in various vertebrates.

Schema illustrating the position of stem cells in Xenopus tadpole, fish, post-hatched chick and adult mouse retinae. Multipotent retinal stem cells are found in the most peripheral zone of the ciliary marginal zone of Xenopus, zebrafish and chick retinae. Stem cells specialized in rod production may be present in the inner nuclear layer of fish retina. In the adult mouse retina, cells with stem cell properties when cultured in vitro are found in the pigmented ciliary epithelium. CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer, IPL, inner plexiform layer; ONL, outer nuclear layer, OPL, outer plexiform layer; PCM, pigmented ciliary margin; RPE, retinal pigment epithelium. Adapted from (Amato, Boy, et al., 2004).

1.3 Retinal stem cells

Each OV consists of RSCs that give rise to all the neuroectoderm-derived cells of the eye. RSC patterning occurs along the dorso distal/proximal–ventral axis of the OV prior to optic cup formation. The RSCs of the OV are capable of producing NR, RPE, or OS when provided with the appropriate combination of signals (Hodgkinson et al., 1993; I. S. C. Liu et al., 1994; Nornes, Dressler, Knapik, Deutsch, & Gruss, 1990). Early during development, RSCs tend to divide symmetrically to increase the pool of progenitors in the newly formed optic cup. Upregulation of *Pax6, Chx10,* and *Sox2* may be important for sustaining the proliferative capabilities of these early RPCs, and mutations in these genes can result in proliferation defects characterized by small or absent eyes (Graw, 2019; Hill et al., 1991; Mathers, Grinberg, Mahon, & Jamrich, 1997b; Taranova et al., 2006). Other transcription factors as Notch1, Hes1and Hes5 were found to be expressed during the same period (Czekaj et al., 2012).

Following this early expansive phase of RPC proliferation, RSCs begin to divide asymmetrically producing one daughter cell that differentiates and a second
daughter cell that continues to divide as a stem cell. Importantly, individual RSCs display a considerable amount of heterogeneity across development in relation to their patterns of cell division and this may hint at either, a stochastic component of RSC proliferation, or a considerable intrinsic heterogeneity of RSCs (Dyer & Cepko, 2001).

In general, stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that are the building blocks of tissue and organs (Kolios & Moodley, 2013). In adult tissue these cells are usually rare, quiescent cells and live within certain compartments in their specific organ or tissue called the niche (Chagastelles & Nardi, 2011). They are responsible for replenishing cells that die within a given organ, either due to physiological wear and tear, disease or following injury (Chagastelles & Nardi, 2011). These cardinal properties describe the stemness of any stem cell. Stemness can be: 1) categorical (an intrinsic property of a stem cell, independent of its environment), 2) dispositional (an intrinsic property of a stem cell that emerges only in the right environment), 3) relational (an extrinsic property induced in a cell that would otherwise be a non-stem cell by its microenvironment) or 4) systemic (an extrinsic property of a system such as tissue, rather than an individual cell) (Laplane & Solary, 2019).
Stemness in RSCs is both, an intrinsic trait, as well as a dispositional property that emerges only in the proper environment. In the adult eye, RSCs are quiescent (they do not proliferate to give new cells nor to replace lost cells in case of disease or injury) as a result of factors secreted from other cells in their niche (Balenci, Wonders, Coles, Clarke, & van der Kooy,

2013; Ballios, Clarke, Coles, Shoichet, & van der Kooy, 2012; Tropepe et al., 2000). On the other hand, when cultured at a clonal density in vitro away from the inhibitory signals, RSCs form clonal spheres within 7 days exhibiting the cardinal stem cell properties of self renewal and multipotentiality (Tropepe et al., 2000). These inhibitory factors were found to be secreted from cornea and lens and include: BMP2, BMP4 and Frizzled-related protein (sFRP). The combination of Inhibiting BMP signaling through Noggin as well as functionally blocking sFRP2 restores RSC stemness by antagonizing the effects of inhibitory signals from the cornea and lens (Balenci et al., 2013). In chapter two of this thesis, I will work on achieving better stem cell expansion by culturing RSCs in vitro in an adjustable environment devoid of niche-specific inhibitory signals.

Progenitor cells (including RPCs) are descendants of stem cells that differentiate further to create specialized cell types. When compared to stem cells, progenitor cells have a limited ability to self-renew. Additionally, the cell potency of progenitor cells is more restricted. While some progenitor cells are multipotent, other progenitor cells are unipotent and only capable of differentiating into one cell type.

Some studies have proposed that the RSC population does not represent a true stem cell population (Cicero et al., 2009; Gualdoni et al., 2010). Rather, the observed multipotentiality is claimed to be the result of transdifferentiation of a pigmented ciliary epithelial cell by induction of stem/progenitor markers in response to growth factors in the culture media. Transdifferentiation implies differentiation of a single cell without proliferative divisions to another cell type, which is not the case with RSCs derived from the ciliary epithelium, the multipotency of the RSC and its ability to differentiate into various retinal cell types, including photoreceptors, has been corroborated by a number of independent reports (Ahmad et al., 2000; Ballios et al., 2012; Tropepe et al., 2000). Depletion of pigmented RPE in mice with blocked Mitf (a highly expressed pigmented cell transcription factor) led to an increased (instead of decreased) number of produced RSC spheres, a point of argument against the transdifferentiation hypothesis (Coles, Horsford, McInnes, & van der Kooy, 2006). RSCs are rare cells embedded within the pigmented CE and posses defined characteristics making them possible to distinguish from vast majority of other pigmented CE cells. RSCs can be prospectively isolated from the ciliary epithelium, they tend to be comparatively larger and be the most heavily pigmented cells in the CE (Ballios et al., 2012; Tropepe et al., 2000).

In addition to adult CE, Other sources of RSCs include pluripotent stem cells i.e. embryonic stem cells (ESCs) and induced pluripotent stem cells (iPCs) (Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Thomson, 1998; Yu et al., 2007). ESCs and iPSCs were successfully used

to differentiate retinal cells including: RPE cells (Brandl, 2019; Choudhary et al., 2017; García Delgado et al., 2019; Kharitonov, Surdina, Lebedeva, Bogomazova, & Lagarkova, 2018; Kuroda, Ando, Takeno, Kishino, & Kimura, 2019; Regent et al., 2019; Uygun, Sharma, & Yarmush, 2009), photoreceptor cells (Barnea-Cramer et al., 2016; Gonzalez-Cordero et al., 2017; S. Zhou et al., 2015; J. Zhu et al., 2018), and ganglion cells (Ji & Tang, 2019; Ohlemacher et al., 2019; Tanaka et al., 2015; S. Wu, Chang, Nahmou, & Goldberg, 2018).

ESC isolated from the inner cell mass of the preimplantation blastocyst can give rise to differentiated cell types of all three germ layers of the organism and rapidly expand in culture, providing a potentially unlimited starting population for cell therapy (Thomson, 1998). iPSCs, which have all of the characteristics of ES cells, can be generated by introducing four genes called as Yamanaka factors (*Oct4*, *Sox2*, *Klf4*, *c-Myc*) into somatic cells (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). These somatic cells include adult fibroblasts, among other tissues such as liver and stomach. iPSCs benefit from being immune compatible as a result of their derivation from adult somatic cells of the same patient, and they overcome many of the ethical issues faced by the derivation and use of ES cells. While ES and iPSCs can be differentiated into cells of any of the three germ layers of the adult organism, they are inefficient in differentiation along a particular lineage and their uncontrolled proliferative ability is a safety concern in clinical application (Shah, 2016). There are other significant limitations around the use of iPSCs in cellular therapy. Heterogeneity among reprogrammed cell populations has made it difficult to understand this reprogramming. For example, researchers recently hypothesized that adult cells may retain memory of their original fates after reprogramming, favouring their differentiation back into lineages related to the original iPS colony-forming cell, through harbouring residual DNA methylation signatures characteristic of their somatic tissue of origin (Kim et al., 2010; Nashun, Hill, & Hajkova, 2015; Noguchi, Miyagi-Shiohira, & Nakashima, 2018).

Müller glial cells have been shown to be able to undergo a reprogramming process to re-acquire a stem/progenitor state, allowing them to proliferate and generate new neurons for repair following retinal damage (Hamon, Roger, Yang, & Perron, 2016). Although müller glia–dependent spontaneous regeneration is remarkable in some species such as fish, it is extremely limited and ineffective in mammals. MG in the injured fish retina, produce not only rods but also other neurons that have been lost, suggesting that they are multipotent (Bernardos, Barthel, Meyers, & Raymond, 2007; Fausett & Goldman, 2006). Müller glia also can proliferate in the chick retina after injury or following activation by growth factor infusion (A. J. Fischer, McGuire, Dierks, & Reh, 2002; A. J. Fischer & Reh, 2001). Similarly,

mammalian müller glia will proliferate in response to injury in the rodent retina, but few neurons are generated without the addition of soluble factors that are needed for the expression of transcription factors that drive differentiation (Ooto et al., 2004; Osakada et al., 2007; Wan, Zheng, Xiao, She, & Zhou, 2007). Humans have not been shown to display endogenous regeneration from this cell source. However, studies done *in vitro* have identified a population of human müller glial cells that display stem cell characteristics (hMSCs) and a relative capacity to generate retinal neurons, including rod photoreceptor cells (Giannelli, Demontis, Pertile, Rama, & Broccoli, 2011; Roesch et al., 2008). Furthermore, on transplantation into a rodent model of photoreceptor degeneration, hMSC-derived photoreceptor-like cells were able to migrate and integrate into the host outer nuclear layer, leading to an improvement in photoreceptor function as assessed by electroretinography (ERG) (Bhatia, Singhal, Lawrence, Khaw, & Limb, 2009; Giannelli et al., 2011; Jayaram et al., 2014). iPSC-derived müller glia were able to differentiate toward retinal fate and generate retinal pigmented epithelial cells (Slembrouck-Brec et al., 2019).

1.4 Heterogeneity of retinal stem cells and progenitors

Since the discovery of RSCs in 2000 (mouse) and 2004 (human), RSC clonal spheres were thought to be similar in their proliferation and the type of progenitors they produce as well as their differentiation potential (Ahmad, Das, James, Bhattacharya, & Zhao, 2004; Ahmad et al., 2000; Ballios et al., 2012; Coles, Inoue, et al., 2004; Das et al., 2005; Tropepe et al., 2000). Researchers studied the heterogeneity of retinal stem cells and progenitors on the cellular level but not on clonal sphere level. Many studies showed that the division of RSCs is heterogeneous; 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 stochasticity (Cayouette, Barres, & Raff, 2003; Cepko, 2014; Fekete, Perez-Miguelsanz, Ryder, & Cepko, 1994; Harris, 1997; He et al., 2012; Trimarchi, Stadler, & Cepko, 2008; Turner & Cepko, 1988; Wetts & Fraser, 1988). RPCs will 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 müller glial cells born last (Bassett & Wallace, 2012; Cepko, 2014; Dyer & Cepko, 2001; Prada et al., 1991; Wong & Rapaport, 2009; Young, 1985a, 1985b). Researchers have also observed this 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 (e.g. cone photoreceptors, horizontal cells (HCs)) (Alexiades & Cepko, 1997;

Buenaventura, Ghinia-Tegla, & Emerson, 2018; Schick, McCaffery, Keblish, Thakurdin, & Emerson, 2019; Trimarchi et al., 2008).

Birth dating of retinal cells can be determined by exposing the cells to a radioactive nucleotide analog (thymidine) or bromodeoxyuridine (BrdU) for a period of time (Alexiades & Cepko, 1996). Another method involves injecting a developing retina with a low titer retrovirus expressing a reporter, such as GFP, or a fluorescent dye to allow visualization. Investigations of progenitor lineages demonstrated that retinal progenitor cells are multipotent throughout development and capable of generating multiple kinds of cells including neurons and glia (McFarlane, Zuber, & Holt, 1998; Turner & Cepko, 1988).

The likelihood of a progenitor cell producing one cell type over another could be determined by intrinsic factors (the genes expressed by that progenitor cell), extrinsic factors (signals contributed by the progenitor's environment), combination of the two or stochastic mechanisms that may operate within each of these models. Intrinsic hypothesis states that: RPCs must follow an intrinsic program controlled by gene expression and transcription factors with stepwise changes in competence to produce one cell type at a time in a conserved order (Table 1.1). Several studies have confirmed this hypothesis and showed that early RPCs produce cells with early fates when transplanted into (J. N. Kay, Link, & Baier, 2005) late retinal environment (Belliveau & Cepko, 1999), and late RPCs produce cells with late fates even when placed in an early environment (Belliveau, Young, & Cepko, 2000). Similarly, RPCs produced cells with temporally appropriate fates when isolated in culture (Austin, Feldman, Ida, & Cepko, 1995; Gomes et al., 2011) and heterochronic mixing experiments (in which cells derived from different developmental stages are co-cultured) also showed that intrinsic factors contribute to proliferation and the timing of the onset of differentiation (Morrow, Belliveau, & Cepko, 1998; Watanabe & Raff, 1992).

Table 1.1: Temporal order of generation of mammalian retinal cell types and Transcription factors required for the development of specific retinal cell types.
In mice from around embryonic day (E) 10.5 onward, retinal progenitor cells generate retinal neurons in a temporal order conserved among many species: retinal ganglion cells first, Müller glia last. Ganglion cells, amacrine cells, cone photoreceptors, and horizontal cells differentiate at relatively early stages, while bipolar cells and rod cells are mainly generated at later stages. Combinations of multiple transcription factors, such as bHLH-type and homeobox-type factors, are required for proper specification of retinal cell types.

Cell type	Birthdate (developmental age in mouse)	Transcription factor
Ganglion cells	E 11 – P 0	Math5 – Bm3b – Pax6 – Atoh7 – Pou4F1/2/3 – lsl1 – Neurod1
Horizontal cells	E 10 – E15	Prox1 – ptf1a – Foxn4 – Lhx1 – Pax6 – Neurod4 – six3
Amacrine cells	E 11 – P 3	Neurod1/2/4/6 – ptf1a – pax6 – six3 – Foxn4 – Bhlhb5 – Barhl2 – lsi1 – Nr492 – Satb2
Cone	E 11 – E18	Crx – Otx2 – TRB2 – Neurod1 – Mash1 – ASCl1 – Rorb – PRdm1 – Sall3 – Pias3 – Thrb – Rxrg – Rora – Nr2f 1/2
Rod	E 12.5 – P 10	Neurod1 – CRX – Otx2 – Nrl – Nr2e3 – ASCl1 – Rorb – Prdm1 – Pias3 – Chx10
Bipolar cells	E 18 – P 9	Otx2 – Neurod4 – ASCl1 – Irx5 – BHLHB5
Müller glial cells	E 18 – P 7	Hes1 – Hes5 – Hesr2 - Rx

It is likely that transcription factors also contribute to the temporal progression of cell fates. The zinc finger transcription factor *Ikzf1* (*Ikaros*) is a vertebrate homologue of Hunchback, a transcription factor that is required for the specification of early fates in the D. melanogaster ventral nerve cord (Elliott, Jolicoeur, Ramamurthy, & Cayouette, 2008). More recently, the ortholog of *Drosophila castor*, *Casz1*, was found to function as a mid/late temporal identity factor that is negatively regulated by *Ikzf1* (Mattar & Cayouette, 2015). *Ikzf1* represses *Casz1* expression in the early RPCs, whereas *Casz1* is de-repressed by the loss of *Ikzf1* in late progenitors. These factors are both necessary and sufficient to drive their respective cell fates in the developing retina, much like they are in *Drosophila* neuroblast lineages, suggesting a conservation of the temporal cascade from invertebrates to vertebrates (Mattar & Cayouette, 2015). Ikaros-deficient retinae exhibit a transient decrease in proliferation at embryonic day E13 and a permanent reduction of most early born cell types (ganglion cells, amacrine cells, and horizontal cells) but interestingly, not cones, which is not accompanied by an increase in late-born types. Accordingly, ectopic expression of *Ikaros* in late-stage progenitors is sufficient to promote development of most early born retinal neurons but again, not cones alongside late-born neurons (Mattar & Cayouette, 2015). The fact that cones are not affected by the gain or loss of Ikaros suggests that different regulatory mechanisms control the timing of cone production. Notably, Ikaros overexpression also causes a slight reduction of bipolar cells and prevents müller glia formation, suggesting that loss of Ikaros is required for the progression to a late temporal state (Javed & Cayouette, 2017). Ikzf1 also contributes to the establishment of the temporally restricted cell fates in the developing mouse neocortex, suggesting that Ikzf1 might have a role as an intrinsic temporal identity factor in other progenitor contexts (Alsiö, Tarchini, Cayouette, & Livesey, 2013).

Extrinsic hypothesis on the other hand, suggests that progenitor cells are controlled by environmental cues, which would direct their choice of cell fate. In the developing retina, however, even though extrinsic signaling has been shown to alter progenitor output, these factors are thought to mostly control proliferation and act as negative feedback inhibition to refine the size of certain population, rather than regulate temporal identity in retinal progenitors (Cepko, 2014; Mattar & Cayouette, 2015). Some of these factors include: Shh, FGF2, Ciliary neurotrophic factor (CNTF) and (Goetz, Farris, Chowdhury, & Trimarchi, 2014).

For example, 1) blocking all *Shh* family activity with the drug cyclopamine led to a complete inhibition of retinal neurogenesis in the zebrafish, while deletion of *Shh* from the mouse retina resulted in a premature induction of photoreceptors and an overproduction of ganglion cells

(Neumann & Nuesslein-Volhard, 2000; Y. Wang, Dakubo, Thurig, Mazerolle, & Wallace, 2005). These observations suggest a key role for *Shh* in retinal cell-fate acquisition. 2) FGF signaling: Injection of the mutant FGF receptor into Xenopus resulted in a 50% loss of amacrine cells and photoreceptors with a corresponding increase in müller glia (McFarlane et al., 1998). This effect appears to be specific to these cell fates since no alterations in either cell death or cell proliferation were observed in this system (McFarlane et al., 1998). On the other hand, overexpression of FGF2 in Xenopus increased ganglion cells by 35% while decreasing müller glia by 50% (Patel & McFarlane, 2000). Additionally, FGF2 changed the relative proportions of rods and cones, but did not alter the overall number of photoreceptors (Patel & McFarlane, 2000). 3) *in vitro* treatment of retinae with CNTF led to a decrease in rod photoreceptor cells, a slight increase in amacrine cells and müller glia and a substantial increase in bipolar cells (Ezzeddine, Yang, DeChiara, Yancopoulos, & Cepko1, 1997). Furthermore, removal of the CNTF receptor yielded the opposite result of additional rod photoreceptors being produced (Ezzeddine et al., 1997). 4) In the chick retina, a decrease in Notch levels led to a corresponding increase in retinal ganglion cells produced (Austin et al., 1995; Silva, Ercole, & McLoon, 2003). Conversely, retroviral introduction of a constitutively active form of Notch decreased the number of ganglion cells (Austin et al., 1995). Introduction of activated Notch into newborn rat retinae led to a block in differentiation of all cell types (Bao & Cepko, 1997; Furukawa, Mukherjee, Bao, Morrow, & Cepko, 2000). Similar experiments performed in Xenopus and zebrafish showed either a similar block in differentiation or an increase in müller glia (Dorsky et al., 1995; Scheer, Groth, Hans, & Campos-Ortega, 2001).

Even though extrinsic signaling has been shown to alter progenitor output as mentioned above, these factors are thought to mostly control proliferation and act as negative feedback inhibition signals to refine the size of a specific neuronal population, rather than regulate temporal identity in retinal progenitors (Bassett & Wallace, 2012; Cepko, 2014).

Many extrinsic and intrinsic factors that we have discussed influence the cell cycle in addition to cell fate. One well-studied factor that is critically involved in the regulation of cell cycle exit is the retinoblastoma tumor suppressor protein (Rb) (Weinberg, 1995). Conditional knockout mice where Rb is deleted from the retina do in fact show a defect in the ability of progenitor cells to exit the cell cycle ((J. Zhang et al., 2004). Interestingly, loss of Rb also leads to the production of fewer rod photoreceptors specifically (J. Zhang et al., 2004). This cell-fate phenotype occurs only in rod photoreceptors and is separate from the effect of Rb loss on the cell cycle. Instead, transcription factors that are important for rod development,

such as Nrl, are absent in Rb-deficient retinae (J. Zhang et al., 2004). The cyclin kinase inhibitors (CKIs), can block the cell cycle at several places and, in some contexts, promote differentiation. Overexpression of a CKI from Xenopus ($p27^{Xic1}$) inhibits retinal progenitor cell proliferation, but also shows a specific cell-fate effect. Müller glia are increased, while bipolar cells are decreased (Ohnuma, Philpott, Wang, Holt, & Harris, 1999). All of these experiments point to the precise regulation of intrinsic factors in response to extrinsic cues leading to a coordinated exit from the cell cycle and the acquisition of specific retinal cell fates.

Mathematical modeling combined with live microscopy to visualize late rat RPCs in cell culture showed that these RPCs randomly choose between three modes of division – two daughter progenitor cells (PP), a progenitor and a differentiating daughter cell (PD) and two differentiating daughter cells (DD) in a stochastic manner, and as development progresses, RPCs shift from a PP to PD bias (He et al., 2012) (Figure 1.5). These experiments were further supported by lineage tracing experiments in zebrafish, where an inducible photoconvertible fluorescent protein was used to monitor the progression of individual RPCs through the cell cycle. Developmental program in which a wave of symmetrical proliferation (PP) followed by asymmetrical (PD) and then terminal (DD) differentiative divisions spreads around the retina. However, if all RPCs at 24 hpf went through exactly the same program (e.g., two rounds of PP to produce four P cells, followed by one round of PD to produce four D and four P cells, followed by one round of DD), all clones would end up being exactly the same size, i.e. 12 differentiated cells. This would generate a retina of approximately the right total number of cells. However, such a stereotypical pattern of RPC lineage progression is not consistent with the large variability in clone sizes of 24 hpf RPC (He et al., 2012). The type of cells generated, with the exception of a few outliers, fits a stochastic model of cell behavior where the probability of generating a particular cell type is the same as the frequency of that cell type in the mature retina. Thus, with every cell division, there is a higher probability of generating a rod than a bipolar cell because the frequency of rods is greater than the frequency of bipolar cells (Bassett & Wallace, 2012; Gomes et al., 2011).

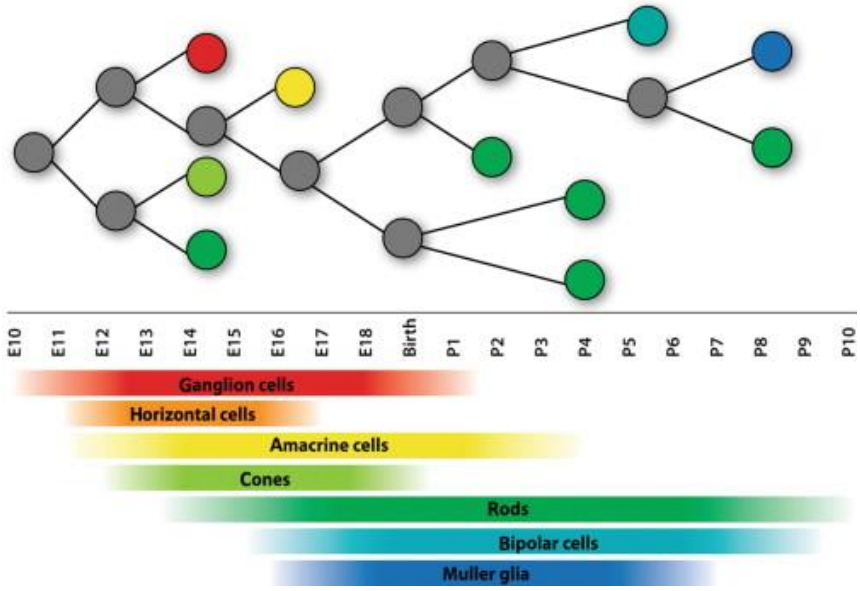

Figure 1.5: RSCs mode of division.

In vertebrate retinogenesis, there is no predefined order of modes of cell division. RPCs stochastically choose one of three modes of division (PP, PD and DD). As retinogenesis progresses, RPCs shift from mostly PP divisions to PD and DD divisions. (PP): two daughter progenitor cells; (PD): progenitor and a differentiating daughter cell; (DD): two differentiating daughter cells. Adapted from (Goetz et al., 2014).

In chapter 3 of this thesis, I am going to investigate the intrinsic and extrinsic factors controlling heterogeneity, proliferation and distribution of pigmented cells in clonally derived RSC spheres.

1.5 Development and Differentiation of Retinal Pigment Epithelium

RPE is a monolayer of pigmented cells situated between the neuroretina and the choroid. The RPE is of neuroectodermal origin and is therefore considered to be part of the retina. RPE is specified at the early optic vesicle stage, long before pigmentation is observed.

Two key players in RPE specification are the transcription factors Mitf and Otx2. Mitf is a key regulator of pigment cell development in the RPE and neural crest; it transactivates crucial genes for terminal pigment differentiation (e.g., *Dct, Tyrp1*, and tyrosinase). *Otx2* is expressed in the eye field and expression appears to persist until the late optic vesicle stage when it is downregulated in the presumptive retina. *Otx2* is required for *Mitf* expression and it transactivates expression of pigment genes in cooperation with Mitf (Martínez-Morales et al., 2003; Martínez-Morales, Rodrigo, & Bovolenta, 2004; Martinez-Morales et al., 2001). In the following model suggested by Martinez-Morales et al. (2004), the RPE induction is triggered by *Pax6* and *Otx1/Otx2* combined activity, *Pax6* and *Wnt* signaling induces expression of *Mitf*

which plays an important role in the development of melanin-containing cells, melanocytes, and RPE, it can support the further formation of RPE together with Otx proteins, according to Baumer and co-authors, expression of *Mitf* is controlled by the redundant activities of *Pax6* and *Pax2*. *Pax6* activity seems to be sufficient to induce RPE formation (Bäumer et al., 2003), and in mature RPE it is downregulated, remaining in the epithelia of the lens, cornea, conjunctiva, iris, amacrine cells and ganglion cells (Gruss & Walther, 1992).

Presumptive RPE progenitors control the initial proliferation and establishment of RPE in the optic vesicle. After proliferation in the presumptive RPE ceases, definitive RPE progenitors will control the expansion step leading to the formation of a single layer of cuboidal cells that become pigmented (Cechmanek & McFarlane, 2017). As development proceeds, a period of differentiation and further maturation follows that results in dramatic morphological, structural, and functional changes of the RPE tissue such as formation of tight junctions, expansion of apical microvilli and invagination of the basal membrane, establishment of polarity and retinoid recycling machinery (Burke & Hjelmeland, 2005; Marmorstein, 2001; Marmorstein, Finnemann, Bonilha, & Rodriguez-Boulan, 1998a; Rizzolo, 2007; Strauss, 2005). In Chapter three of this thesis I will characterize two RPE progenitor populations. Both populations are the product of the same mother stem cell and contribute to the formation of immature and mature RPE cells.

The RPE fate in the optic cup is maintained through several pathways that include: Shh, retinoic acid, BMP and Wnt/b-catenin pathways. In chick and mouse, reduced *Shh* signaling may not affect RPE specification but results subsequently in loss of RPE marker expression, increased proliferation of RPE and transdifferentiation into retina (S. Huh, Hatini, Marcus, Li, & Lai, 1999; X. M. Zhang & Yang, 2001). RPE differentiation defects are also observed in frog, when *Shh* signaling is downregulated (Perron et al., 2003).

Retinoic acid signal transduction occurs via the retinoic acid receptors (RARa, b, g) that bind to retinoid X receptor (RXR) and form heterodimers when bound to the RA response element in target genes. RAR mutants display a range of severe eye defects, including microphthalmia and transdifferentiation of RPE into NR (Cvekl & Wang, 2009; Lohnes et al., 1994; Matt, Ghyselinck, Pellerin, & Dupé, 2008). Furthermore, disruption of BMP signaling by overexpression of the BMP antagonist noggin caused transdifferentiation of the ventral RPE (Adler & Belecky-Adams, 2002).

Wnt/b-catenin pathway also controls differentiation of the RPE in the optic cup. In brief, activation of the Wnt/b-catenin pathway results in cytoplasmic stabilization of b-catenin,

ultimately converting T-cell factor/lymphoid enhancer factor (TCF/LEF transcription factors) from repressors into activators (Fuhrmann, 2010a). Interference with Wnt/b-catenin signaling in chick and mouse RPE of the optic cup causes loss of TCF/LEF reporter activation and severe eye defects such as microphthalmia and transdifferentiation of the RPE into NR (Fujimura, Taketo, Mori, Korinek, & Kozmik, 2009; Westenskow, McKean, Kubo, Nakagawa, & Fuhrmann, 2010; Westenskow, Piccolo, & Fuhrmann, 2009).

Mature RPE cell exhibits an apical–basal polarity characteristic of a transporting epithelium, with apical tight junctions binding neighbouring RPE cells together to form the outer blood-retinal barrier (Rizzolo, 1997) (Figure 1.6). This barrier induces transcellular transport and in conjunction with asymmetrical membrane protein distribution, allows directional transport of glucose, retinol (Vitamin A) and fatty acids to the photoreceptors. Equally important, this active transport process removes ions, metabolic waste products and water from the subretinal space (Strauss, 2005). The removal of water from the subretinal space to the choroidal circulation is vital for photoreceptor health (la Cour & Tezel, 2006). The photoreceptors, neurons and glial cells have a high metabolic turn-over rate leading to production of metabolic water which accumulates in the retina. In addition, intraocular pressure drives the movement of water from the anterior eye towards the retina where it accumulates. Water is eliminated from subretinal space by active transport via the RPE through Cl^- transport that is driven by the activity of the apically localized Na^+/K^+-ATPase (Hu, Gallemore, Bok, Lee, & Frambach, 1994) . Water transport is light-dependent, the light-dependent hyperpolarisation of the apical membrane reduces the transport rate of Na^+/HCO_3^- co-transporter which in turn leads to intracellular acidification. In the dark cGMP-gated cation channels are open in the outer segments of photoreceptors. The influx of Na^+ and Ca^{2+} through these channels is counterbalanced by an outflow of K^+ at the inner segment. These ionic currents represent the so called dark current (Baylor, 1996). When light falls onto photoreceptor outer segments, the cGMP-dependent cation channels are closed and the K^+ outflow at the inner segment becomes smaller. At the same time Na^+/K^+-ATPase at the inner segment takes up K^+ into the photoreceptor. This results in a reduction of the K^+ concentration in the subretinal space. This decrease is compensated for by the RPE. The mechanism is based on the fact that the ion conductance of the apical membrane of the RPE is dominated by a K^+ conductance (la Cour, 1985). The light-induced decrease in the subretinal K^+ concentration leads to hyperpolarization of the apical RPE membrane. This in turn leads to activation of inward rectifier K^+ channels generating an efflux of K^+ into the subretinal space, and to an increase of the subretinal K^+ concentration back to normal values (la Cour, 1985). Furthermore, the

highly folded basal membrane of the RPE helps to increase the surface area required for absorption and secretion (Strauss, 2005).

Mature RPE are also important for maintaining good retinal and photoreceptor function through different roles. Functional interaction of both the pigmented cell and the photoreceptor cell is essential for visual function (Bok, 1993; Sparrrow, Hicks, & Hamel, 2010; Steinberg, 1985; Strauss, 2005). Starting from the period of embryonic development, the functional differentiation of the photoreceptor layer and RPE depend on each other (Marmorstein, Finnemann, Bonilha, & Rodriguez-Boulan, 1998b; Rizzolo, 1997). The RPE cells extend apical microvilli into the subretinal space surrounding the photoreceptor outer segments. The apically localised transmembrane integrin, αVβ5, mediates retinal adhesion and promotes circadian-controlled phagocytosis of shed outer segments (Nandrot, Anand, Sircar, & Finnemann, 2006). Once engulfed, the segments are broken down by lysosomal activity within the RPE cell. This process is integral to the visual cycle, allowing the regeneration of the Vitamin A-based chromophore, trans-retinol, using the RPE-specific cytoplasmic isomerohydrolase, RPE65 (Strauss, 2005). The chromophore, oxidised to 11-cis-retinal, is bound to the cellular retinaldehyde-binding protein (CRALBP), before being released by the RPE cells to be taken up by the photoreceptors. Mutations in the phagocytic signalling protein MER proto-oncogene tyrosine kinase, commonly referred to as MerTK, the cytoplasmic RPE65, or the binding protein CRALBP induce significant pathology of the photoreceptors, as seen in retinitis pigmentosa (Travis, Golczak, Moise, & Palczewski, 2007). I will investigate the relation between RPE and photoreceptors in this thesis (chapter 3) and how it affects the proliferation and survival of RPE.

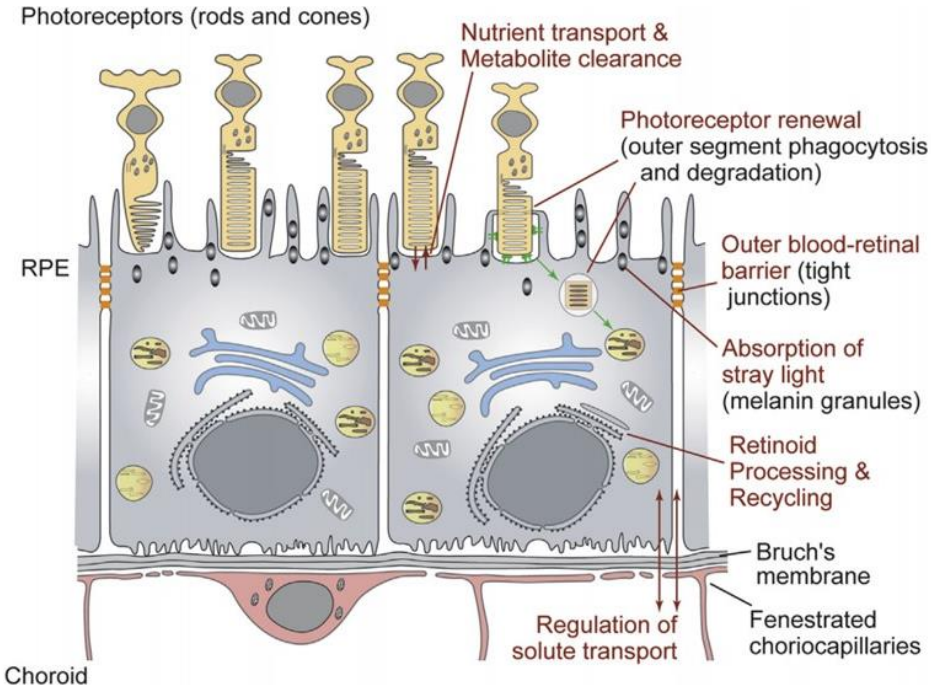

Figure 1.6: Different roles the RPE cells play to support the retina.

Adapted from (Lehmann, Benedicto, Philp, & Rodriguez-Boulan, 2014).

Light transduction starts with the absorption of photons by the chromophore of rhodopsin, 11-cis retinal (Baylor, 1996; Baylor & Burns, 1998; Okada, Ernst, Palczewski, & Hofmann, 2001). After absorption of photons, 11-cis retinal will change its conformation into all-trans retinal. All-trans retinal is metabolized into all-trans retinol and transported to the RPE. After uptake into the RPE all-trans retinol is transferred to CRBP (cellular retinol binding protein) which delivers all-trans retinol to a protein complex of several enzymes. This complex consists of LRAT (lecithin retinol transferase), RPE65 (RPE protein with 65 kDa) and RDH5 (11-cis-retinol dehydrogenase). The three enzymes catalyze the three-step reaction from all-trans retinol to 11-cis retinal: esterification of retinol by adding an acyl-group (LRAT), re-isomerisation to 11-cis using the energy from ester-hydrolysis (RPE65) and oxidation to 11-cis retinal (RDH5). The reaction is accelerated by CRALBP (cellular retinaldehyde-binding protein) which is also part of the enzyme complex and to which 11-cis retinal is immediately transferred. From CRALBP, 11-cis retinal is released to interphotoreceptor retinoid binding protein (IRBP) and is transported back to photoreceptors. All-trans retinal needs to be re-isomerized to ensure a sufficient delivery of 11-cis retinal for all visual needs and proper visual function (Lamb, Collin, & Pugh, 2007; Lamb & Pugh, 2004). Since photoreceptors do not

express a re-isomerase for all-trans retinal, the re-isomerization takes place in the RPE. For this purpose, all-trans retinal is delivered to the RPE, re-isomerised to 11-cis retinal and delivered back to photoreceptors. This process is called the visual cycle of retina. Thus, the RPE plays a crucial role in visual function and light adaptation. RPE65 plays a double crucial role in the visual cycle (M. Jin, Li, Moghrabi, Sun, & Travis, 2005; Strauss, 2011). On one hand it catalyses the re-isomerization reaction at light conditions. In the dark it functions as a retinol binding protein and represents an important retinol pool for the visual cycle. RPE65 combines both essential functions for adaptation of the visual cycle: storage as well as enzyme reaction (Strauss, 2011).

The melanin granules within the RPE cytoplasm help to absorb radiant energy focused by the lens onto the retina, which is beneficial to visual acuity by preventing light reflection and dispersed light from interfering with the signal received by the photoreceptors (Boulton & Wassell, 1998). RPE also play an important role in maintaining the structural integrity of the retina by an efficient defense against free radicals, photo-oxidative exposure and light energy (Beatty, Koh, Phil, Henson, & Boulton, 2000; Obana, 2010).

The RPE is made of specialized epithelial cells that interact and support photoreceptors on one side (apical) and also interact with different types of cells on the basal (blood) side, such as endothelial cells and cells of the immune system. In order to communicate with the neighboring tissue, the RPE is able to secrete multitude of factors and signaling molecules (figure 1.7).

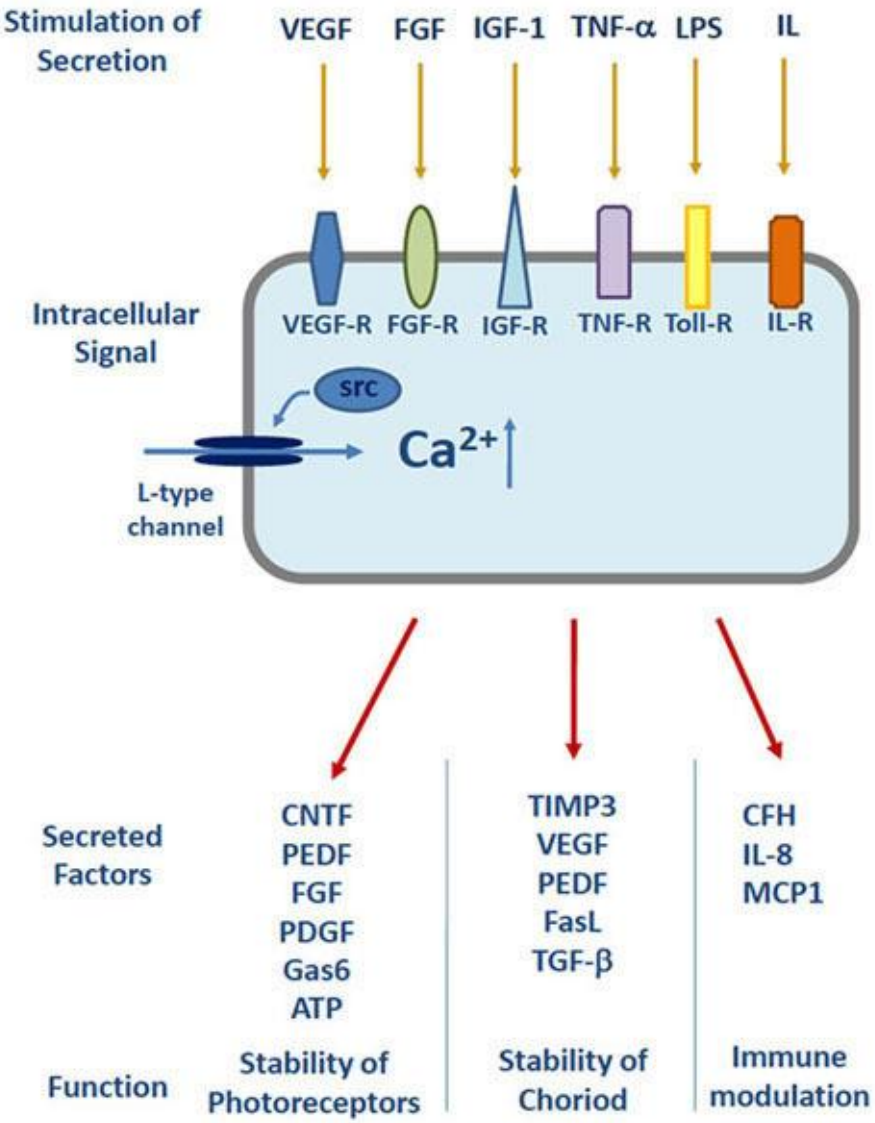

Figure 1.7: Growth-factor signaling and secretion in the RPE.

ATP, Adenosine triphosphate; CFH, complement Factor H; CNTF, ciliary neurotrophic factor; FASL, CD95 apoptosis receptor ligand; FGF, fibroblast growth factor; IL, interleukin; IGF-1, insulin-like growth factor-1; LPS, lipopolysaccharides; PEDF, pigment epithelium-derived factor; PDGF, platelet-derived growth factor; TIMP3, tissue inhibitor of metallo matrix proteases; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β. Receptors: FGF-R, fibroblast growth factor receptor; IGF-R, insulin-like growth factor receptor; IL-R, interleukin receptor; MCP-1, monocyte chemotactic protein 1; TNF-R, tumor necrosis factor receptor; Toll-R, toll-like receptors; VEGF-R, vascular endothelial growth factor receptor. Adapted from (Strauss, 2009).

It secretes ATP, fast-ligand (fas-L), fibroblast growth factors (FGF-1, FGF-2, and FGF-5), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), lens epithelium-derived growth factor (LEDGF), members of the interleukin family, tissue inhibitor of matrix metalloprotease (TIMP) and pigment epithelium-derived factor (PEDF) (Strauss, 2005). In the healthy eye some of these factors are constantly released and help to maintain the structural integrity of the neighboring tissues. PEDF is for example a neurotrophic factor which is secreted to the apical/retinal side of the RPE to stabilize the neuronal retina by prevention of apoptosis (Becerra et al., 2004; Cayouette, Smith, Becerra, & Gravel, 1999; Ogata et al., 2001). VEGF and TIMP are secreted to the basolateral side of the RPE and are required to stabilize the fenestrated structure of the endothelium of the choroid (Witmer, Vrensen, Van Noorden, & Schlingemann, 2003). Photoreceptor and vasculature health are maintained by polarized secretion of various trophic factors by the RPE. In particular, the secretion of VEGF and PEDF by the RPE has been implicated in the maintenance of photoreceptor health, immune privilege and the integrity of the surrounding vasculature (C. N. Kay, Ryals, Aslanidi, Min, & Ruan, 2013; Strauss, 2005).

RPE cells are connected at their basal side through adherence junctions and through tight junctions at their apical side. The tight junctions enable the epithelium to form a barrier by joining neighboring cells together and regulating transepithelial diffusion through the paracellular spaces. Tight junctions help establish epithelial polarity and maintain different protein compositions of the apical and basolateral membranes (Zahraoui, Louvard, & Galli, 2000). Tight junctions also act as a "fence" to retard intermixing of the phospholipids of the apical and lateral membranes. Number of tight junction-associated proteins have been identified and cloned, including PAR3, ZO-1, -2, and -3, claudin, and occludin (Rizzolo, 2007).

Occludin has a domain that binds to both ZO-1 and -2, which in turn bind to the cytoskeleton. The extracellular domain of occludin binds to another occludin molecule on an adjacent cell, forming the tight junction responsible for the permeability barrier (Hartsock & Nelson, 2008; McNeil, Capaldo, & Macara, 2006).

The claudin family of transmembrane proteins has also been identified as a critical component of the barrier function of tight junctions which plays a key role in maintaining tissue integrity and morphology of RPE cells (Hartsock & Nelson, 2008; Rahner, Fukuhara, Peng, Kojima, & Rizzolo, 2004; Rizzolo, Peng, Luo, & Xiao, 2011). In mouse, RPE appeared to express mRNA for claudins AL, 1, 2, 5, 11 and 12. Rahner et al. showed that claudin-1 increased 1000-fold

between E7 and E18, claudins 2 and AL increased only 10- and 60-fold, respectively (Rahner et al., 2004). On E18, claudin-1 represented approximately 83% of the total claudin mRNA (Rahner et al., 2004).

Adherens junctions bind to a continuous belt of actin filaments (the adhesion belt), where they function to hold neighboring cells together through the family of Ca^{2+}-dependent cell-cell adhesion molecules known as cadherins (Halbleib & Nelson, 2006; Nose & Takeichi, 1986; X. Yang, Chung, Rai, & Esumi, 2018). There are many members of the classic cadherin family, but E-cadherin in epithelial tissue is the best studied member in the context of stable adhesion (L. Xu, Overbeek, & Reneker, 2002). To function, cadherins must form complexes with cytoplasmic plaque proteins called catenins and with the actin cytoskeleton (N. Wang, Tytell, & Ingber, 2009). In this thesis, I will show how the expression of certain tight junction proteins in early RPE progenitors affect the distribution of RPE progenitors in clonal RSC spheres.

1.6 Static culture

Test tubes, T-flasks and well-plates are traditional means of facilitating static cultures (Ramlogan, 2010). Conventional 2D cell culture relies on adherence to a flat surface, typically a petri dish of glass or polystyrene, to provide mechanical support for the cells. Cell growth in 2D monolayers allows for access to a similar amount of nutrients and growth factors present in the medium, which results in homogenous growth and proliferation (Edmondson, Broglie, Adcock, & Yang, 2014). The advantages of 2D cultures are: simple and low-cost maintenance of the cell culture and ease of performing functional tests. (Hickman et al., 2014; Kapałczyńska et al., 2018).

However, 2D cultures are associated with several disadvantages like suboptimal expansion of cells in these monolayer culture systems as well as being labour intensive, requiring increased handling that can produce unintended cell loss and carry a bigger risk of contamination (D. Huh, Hamilton, & Ingber, 2011). Furthermore, culture conditions are suboptimal due to lack of monitoring and control over the cultivation (D. Huh et al., 2011; Kapałczyńska et al., 2018). Also, in terms of logistics and costs, expansion of stem cells in 2D systems is unfavoured as this will require extra costs for the labour needed to transport and passage the cells in research laboratory. The majority of 2D tissue culture systems are grown statically without any mixing. These static systems have limitations that arise from their non-homogeneous nature. Without mixing, concentration gradients of pH, nutrients, toxins, gases and growth factors will occur in the culture medium (Collins, Nielsen, Patel, Papoutsakis, & Miller, 1998). This will give

rise to different cell behaviour in different spatial locations in the culture and will also reduce culture reproducibility.

To expand stem cells in a controlled, reproducible, cost reducing and more efficient way, more and more research is being performed on the development of controlled bioreactors for both tissue engineering and cell therapy applications (King & Miller, 2007).

These dynamic systems offer distinct advantages: superior rates of oxygen transfer, waste removal and nutrient supply, achieving homogeneity of nutrient concentration, temperature profiles and pH equilibrium (Ellis, Jarman-Smith, & Chaudhuri, 2005).

In the next section I will compare static culture to stirring bioreactor systems and demonstrate why bioreactors are the preferred method for cell expansion. In chapter two of this thesis I will apply this comparison to our RSCs culture.

1.7 Bioreactors

A bioreactor can be defined as a device that uses mechanical means to influence biological processes. In tissue engineering, bioreactors can be used to aid the *in vitro* development of new tissue by providing biochemical and physical regulatory signals to cells to encourage them to undergo differentiation prior to *in vivo* implantation (Ellis et al., 2005; Plunkett & O'Brien, 2011) (Figure 1.8).

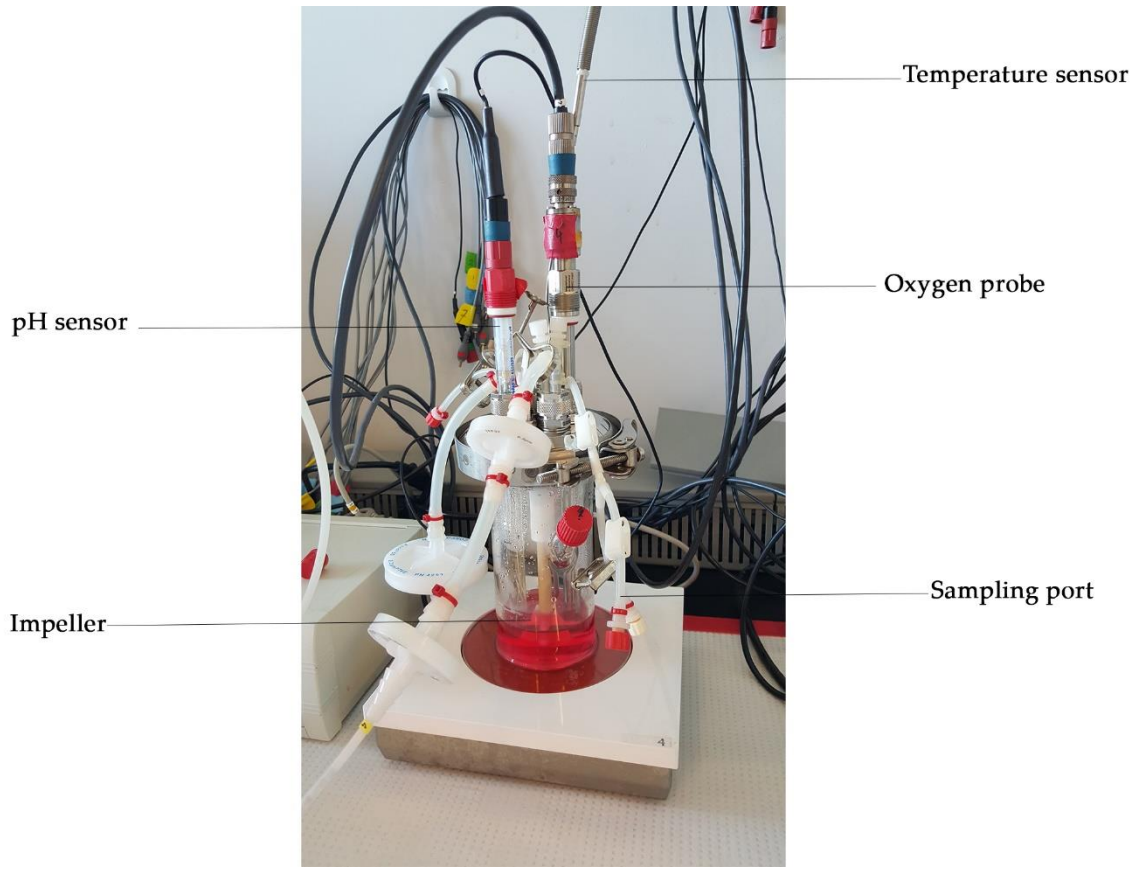

Figure 1.8: Stirring suspension bioreactor system.
Bioreactor vessel illustrating some important component.

Using bioreactors, scientists were able to control different cellular microenvironment parameters and adjust pH, oxygen tension to levels similar to those present at the *in vivo* niche, add nutrients and remove unwanted metabolites (e.g ammonia, urea, lactate) to improve cell growth, viability and subsequently expansion (Ramlogan, 2010). Bioreactors do not only provide culture control but can also provide optimisation, standardisation, scale up feasibility, and an automatic operation for cultivation of cells. Tissue-engineered products from bioreactor systems are more likely to be dependable, predicable and free of contaminants. Therefore, bioreactors provide a culture environment that could potentially meet the requirements for clinical application of tissue engineered products (Placzek et al., 2009).

Many types of bioreactors that are currently used for tissue engineering applications include stirred-tank, perfusion, spinner flask, biowave, rotational wall, roller bottle, radial flow, packed-bed, and rotating (Bancroft, Sikavitsas, & Mikos, 2003; Cameron, Hu, & Kaufman, 2006; Dang, Gerecht-Nir, Chen, Itskovitz-Eldor, & Zandstra, 2004; Goodwin, Schroeder, Wolf, & Moyer, 1993; Schroeder et al., 2005; F. Zhao & Ma, 2005). The mentioned bioreactors have been used primarily for tissue culture, but spinner flasks (Frauenschuh et al., 2007; Schop, Janssen, Borgart, De Bruijn, & Van Dijkhuizen-Radersma, 2010a; Schop et al.,

2009) and biowave bioreactors are gaining prominence in cell expansion (Eibl, Werner, & Eibl, 2010; Schlaeppi et al., 2006).

Different bioreactor systems have been developed for adult stem cells, either to propagate undifferentiated cells and/or to induce differentiation into a particular tissue lineage (Rodrigues, Fernandes, Diogo, Lobato Da Silva, & Cabral, 2011). Bioreactor has been successfully used to expand some types of stem cells including: mesenchymal stem cells (Godara, McFarland, & Nordon, 2008), hematopoietic stem cells (Cabral, 2001; Cabrita et al., 2003; Safinia, Panoskaltsis, & Mantalaris, 2005), neural stem cells (Kallos, Sen, & Behie, 2003) and pluripotent stem cells (Kehoe, Jing, Lock, & Tzanakakis, 2010).

Table 1.2: Different types of bioreactors and their applications.

Type of Bioreactor	Type of stem cell	Reference
Stirred-tank	Human neural stem cells	(Simaõ et al., 2016)
	Human pluripotent stem cells	(Kropp et al., 2016)
	Human mesenchymal stem cells	(Dufey et al., 2017)
Spinner flask	IPSCs	(Ismadi et al., 2014)
	Human mesenchymal stem cells	(Nuno, Fonte, Alexandra, Lobato Da Silva, & Rafiq, 2014)
	Embryonic stem cells	(Kehoe, Lock, Parikh, & Tzanakakis, 2008)
	Neural stem cells	(Sen, Kallos, & Behie, 2002)
Perfusion	Human mesenchymal stem cells	(F. Zhao & Ma, 2005)
	Embryonic stem cells	(Yeo et al., 2013)
	Hematopoietic stem cells	(Housler et al., 2014)
Biowave	Mesenchymal stem cells	(Jossen, van den Bos, Eibl, & Eibl, 2018)
	Human pluripotent stem cells	(Kropp, Massai, & Zweigerdt, 2017)
Rotating wall vessels	Mesenchymal stem cells	(Z. Y. Zhang et al., 2009)

	Human umbilical cord cells Human epidermal stem cells Retinal organoids	(Reichardt et al., 2013) (Lei et al., 2011) (DiStefano et al., 2018)
Roller bottle	Human mesenchymal stem cells Embryonic stem cells Human hematopoietic stem cells	(Panchalingam, Jung, Rosenberg, & Behie, 2015) (Unger, Muzzio, Aunins, & Singhvi, 2000) (Andrade-Zaldívar, Kalixto-Sánchez, de la Rosa, & De León-Rodríguez, 2011)
Packed-bed	Placental derived mesenchymal stromal cells Human mesenchymal stem cells Hematopoietic stem cells	(Osiecki et al., 2015) (Weber et al., 2010) (Meissner, Schröder, Herfurth, & Biselli, 1999)

Selection of a bioreactor system for mammalian cell cultivation beyond bench scale is dependent on whether the cells are adherent, in a suspension grown as single cells or aggregates (Ulloa-Montoya, Verfaillie, & Hu, 2005).

Suspension cells can be grown in stirred vessel (spinner flask or stirred-tank), rotating wall vessels, perfusion and biowave. Adherent cells can be expanded using roller bottle, packed-bed or parallel plate bioreactors. For example: murine embryonic stem cells are adherent cells traditionally grown on tissue culture flasks. For the purpose of increasing cell number, surface culture and microcarriers are needed. Surface culture includes roller bottle and multiplate parallel plates (Merten, 2015).

1.8 Microcarriers

Microcarriers offer the advantage of providing a large surface area for monolayer cell growth during proliferation in a homogenous suspension culture. Since its introduction in 1967, microcarrier culture has been applied successfully for growing primary cells and anchorage-dependent cell lines for the production of vaccines, pharmaceuticals and cell population expansion (Van Wezel, 1967). van Wezel cultured fibroblast-like cells derived from rabbit embryonic skin cells and human embryonic lung cells on positively charged DEAE-Sephadex beads suspended in stirred culture. Since then, microcarrier cultures have been commonly used

for large-scale expansion of anchorage-dependent cells in vaccine and pharmaceutical production.

Microcarriers (MCs) are small spherical beads (diameters between 90 – 300μm) made of different substrates and suspended in growth medium (B. Li et al., 2015). Different coatings provide adherent surfaces for cells to attach, grow and expand. Detaching cells from microcarriers does not require the use of Trypsin and other aggressive enzymes, from which approximately only 30% of the cultured retinal cells can survive (Tropepe et al., 2000). There are several types of microcarriers that are commercially available made from wide variety of materials (e.g. plastics, glass, dextran, cellulose, gelatin and collagen), each with different characteristics. The choice of microcarriers depends on their applications, as it has been reported that different cell types grow better on one microcarrier type over another. For example, several adult cell types have been shown to prefer Cytodex 1 MCs (Q. Fang et al., 2019; Rafiq, Coopman, Nienow, & Hewitt, 2016; Schop, Janssen, Borgart, De Bruijn, & Van Dijkhuizen-Radersma, 2010b), Cytodex 3 MCs (Hewitt et al., 2011; Nie, Bergendahl, Hei, Jones, & Palecek, 2009; L. Zhou et al., 2013) or CultiSphere MCs (Santos et al., 2011).
Regarding their structure, they can either be micro or macroporous. In microporous MCs, the diameters ranging from 150-200 μm and pore sizes smaller than 10 μm. Cells proliferate in a monolayer when attached to the external surface of the carrier, being directly exposed to the shear stress caused by agitation and aeration that can be harmful for cell expansion (Butler, 1996). In macroporous MCs with diameters ranging from 200-400 μm and pore sizes between 10-50 μm (sometimes even greater), despite growing on the outer surface, cells are also able to grow inside the carrier pores since the mean cell diameter for single cells in suspension is about 10 μm (Nilsson, Buzsaky, & Mosbach, 1986), being shielded against hydrodynamic forces experienced within the system. However, cell growth within the microcarrier pores raises some doubts as whether it has created a diffusional gradient instead of an equally homogeneous environment for cell proliferation compared to the outer matrix surface with an efficient mass transfer and gaseous exchange. Cells can also grow in three dimensions at high densities and that confers more stability and improves the longevity of the culture, making them suitable for long-term culture (Storm, Orchard, Bone, Chaudhuri, & Welham, 2010). Beside these advantages, some disadvantages of carriers have also to be considered: 1) some carriers have to be prepared before use, 2) cell harvest from macroporous carriers can be difficult, 3) cell count, especially in the case of macroporous carriers, requires special techniques, 4) for larger macroporous carriers, mass transfer limitations have to be considered (Eibl, Eibl, Pörtner, Catapano, & Czermak, 2009).

The ideal microcarrier should be non-toxic, biocompatible, promote favourable cellular interactions and tissue development and at the same time fulfil adequate physical and mechanical properties (Mano et al., 2007). Non-toxic microcarriers are required to obtain good cell growth and also when the cell culture products are used clinically. The following criteria are essential for microcarriers: 1) They must have surface properties that allow the cells to adhere and spread in order for the cells to proliferate. 2) The density of the microcarriers should be slightly greater than that of the surrounding medium so that cells and medium can be separated easily and it should allow for complete suspension of the microcarriers with only gentle stirring. 3) To make the cells reach confluence at approximately the same time ,the size distribution should be narrow. The size of the microcarriers should also allow easy sampling and handling. 4) The optical properties should permit studying the cells throughout the culture using standard microscopy techniques and without having to remove the cells from the carriers. 5) The stirring by waving of the microcarriers during cultivation requires a microcarrier that is not too rigid to avoid damage when a collision occurs.

Microcarriers offer several advantages when culturing anchorage-dependent cells. Compared to conventional methods the microcarriers provide growth in several dimensions and at high densities, which makes it possible to grow several million cells/ml (Boo, Selvaratnam, Tai, Ahmad, & Kamarul, 2011). This means that the laboratory and storage space can be reduced markedly, which will lower the costs and save time. The cell culture techniques where microcarriers are used have become very important when large amounts of biological materials are produced, such as vaccines, antibodies, enzymes, hormones, nucleic acids and interferons (Mano et al., 2007).

Taking all the above-mentioned MC advantages in consideration, I will test different types of MCs and monitor how they affect RSC survival and expansion in chapter two.

1.9 Aim and research summary

Research aims:

This research aims to establish an expansion protocol that will provide large number of stem cells that can be later differentiated into photoreceptors and RPE. The current available protocol involves growing RSCs in static monolayer culture which, even with the addition of several mitogens, still considered inefficient. The first aim of this thesis is to investigate and compare the possible methods available to expand the numbers of RCSs. Taking in consideration the challenges associated with the current available transplantation protocols, the protocol published in this thesis will not only provide large number of stem cells that can be directed toward photoreceptors and RPE differentiation, in addition, these adult stem cell derived photoreceptor cells will provide a great source for autologous transplantation. Expansion level across platforms (static, SSB and SSB plus MCs) will be tested using RCS clonal assay. The number of tertiary clonal spheres will be counted, and other stem cell properties will be addressed including: survival, proliferation, self-renewal, and differentiation. RCS behaviour will be monitored at normoxia vs niche-like hypoxia (21% O_2 vs 5% O_2) to determine which condition is more effective in promoting symmetrical stem cell division.

The second aim of this thesis is to study the behaviour of RSCs and RPCs on clonal sphere level. RSC spheres will be classified into three groups depending on their pigment distribution. I will study the intrinsic and the extrinsic factors affecting the proliferation of pigmented and non-pigmented cells and to define and better understand the heterogeneity of RSCs and their precursors.

General hypothesis:

RSCs are multipotent and self-renewing cells. They can divide symmetrically giving rise to two identical daughter cells or asymmetrically producing an identical daughter cell and a progenitor cell. There are about 100 RSCs per mouse eye and 10000 per human eye. These stem cells are multipotent i.e can give rise to all retinal cell types. In practice, there are many problems associated with growing stem cells and differentiated cells *in vitro*. First, RSCs are quiescent in adult due to the inhibitory niche *in vivo*. Second, when cultured *in vitro,* most of these stem cells get lost during dissection and passaging due to the use of strong enzymatic dissociation protocol. Third, RSCs *in vitro* divide asymmetrically; with subsequent passages, each stem cell will give rise to one RSC sphere. Fourth, when stem cells differentiate into retinal cells *in vitro*, most will die after transplantation and few will get integrated into the

recipient retina. Fifth, RSCs under pan-retinal conditions give rise to low percentage of photoreceptors and RPE cells, factors like RA/taurine and coco can be added to the culture to increase the production of rods and cones respectively. However, growing RPE cells from adult RSCs remains a challenging process. Establishing a protocol that can increase the number of RSCs by enhancing their survival and facilitating their symmetrical division *in vitro* is a growing demand.

Specific research questions:

The main hypothesis of this work:

1) Culturing RSCs in stirring suspension bioreactor in hypoxia will improve survival and enhance symmetrical division.

To test the first hypothesis, I compared RSC growth and proliferation across different platforms: static culture, stirring bioreactor and stirring bioreactor loaded with microcarriers. In each platform I compared the growth and viability of RSCs in normoxic (21% O_2) and hypoxic (5% O_2) cultures. In bioreactors, I tested the effects of different agitation speeds, microcarrier types and different seeding density. Then, conducted a tertiary sphere assay to measure RSC expansion and in the end, I combined the best conditions that resulted in greater expansion.

2) All RSC clonal spheres arise from the same type of RSC.

To test for the second hypothesis, RSC clonal spheres were classified into three groups based on their pigment level and distribution. The differentiation potential for each group was observed and the groups were subsequently tested for self renewal and multipotentiality. Several experiments were performed to test for intrinsic and extrinsic factors influencing fate decision in the downstream progenitors in all sphere types. I classified the pigmented progenitors in each sphere type into early and late RPE progenitors depending on their proliferation in response to signals received from co-cultured non-pigmented cells and based on their qPCR gene expression analysis.

Chapter 2

Expansion of Retinal Stem Cells and their Progeny Using Cell Microcarriers in a Bioreactor

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2.1 Abstract

Blindness as a consequence of degenerative eye diseases (e.g., age-related macular degeneration and retinitis pigmentosa) is a major health problem and numbers are expected to increase by up to 50% by 2020. Unfortunately, adult mouse and human retinal stem cells (RSCs), unlike fish and amphibians, are quiescent *in vivo* and do not regenerate following disease or injury. To replace lost cells, we used microcarriers (MCs) in a suspension stirring bioreactor to help achieve numbers suitable for differentiation and transplantation. We achieved a significant 10-fold enrichment of RSC yield compared to conventional static culture techniques using a combination of FACTIII MCs and relative hypoxia (5%) inside the bioreactor. We found that hypoxia (5% O_2) was associated with better RSC expansion across all platforms; and this can be attributed to hypoxia-induced increases in survival and/or symmetric division of stem cells. In the future, we will target the differentiation of RSCs and their progeny toward rod and cone photoreceptor phenotypes using FACTIII MCs inside bioreactors to expand their populations in order to produce the large numbers of cells needed for transplantation.

2.2 Introduction

Retinal stem cells (RSCs) are a rare population (1 in 500) of large, multipotent, heavily pigmented cells that reside in the pigmented layer of the ciliary epithelium (CE) of the adult human and mouse eye. Embryonic RSCs can divide symmetrically to expand and maintain the stem cell pool by producing two new stem cells (Ahmad et al., 2000; Coles, Angenieux, et al., 2004; Tropepe et al., 2000) or asymmetrically giving rise to a daughter stem cell and a retinal progenitor cell (Balenci & van der Kooy, 2013; Burton & Caniggia, 2001; Otteson & Hitchcock, 2003; Saito et al., 2003; Tropepe et al., 2000). Retinal progenitors can differentiate to either retinal pigmented epithelium (RPE) or neural retina cell types: photoreceptors, horizontal, amacrine, bipolar and retinal ganglion cells and müller glial cells (Ballios et al., 2012). There are approximately 10,000 RSCs in a single human eye (Coles, Angenieux, et al., 2004) and 100 per mouse eye (Ahmad et al., 2000; Tropepe et al., 2000). It could be said that the stem cell status of the RSC is still contested; in particular two labs (Cicero et al., 2009; Gualdoni et al., 2010) have argued against the stemness of RSCs, one of them (Cicero et al., 2009) suggested that RSCs are a product of trans-differentiation from RPE cells to neural cells. This remains, however, a remote possibility given that RSCs can be prospectively isolated and that RSCs can self-renew over multiple passages and produce progenitors that differentiate to all neural retinal cell types (Abdouh & Bernier, 2006; Ahmad et al., 2000; Ballios et al., 2012;

Coles, Angenieux, et al., 2004; Del Debbio, Peng, Xiong, & Ahmad, 2013; Demontis, Aruta, Comitato, de Marzo, & Marigo, 2012; Y. Fang et al., 2013; Inoue et al., 2010).

Clinical applications of adult-derived mammalian RSCs are associated with several problems. Unlike fish and amphibians, those mammalian RSC cells are quiescent *in vivo* in adults and do not regenerate following disease or injury (Balenci & van der Kooy, 2013; Otteson & Hitchcock, 2003). In many fish and amphibians, the CMZ is considered a proliferative niche, where RSCs contribute to retinal regeneration throughout life (Balenci et al., 2013). In mammals, including humans and mice, no such proliferation is observed within the adult CE beyond early postnatal day (PND) 10 in mice and past birth in humans, nor following disease or injury (Ahmad et al., 2000; Tropepe et al., 2000). Another problem associated with clinical application is that large numbers of cells are needed to overcome cell loss due to degeneration itself, and due to poor cell survival and integration following transplantation (Warre-Cornish, Barber, Sowden, Ali, & Pearson, 2014). To produce large quantities of retinal precursors and subsequently their progeny of photoreceptors and RPE, some research groups have used pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which have an infinite ability for self-renewal as well as the potential to differentiate into every cell type in the body (Schwartz, Tan, Hosseini, & Nagiel, 2016; Sowden, 2014; Uygun et al., 2009; N. Wu, Doorenbos, & Chen, 2016; Yue et al., 2010). ESCs are derived from the inner cell mass of blastocyst-stage embryos; hence their ability to differentiate into all adult cell types derived from the three embryonic germ layers. Many labs have been successful in differentiating photoreceptors and RPE from ESCs (Cowan, 2016; Schwartz et al., 2016; Sowden, 2014; Uygun et al., 2009; Yue et al., 2010), but the ethical issues and immune rejection issues remain problematic, and the clinical application of ESCs carries a risk of teratoma formation as well. Similar to ESCs, iPSCs are pluripotent cells, but they differ from ESCs in being produced *in vitro* by reprogramming somatic cells through transduction of four transcription factors: Oct3/4, Sox2, Klf4 and c-Myc (N. Wu et al., 2016). The use of defined reprogramming factors for generating specific iPSCs offers: 1) an autologous source, eliminating rejection; 2) an opportunity to repair genetic defects; 3) absence of ethical problems faced with the use of ESC derived cells (Geng et al., 2017; N. Wu et al., 2016). However, issues including the risk of viral integration producing oncogene expression are still concerning (Geng et al., 2017). Ciliary epithelium derived human RSCs and RSCs derived from iPCs are potential autologous sources to bypass the ethical and immune rejection concerns associated with the use of ESCs (Clarke, Ballios, & van der Kooy, 2012; Ramsden et al., 2013).

RSCs traditionally have been grown as clonal spheres of cells in static tissue culture flasks (T-flasks) or in 24 well plates, However, scaling-up cell production by using T-flasks is not effective: it can be very labor intensive and there is no control over many culture parameters. Moreover, there is significant cell loss that results from using the current enzymatic dissociation protocol (Tropepe et al., 2000) on clonal spheres of cells. In this report, we studied the effect of systematic variation of factors such as agitation speed and oxygen concentration on RSC behavior inside bioreactors. We hypothesize that using SSBs (suspension stirring bioreactors) that are scalable will enhance RSC expansion through facilitating symmetrical division and allowing greater survival of cells by controlling conditions such as pH, temperature, agitation rate, nutrient level and O_2 concentration. Another advantage of SSBs over static T-flask cultures is that we can more easily manipulate factors that are known to impact cell behavior (e.g. shear stresses, hydrodynamic pressure and medium flow patterns) (Sen, Kallos, & Behie, 2001). Suspension culture bioreactors have been previously used to expand embryonic stem cells (Cormier, Zur Nieden, Rancourt, & Kallos, 2006; Fernandes et al., 2009; King & Miller, 2007), neural stem cells (Gilbertson, Sen, Behie, & Kallos, 2006), mammary stem cells (B. S. Youn et al., 2005), hematopoietic stem cells (Choi, Noh, Lim, & Kim, 2010) and cancer stem cells (Benjamin S. Youn, Sen, Behie, Girgis-Gabardo, & Hassell, 2006). To increase viability by reducing the cell death associated with the use of dissociative enzymes, we employed cell microcarriers (MCs) (Forestell, Kalogerakis, Behie, & Gerson, 1992). Detaching cells from MCs does not require the use of Trypsin and other aggressive enzymes, from which approximately only 30% of the retinal cells can survive (Coles et al., 2006). Instead 1X TrypLE select solution (Cat. No.12563-011, Gibco) can be used to detach cells in a safer and faster way because: 1) it is an animal free compound and is less harsh to cells, 2), can be diluted using either buffer or media, 3) is stable at room temperature and can last on the shelf for up to 6 months and 4) it is free of contaminating viruses and prion proteins (Rourou, van der Ark, van der Velden, & Kallel, 2007). There are several types of MCs that are commercially available, each with different characteristics. For example, several adult cell types have been shown to prefer Cytodex 1 MCs (Frauenschuh et al., 2007; Ng, Berry, & Butler, 1996; Schop, an Dijkhuizen-Radersma, et al., 2010; Schop, Janssen, et al., 2010a), while mouse embryonic stem cells have been reported to grow well on Cytodex 3 MCs (Abranches, Bekman, Henrique, & Cabral, 2007; Alfred et al., 2011) and human embryonic stem cells show enhanced attachment and growth on Hillex and CultiSphere MCs (Phillips et al., 2008). Cytodex 1 and Cytodex 3 microcarriers have been shown to support human MSC growth in suspension culture (Schop, an Dijkhuizen-Radersma, et al., 2010; Yuan, Kallos, Hunter, & Sen, 2014). Another method of large scale expansion using MCs in SSB can be

done by using bead-to-bead cell transfer either by adding new bare MCs to already confluent MCs, or by transferring old MCs to a new bioreactor containing new MC beads (Luo, Sun, Geng, & Qi, 2008; Merten, 2015; Ohlson, Branscomb, & Nilsson, 1994). However, this method might not work with all cell types and can lead to microcarrier aggregation, cell multilayering and heterogeneity within the aggregates (Merten, 2015).

We have developed a novel RSC enrichment protocol using cell MCs inside suspension bioreactors spinning at lower agitation rates and with lower oxygen concentrations to achieve the best possible cell survival.

2.3 Materials and Methods

2.3.1 Mouse Strain

RSCs were derived from the ciliary epithelium of adult (7 – 8-week-old) C57BL mice. 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.

2.3.2 Primary cell isolation and culture

Cells were plated in SFM on non-adherent tissue culture plates (Nunc; Thermo Fisher Scientific, Rochester, NY) at a density of 20 cells/μL in serum-free media with FGF2 (10 ng/mL, human recombinant; Sigma-Aldrich) and heparin (2 ng/mL; Sigma-Aldrich) in humidified 5% CO_2 incubator with temperature of 37ºC.

For low density experiments we dissected 7-10 mice (14-20 eyes) per individual bioreactor experiment. For high density bioreactor and microcarriers experiments we dissected 15-17 mice (30-34 eyes) per individual experiment.

2.3.3 Bioreactor preparation

Following 7 days of primary culture in serum free media, clonal spheres were dissociated into a single cell suspension using an enzymatic solution (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, Oakville, ON). Cells were then transferred to (125 ml / paddle) stirring bioreactor vessels (Das gip, SR0250ODLS, Eppendorf) with a 65ml working volume of serum free medium with FGF2/heparin (FH). Prior to use, all inner surfaces of the glass suspension bioreactor vessels and outer surfaces of the impellers were siliconized using a 1:9 ratio of Sigmacote (Cat. No. SL-2, Sigma-Aldrich Toronto, ON, Canada) to hexane (Cat. No. B90210, Omnisolv from VWR International), sterilized and calibrated (oxygen and pH

calibration). Bioreactor parameters were set as following for all experiments: pH: 7.3 Temp: 37ºC and O_2 concentration: 21% & 5%. Semi-fed batch (30%) culture was used by adding 15 ml every two days. Cells were cultured at a density of 7 cells/µl. Static control culture was kept in a humidified 5% CO_2 incubator at 7 cells/µl density for 7 days.

2.3.4 Tertiary RSC Clonal Sphere Assays

Samples (15ml) were taken every two days over 7 days duration. 37µm filter (Stem cell technology, cat#27215) was used to separate single cells from aggregates. Dissociative enzymes similar to those used above were added to aggregates only. Then, cells (single cells and aggregates) were cultured separately at 10000 cells/well density in a 5% CO_2 humidified incubator to determine the tertiary clonal sphere percentage frequencies of total cells. 10 µl for Cell counting performed by hemocytometer (Cat. No. B3175, VWR) using Trypan blue dye (Cat no. T8154, Sigma-Aldrich).

2.3.5 Cells counts and viability

After dissociating bioreactor and static samples, 10 µl samples were prepared for cell counting performed by hemocytometer (Cat. No. B3175, VWR) using Trypan blue dye (Cat. No. T8154, Sigma-Aldrich). Viability was analyzed using Ethidium homodiamer-2 (Cat. No. E3599, Invitrogen) and hochest staining. Samples were diluted, if necessary, using Ca2þ- and Mg2þ-free phosphate-buffered saline (DPBS) (Gibco).

2.3.6 Flow cytometry

Secondary spheres from 5% O_2 bioreactors were dissociated. EdU (Click-iT EdU kit, Cat. #C10083 Invitrogen) was diluted in DMSO, 1X PBS at 5 mg in 2 ml giving a stock concentration of 10 mM. Working dilutions were made in 1X PBS at 10 µM. Cells were treated with 10 µM EdU for 3 hours duration, after 24 hours of initial culture. Cells then were washed using DPBS 1x and dissociated using the previously described enzymes, after which they were fixed using 4% PFA for 15 minutes and permeabilized using 0.5 µl TritonX-100. Reaction cocktails were prepared according to protocol instructions and cells incubated for 30 minutes. Cells were then analyzed using flow cytometry (BD LSRFORTESSA).

2.3.7 Microcarrier Surface Screening

We conducted the screening experiment using three different coatings: FACTIII (cationic charged collagen), ProNectin-F (recombinant protein coated) and HillexII (modified polystyrene) purchased as a starter kit from Solohill Inc. These microcarriers do not require

the overnight soaking step and can be sterilized along with the bioreactor during the autoclaving step. For preliminary testing of the microcarriers, primary RSCs were dissociated and seeded on different types of microcarriers (FACTIII, NectinF,HillexII, Solohill INC.) in 6 wells plate seeded with 20000cells/ml (4-5 cells/bead) in a 21% static humidified incubator with the shaker adjusted to 50 rpm, in addition to static control culture without microcarriers. 2 ml samples were taken every two days to monitor cell growth, attachment, viability and do RSC sphere clonal assay (table 1). Microcarriers were allowed to settle down in the falcon tube then washed two times using Dulbecco's Phosphate-Buffered Saline (DPBS). Cells then were detached from the microcarriers using 1x TryplE solution for 15-20 minutes in 37ºC, and then they were filtered and washed with Serum free media. Static control samples were dissociated using the trypsin/collagenase enzyme mix. Cell counts were done using Trypan blue stain and viability was assessed using Ethidium homodiamer-2 and Hoechst staining. Cells then were cultured in SFM+FH at a density of 10000 cells/well at 21% oxygen concentration in incubators for 7 days for tertiary clonal sphere assays.

2.3.8 Continuous versus intermittent Agitation

To test the effect of agitation mode (continuous versus intermittent) on RSCs attachment and expansion, we dissociated primary RSC spheres and seeded them on FACTIII MCs (10000 cells/ml) in either continuous agitation (50rpm) or intermittent agitation (50 rpm, 3min agitation followed by 30 min no agitation for 6 hours followed by continuous agitation thereafter) under conditions of 5% oxygen, pH 7.34 and temp 37ºC. Samples were taken every 2 days for growth analyses and tertiary clonal sphere assays. Microcarriers were allowed to settle down in the falcon tube, and then washed two times using Dulbecco's Phosphate-Buffered Saline (DPBS). Cells were detached from the microcarriers using 1x TryplE enzyme, then filtered and washed with Serum free media and seeded at 10000 cells/well clonal density in a 5% oxygen incubator in SFM+FH.

2.3.9 Low versus High Seeding Density

Primary spheres were dissociated using the Trypsin/Collagenase enzymes mix and then transferred to a bioreactor vessel filled SFM+FH and FACTIII microcarriers (sterilized and autoclaved along with the bioreactor). Cells were cultured either at 10000 cells/ml (2 cells/bead) or 50000 cells/ml (9-10 cells/bead). The bioreactor stirring speed was adjusted to 50rpm continues agitation in both experimental assays. Samples were taken every 2 days for growth analyses and tertiary clonal sphere assays. Microcarriers were allowed to settle down in the falcon tube, then washed two times using Dulbecco's Phosphate-Buffered Saline

(DPBS). Cells were detached from the microcarriers using 1x TryplE enzyme, then they were filtered and washed with serum free media and seeded at 10000 cells/well clonal density in 5% oxygen incubator in SFM+FH.

2.3. 10 1xTryplE versus 10x TryplE

Primary spheres were dissociated using Trypsin/Collagenase enzymes mix and then transferred to a bioreactor vessel filled with SFM + FH and FACTIII MCs (sterilized and autoclaved along with the bioreactor) at 50,000 cells/mL (9–10 cells/bead) seeding density. Samples were taken every 2 days and cells on the MCs were incubated in 1X TrypLE or 10X TrypLE Select (Cat. No. A1217701, Gibco) for 10–15 min at 37ºC. After counting, cells were seeded at a density of 10,000 cells/well (SFM + FH) for 7 days in a 5% oxygen incubator for tertiary clonal spheres assay.

2.3.11 RSC Differentiation and Immunostaining

RSC colonies cultured in 5% SSB were plated on laminin-coated (50 ng/mL, Sigma-Aldrich) 24-well plates (Nunc) in a humidified 5% CO2 incubator, and differentiated using a protocol for rod photoreceptor differentiation involving taurine (100 μm; Sigma-Aldrich) and retinoic acid (RA) (500 nM; Sigma-Aldrich), plus FH for 50 days. Pan-retinal differentiation media supplemented with 1% fetal bovine serum (FBS) (Invitrogen, Burlington, ON) and FH was used as a control. Media and growth factors were replaced every 4 days. The cells were then rinsed with PBS and fixed using 4% PFA for 10 min at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 10 min and preblocked with 2% normal goat serum and bovine serum albumin (BSA) for 1 hr at room temperature. Cells were then incubated overnight at 4ºC in the following primary antibody: anti- Rhodopsin (MAB5316, RetP1, 1:250; Millipore) and next day cells were washed three times with PBS and incubated in the following secondary anti-body: Alexa fluor 568 (1:400; Invitrogen) for 1 hr at room temperature. Nuclei were stained with Hoechst dye (1:1,000; Sigma-Aldrich). Stained cells were then examined under a fluorescence microscope (Axio Observer D1; Carl Zeiss) using AxioVision 4.8 software (Carl Zeiss).

2.3.12 Statistics

Statistical analysis was 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 mean ± SEM. The significance value was set at $p < 0.05$.

2.4 Results and discussion

2.4.1 A lower agitation rate increased the yield of RSCs by 8 times over faster agitation

To engineer the RSC microenvironment in stirring bioreactors, several factors should be taken in consideration: physical forces (mechanical stress and hydrodynamic forces), oxygen tension, extra cellular matrix (ECM) and the regulation of autocrine and paracrine signaling. The hydrodynamic forces generated in a bioreactor depend on: agitation rate, seeding density, impeller and vessel geometry, as well as the presence of oxygen and pH probes that can disturb the radial flow (M. Liu, 2013). We dissociated RSC colonies from primary clonal spheres and then cultured the cells at 7000 cells/ml density under high and low speeds (50 and 80 rpm) in 21% Oxygen, 5%CO_2, pH 7.3 and at a temperature of 37ºC in a DASGIP stirring bioreactor vessels (Cat. No. SR0250ODLS, Eppendorf). Thirty percentage of fresh media and growth factors were added every two days (30% semi-fed batch). Other studies previously have illustrated the advantages of using semi-fed batch (i.e. adding fresh media and growth factors regularly) over batch culture (maintaining the initial starting media inside the bioreactor for the entire experiment length without adding fresh media) on cell growth and proliferation (Kallos et al., 2003). Semi-fed replacement of culture media will ensure an adequate supply of nutrients as well as removal of any by products or cytokines that can negatively affect cell growth and proliferation (Kallos et al., 2003). In our study, samples were taken every 2 days over the 6-day culture period. The average percentage of viable cells for each sample from the three experiments is shown in Figure 2.1A. We observed less viability in cells grown under high agitation compared to those cultured under low agitation or in a static culture, which can be attributed to the effects of shear stress on the cells in the high agitation culture in addition to the negative effects of the dissociating enzymes. We dissociated the cells from each bioreactor sample and cultured them at clonal density in a humidified static incubator for 7 days (tertiary clonal assay) (Coles et al., 2008; Tropepe et al., 2000) in order to calculate the numbers and percentages of RSCs. Although single RSCs start clonal sphere growth, the percentage of RSCs in a sphere is very low; the vast majority of sphere cells are retinal progenitor cells with less proliferative and differentiation abilities. The average tertiary sphere percentages from all samples (Day 2,4 and 6) were significantly higher at 50 rpm 0.01 ± 0.00182 compared to 80 rpm (0.00133 ± 0.00023), but the numbers of RSC spheres produced under 50 rpm conditions were not statistically greater than those seen in the static control cultures (0.00653 ± 0.00323) (Figure 2.1B)**.** The percentages of clonal RSC spheres of total cells cultured per day are shown in Figure A-1. We conclude that culturing RSCs under the higher agitation rate of 80 rpm negatively affected cell viability and the number of tertiary

RSCs. Thus, 50 rpm was chosen as a preferred stirring speed for all of the following experiments.

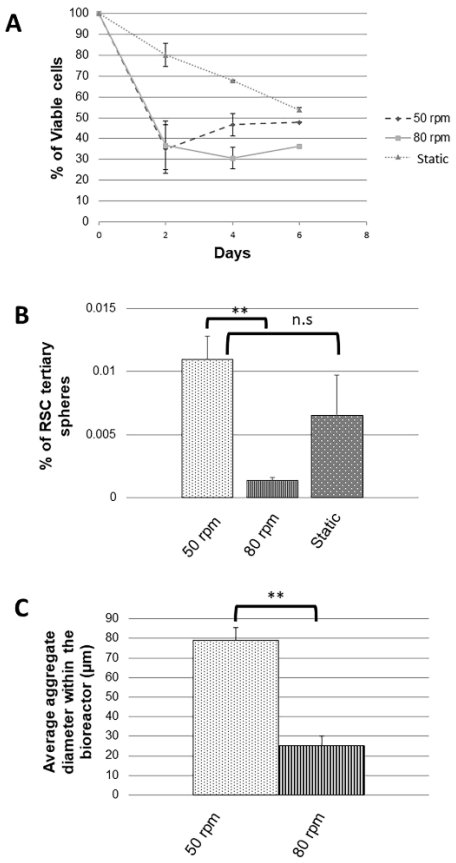

Figure 2.1: Viability of RSCs and their progeny in spinning suspension (50 rpm and 80 rpm) vs stationary cultures.

(A) Average percentage of viable cells cultured in a stirring suspension bioreactor for 6 days under two different agitation rates compared with static control culture. (B) Average percentage of tertiary sphere forming cells in 50 rpm and 80 rpm bioreactors versus static culture. Average tertiary sphere percentages from all samples (day 2,4 and 6) were significantly higher at 50 rpm compared to 80 rpm ($p=0.02$, $t=3.731$, $df=4$, $n=2$) but not to the static control culture ($p= 0.4407$, $t=0.855$, $df=4$). Each bar represents the average percentage of RSCs retrieved from Day 2, 4 and 6 samples in each experiment from $n= 2$ individual bioreactor experiments and $n= 8$-24 technical replicate per sample in each experiment. 14 eyes from 7 mice were dissected for primary spheres growth to seed each bioreactor). (C) Average diameter of aggregates formed inside the bioreactor at both speeds on day 6. Average aggregate diameter was significantly smaller in 80 rpm cultures compared to 50 rpm cultures ($p< 0.004$, $t=5.76$, $df=3$). Results represent the means $n= 2$ individual bioreactor experiments per condition and $n= 2$-9 technical replicate in each experiment) ± SEMs (**$p< .01$).

2.4.2 Hypoxia (5% O_2) increased the survival and the symmetric division of RSCs with no effect on proliferation rate

Next, we examined the effect of different oxygen concentration on RSC growth and proliferation in stirring suspension bioreactor cultures. RSCs within their niche reside in relatively hypoxic conditions (1–5% O_2) (Panchision, 2009). Low oxygen tension is critical for multiple stem cell types to maintain their self-renewal and proliferation (Cheung & Rando, 2013). Hypoxia also is known to stabilize Hypoxia Induced Factor alpha (HIF-1), which in turn activates genes that are implicated in many different cellular functions such as cell survival, cell proliferation, self-renewal, apoptosis, glucose metabolism and angiogenesis (Ke & Costa, 2006). To investigate the effect of relative hypoxia on RSCs, we dissociated primary clonal retinal and transferred them to a Dasgip bioreactor system. The average tertiary sphere percentages (day 2, 4 and 6) from aggregate samples were 0.01%±0.00311 in 5% SSB, 0.0018% ±0.0009 in 21% SSB and 0.0014% ±0.00088 in the static cultures (Figure 2.2A). The tertiary clonal sphere percentages from aggregate samples only per day are shown in Figure A-3A, and data from single cell samples only are shown in Figure A-3B. The average tertiary sphere percentages (day 2, 4 and 6) from aggregate and single cells samples were (0.0068%±0.00197) in 5% SSB, (0.0013% ±0.00055) in 21% SSB and (0.002% ±0.00099) in the static cultures (Figure 2.2B). By culturing in SSB and hypoxia we recovered 5 times more clonal spheres compared to 21% SSB and 7 times more than that which was obtained from 21% static cultures. No significant difference was observed between the numbers of tertiary clonal spheres derived from 21% SSB and 21% static cultures. We conclude that culturing in relative hypoxia was associated with a significant increase in clonal stem cell yields in both static and bioreactor cultures. We suggest that relative hypoxia could have exerted this effect through one or more of the following mechanisms: improving cell survival, enhancing proliferation or increasing the symmetric division of RSCs.

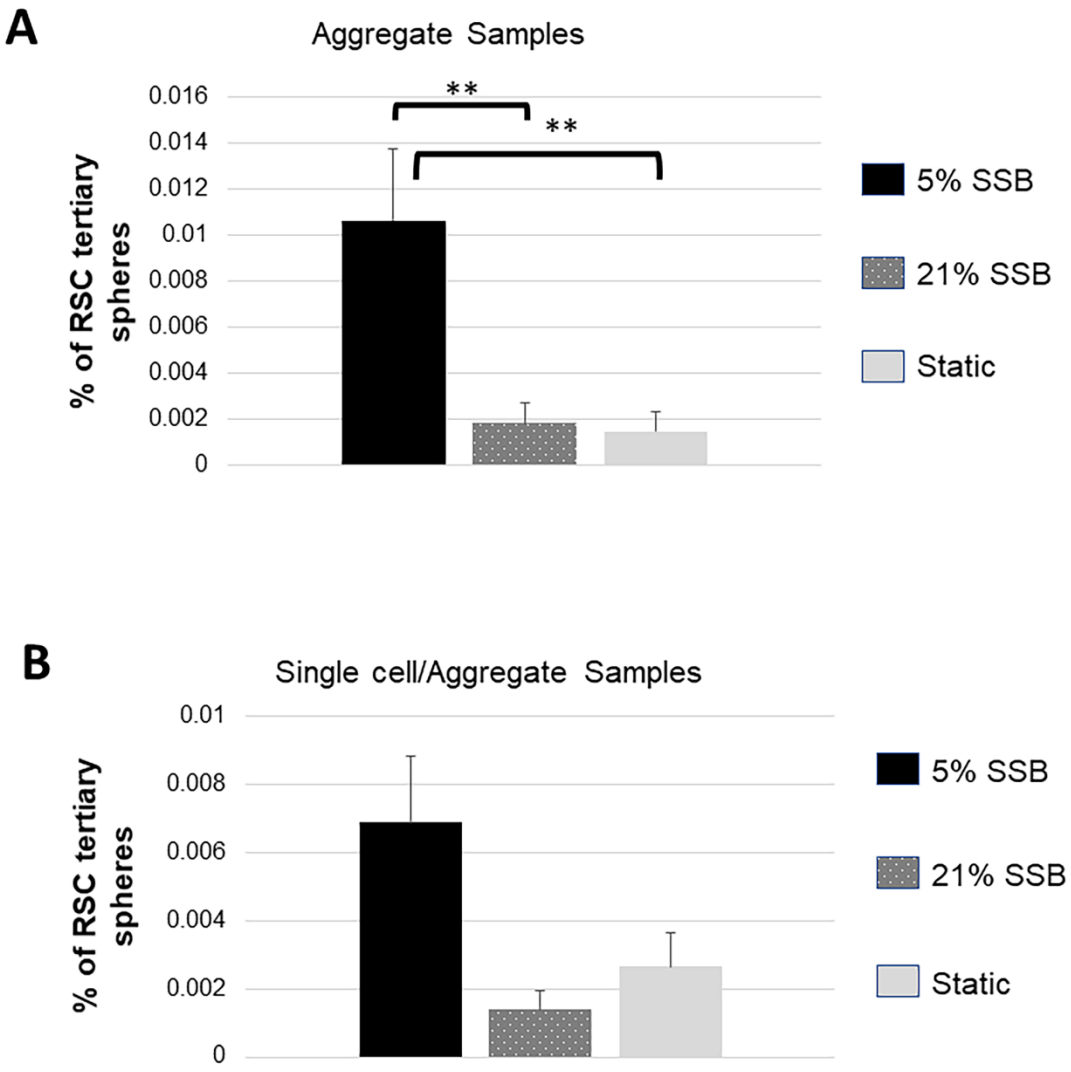

Figure 2.2: Tertiary clonal RSC sphere assays.

Cells from bioreactor and static culture samples were dissociated and plated at 10000 cells/500µl/well in serum free media, FGFB and Heparin for 7 days in 21% oxygen static humidified incubator. (A) Average percentage of clonal sphere forming cells in 5% SSB, 21% SSB and 21% static culture. Culturing in SSB and hypoxia we resulted in a 5-fold increase in spheres compared to 21% SSB ($p=0.0265$, $t=2.719$, $df=4$, $n=3$ individual bioreactor experiments) and 7-fold increase when compared to 21% static culture ($p=0.0235$, $t=2.837$, $df=4$, $n=2$-3 individual bioreactor experiments). Results represent the means ±SEMs. Each bar represents the average percentage of RSCs retrieved from Day 2, 4 and 6 samples in each experiment from $n= 2$ individual bioreactor experiments and $n= 8$-24 technical replicate per sample in each experiment. Fourteen eyes from 7 mice were dissected

for primary spheres growth to seed each bioreactor). (b) Average percentage of RSC clonal spheres from aggregate and single cell samples. Average tertiary spheres percentage (days 2, 4, and 6) from aggregate and single cell samples in 5% SSB, 21% SSB, and static culture.

To further analyze the effect of relative hypoxia on the survival of RSCs, we tested the viability of single cell and aggregate samples every two days using Ethidium homodiamer-2 and Hoechst staining. The average 5% SSB viability (single cells + aggregates) over 6 days was similar to 21% SSB culture and 21% static culture (77.54% ± 15.15, 77.11% ± 9.53 and 62.29% ± 12.31, respectively) (Figure A-2A). On the other hand, there was 1.5-fold increase in the viability of the cells in the aggregates samples in hypoxia (Figure A-2B) compared to normoxia in SSB cultures. To investigate the effect of 5% oxygen on the proliferation of RSCs, cells density was measured every two days using trypan blue and a heamocytometer for both single cell and aggregate samples. The growth rate chart showed non-significant differences between 5% and 21% SSB with expansion of 12.9 ± 3.31 and 12.92 ± 1.59-fold, respectively (Figure 2.3A and 2.3B). The average sphere diameters also were similar at 70µm ±8.08 in 5% SSB, 81.33µm ± 5.81 in 21% SSB and 86.66µm ±6.65 in static culture (Figure 2.3C). To further test the proliferative capacity of RSCs in hypoxic and normoxic cultures, the population doubling rate for each condition was calculated using this equation: N=[log(NH)-log(N1)]/log (2) where N = population doublings, N(H) = cell harvest number, and N(I) = plating cell number. Population doubling time (PDT) was calculated at each passage, PDT= time of culture (hrs)/N, where N = population doubling level. Minimal differences were observed between normoxic and hypoxic cultures suggesting that relative hypoxia did not affect the proliferation of RSCs and their progeny. To further document this observation and test the alternative explanation of increased survival in hypoxia, hypoxic and normoxic RSC spheres were inoculated with Edu 24 hours post culture initiation. The percentages of cells labeled with Edu were determined using a click-it kit (Invitrogen, cat# C-10420) 3 hr post inoculation. Edu incorporates into DNA during S-phase and labels all dividing cells (Figure **2.**4A). No significant difference was seen in Edu labeling between normoxia and hypoxia.

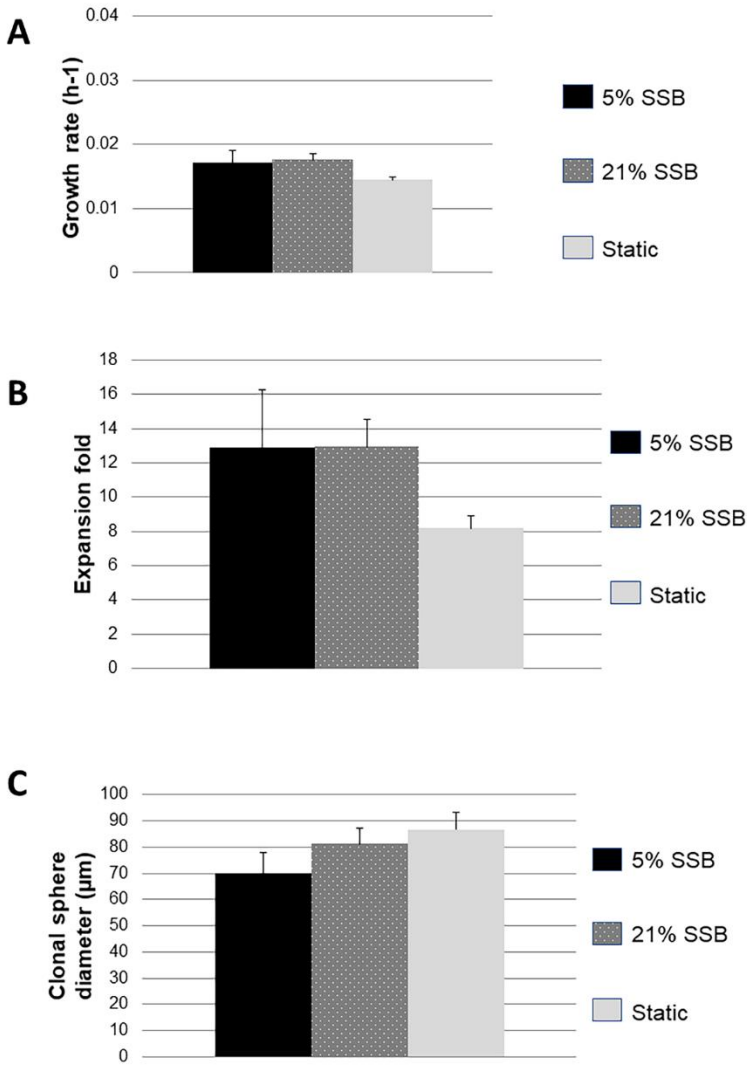

Figure 2.3: Growth Kinetics of RSCs grown in hypoxic and normoxic SSB, and static conditions.

(A) Growth rate. (B) Fold expansion. (C) Average clonal sphere diameter for tertiary spheres derived from 5% SSB, 21% SSB and 21% control static culture. Results represent the means of *n*=2-3 of individual bioreactor experiments ± SEMs.

To investigate if hypoxia affects the mode of RSC division, we added the Notch receptor ligand Delta3 (Dll3) to tertiary RSCs grown from both hypoxia and normoxia in static cultures (Figure **2.**4B). Delta 3 previously was shown to increase RSC symmetrical division in adult and PND2 RSCs, and a number of previous studies have also indicated its critical role in maintaining the precursor cell pool in the developing eye (Artavanis-Tsakonas, Rand, & Lake,

1999; Balenci & van der Kooy, 2013; Jadhav, Cho, & Cepko, 2006). Dll3 increased the number of clonal RSCs by 2.4 times when added to cells from normoxic cultures (0.875 ±0.144 in normoxia alone and 2.14 ± 0.340 in normoxia + Dll3). When added to cells from hypoxia, Dll3 caused a non-significant increase in the number of RSCs compared to hypoxic culture alone (hypoxia 1.54±0.110, hypoxia + Dll3 1.25 ±0.25). We suggest that hypoxia already had increased symmetric divisions of RSCs and that adding Dll3 had no further effect on enhancing symmetric divisions.

From the above observations, we conclude: 1) relative hypoxia did not increase RSC and progenitor proliferation compared to normoxia and 2) relative hypoxia improved cell survival and facilitated RSC symmetrical division. However, RSCs are quite rare cells in our cultures and thus differentiating selective survival versus symmetric proliferation effects on specifically RSCs remains difficult. In light of the above results, relative hypoxia (5% O_2) will be used for all of the following experiments.

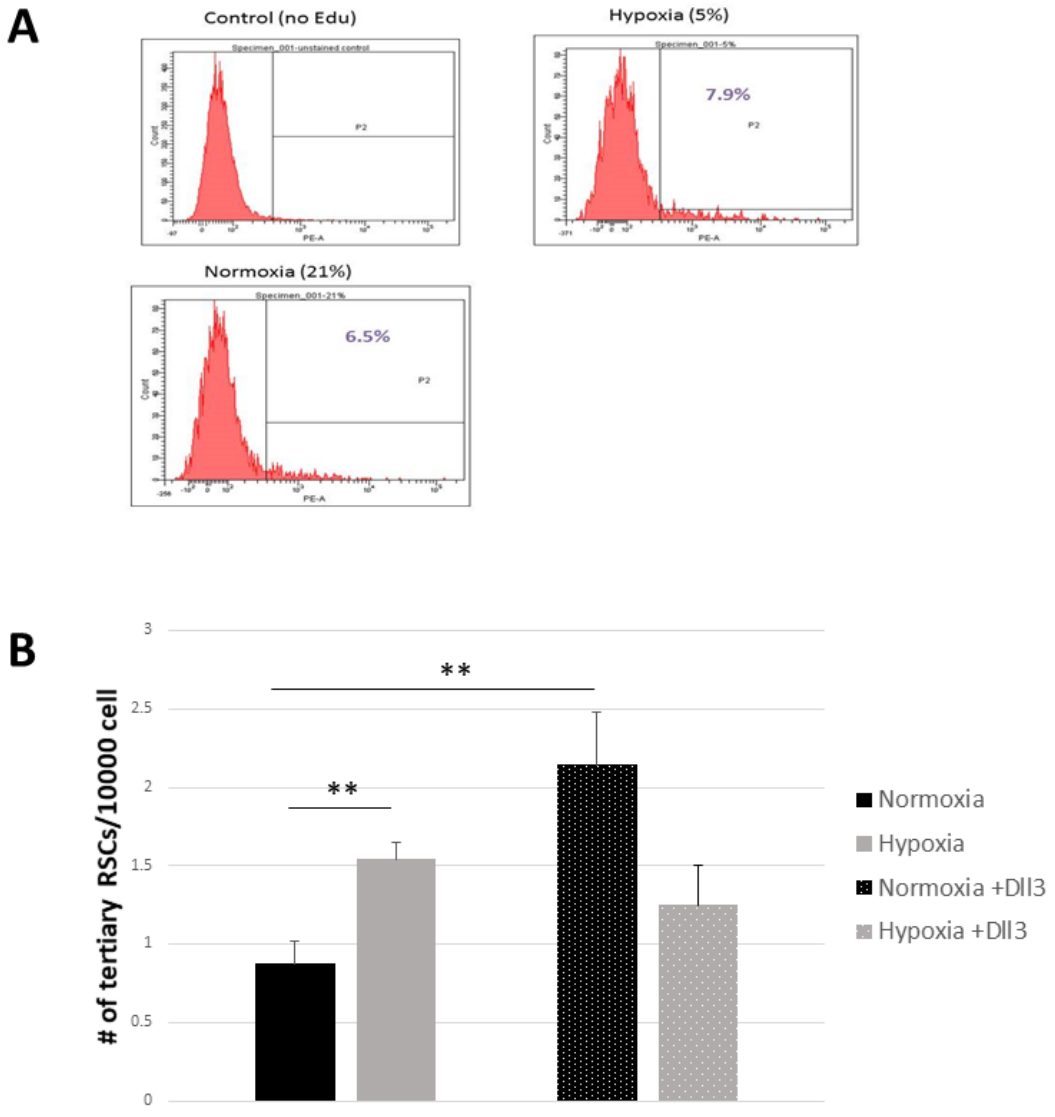

Figure 2.4: Effects of hypoxia on RSCs (symmetrical division rather than proliferation).

(A) Flow cytometric analysis of adult RSC cultured in hypoxic (5%) and normoxic (21%) oxygen. No significant difference in Edu expression (red) was seen between 5% and 21% oxygen cultures. Data shown were gathered on a BD LSRFORTESSA flow cytometer. (B) Effect of adding Dll3 to tertiary RSCs cultured in normoxia and hypoxia. We found 2.4 times increase in the percentage of clonal RSCs when Dll3 was added to normoxic culture ($p=0.002$, $t=4.719$, $df=7$, $n=3$) and 1.7 times when we cultured in hypoxia. Adding Dll3 to hypoxic cultures produced no differences in RSC percentages, $p=0.402$, $t=0.891$, $df=7$, $n=3$. Results represent the means ±SEMs. *n*= number of biological replicates.

2.4.3 A specific cell microcarrier resulted in a 5-fold increase in clonal RSC spheres compared to SSB alone and a 10-fold compared to static culture

To expand the RSCs using MCs, we conducted a screening experiment using three different coatings: FACTIII (cationic charged collagen), ProNectin-F (recombinant protein coated) and HillexII (modified polystyrene) purchased as a starter kit from Solohill. We observed that the viability in all microcarriers stayed above 80% throughout the culture period. FACTIII and HillexII had similar growth rates and expansion (Table 2.1).

Table 2.1: Retinal stem cell growth compared using three different microcarrier types.

	FACTIII	**ProNectin F**	**HillexII**	**Control**
Maximum cell density (cell/ml)	495000 (day2)	275000 (day2)	267500 (day6)	207500 (day6)
Viability percentage	81.47%	81.03%	74.76%	83.66%
Attachment percentage (Day4)	75.13%	41.88%	51.75%	
Average specific growth rate	0.0174	0.015	0.0179	0.0162
Doubling time (hours)	39.82	46.2	38.7	42.77
Expansion fold	12.37	8.8	13.37	10.37
Number of tertiary RSCs/10000 cell	0.442	0.246	0.193	0.193

These values were higher than those observed in Pro-nectinF and control culture. FACTIII reached its maximum cell density on day 2 (495000 cells/ml) and had a higher attachment with an average of 75.13% of cells attached compared to other types of microcarriers. The average clonal RSC percentages from cells taken from days 2, 4 and 6 were 0.0022% ± 0.00046 in FACTIII cultures, 0.001 % ± 0.00042 in FNectin, 0.0009% ± 0.00028 in HillexII and 0.0009% ± 0.00028 in static control (Figure 2.5A). Using FACTIII in shaking static culture produced 2.4 times the RSCs compared to static culture. Thus, the following bioreactor experiments used the FACTIII MCs, as they were shown to affect most positively RSC attachment and expansion. We then tested the effects of agitation mode, seeding density and different dissociation protocols on RSCs seeded on the FACTIII MCs and compare them to the Stirring SSB alone and our static control cultures. We found that the average percentage of RSCs (day 2, 4 and 6) was higher in continuous agitation culture compared to intermittent agitation with 0.01 ±0.00225 and 0.00638 ±0.00144, respectively (Figure 2.5B). More clonal RSC spheres were observed in the continuous agitation culture on day 4, but there were not significant differences on days 2 or 6 (Figure A-4B). We also observed 3 times more clonal RSC spheres (as a % per total number of cells) arising from high-density culture (0.02911±0.0009) compared to low-density culture (0.01 ± 0.00225) (Figure 2.5C). In all experiments, culturing in higher density (50000 cell/ml) gave better RSC yields. Also, a higher but not significantly different frequency of RSCs for the same numbers of cell plated was retrieved from 10xTryplE experiments (0.0351 ± 0.0169) compared to 1x TryplE (0.0291 ±0.00371) (Figure 2.5D). Given that no significant difference was observed in the total cell expansion between the two conditions (Figure A-5A) we used 10x TryplE for the following experiments.

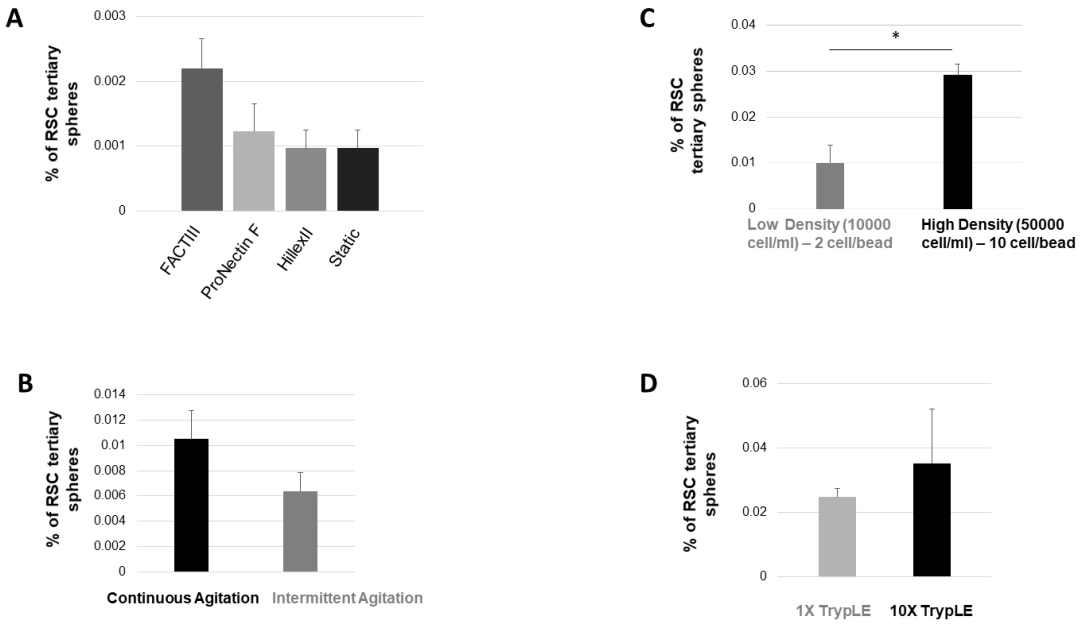

Figure 2.5: Microcarrier screening and testing under different agitation and oxygen tension.

(A) Average tertiary sphere forming cell percentage. Primary RSCs were dissociated and seeded on different types of microcarriers (FACTIII, NectinF and HillexII, Solohill INC) in 6 wells plate placed on a shaker adjusted to a speed of 50 rpm in 21% oxygen humidified incubator. A static culture without MCs was used as a control. (B) Percentage of clonal tertiary sphere forming cells: continuous agitation versus intermittent agitation. Average percentages of RSCs were higher in continuous agitation culture compared to intermittent agitation. (C) Percentage of clonal tertiary sphere forming cells: effect of seeding density (high vs low) on growth and behavior of RSCs. We observed 2.5 times more RSC spheres arising from high density culture compared to low density culture ($p=0.0262$, $t=2.731$, $df=4$, $n=2$-4). (D) Effects of cell detaching enzymes (1xTryplE vs 10x TrylE) on growth and behavior of RSCs. A non-significant RSC percentage increase was observed in the 10xTryplE experiment compared to 1x TryplE ($p=0.365$, $t=0.394$, $df=2$). Results represent the means ± SEMs.

We then studied the effects of using FACTIII MCs in hypoxia, high density, continuous agitation and 10x TryplE on RSCs expansion, Static and SSB preparations without MCs were used as controls. Samples were taken every two days to monitor cell viability (Figure 2.6A**);** SSB and static samples were dissociated using a Trypsin/Collagenase enzymes mix, while MCs were treated with 10x /TryplE. 1x and 10x TryplE failed to dissociate spheres from static and bioreactor samples, but did detach cells from MCs. Using collagenase enzyme to detach

cells from MCs was associated with much lower stem cell yields than 1x and 10x TryplE (data not shown). Cells from each sample were then seeded at 10000 cells/500µl/well in a 5% oxygen and 5% CO_2 humidified incubator for 7 days for clonal sphere assays. The overall viability of the cells on the MCs was higher compared to SSB and static cultures (Figure 6A). The use of MCs resulted in 5 times more tertiary RSCs compared to hypoxic SSB and 10 times more tertiary RSCs in comparison to normoxic static culture (Figure 2.6B, C and D), with the most RSC spheres observed from the day 4 samples (Figure A-6). We noticed that in MC experiments the numbers of stem cell spheres decreased between days 4 and 6 (Figure A-6A and B). Potential explanations for this observation are 1) an increase in toxic metabolites or a decrease in nutrients in the media between days 4-6, 2) more cell expansion was seen on day 4 which could have negatively affected the stem cells through contact inhibition (Figure A-7), or 3) RSCs could have increasingly divided asymmetrically between days 4 and 6. We conclude from the above results that continuous agitation, higher density culture, 10xTryplE (or 1x TryplE) and 5% O_2 increased the expansion of retinal stem cells over static culture.

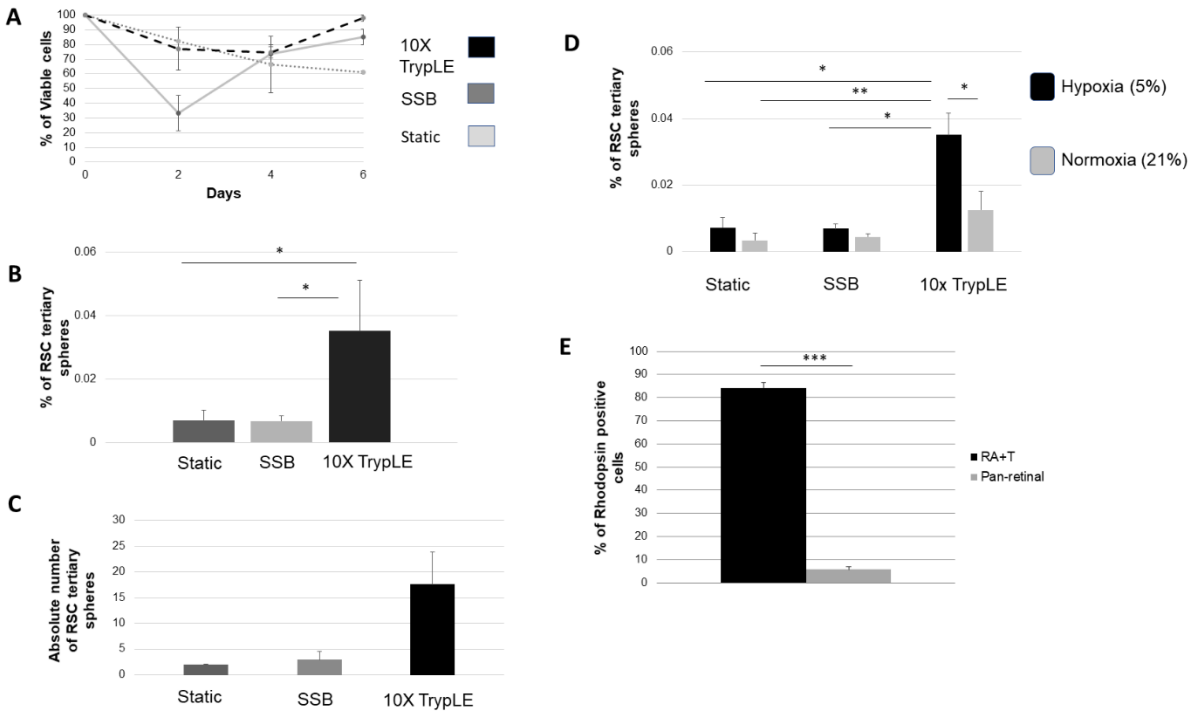

Figure 2.6: Comparison between RSCs expansion in static, SSB and MC cultures.

(A) Viability of cells inside MC, SSB and static culture. (B) The average numbers of clonal tertiary RSCs as percentages of total viable cells in different platform. (day 2, 4 and 6). MC experiment produced 5 times more tertiary RSCs compared to hypoxic SSB and 10 times more when compared to normoxic static culture, p values 0.0327 (t = 3.715, df = 2) and 0.02 (t = 3.92, df = 4) respectively. Each bar represents the average percentage of RSCs retrieved from Day 2, 4 and 6 samples in each experiment from two individual bioreactor experiments (n = 2 individual bioreactor experiments, n = 8-24 technical replicate per sample in each experiment). 15-17 mice (30-34 eyes) were dissected for primary spheres growth to seed each individual experiment. Results represent the means ± SEMs. (C) Average total number of RSCs in each condition. (D) Average numbers of clonal tertiary RSCs as percentages of total viable cells observed in microcarrier, bioreactor and static culture in hypoxia vs normoxia. E) Differentiation of RSC derived from hypoxic bioreactor culture in Taurine and Retinoic acid for 50 days compared to pan-retinal control RSCs differentiated into rods using a protocol developed in the lab (Ballios et al., 2012). The percentage of rod photoreceptors (stained for anti-Rhodopsin antibody) was 84.31 ± 2.08 in taurine and retinoic acid, significantly higher (p = 0.00052, t = 31, df = 2) compared to 5.82% ± 1.23% rhodopsin positive cells in pan-

retinal differentiation control cultures. Results represent the means of *n* = 3-5 biological replicates ± SEMs.

2.4.4 Bioreactor derived RSCs differentiate effectively to rod photoreceptors using Retinoic acid and Taurine

We then cultured bioreactor derived spheres on laminin in retinoic acid and taurine to test their differentiation potential. The percentage of rod photoreceptors (stained for anti-Rhodopsin Immunocytochemistry) was 84.31 ± 2.08 (Figure 2.6E) in taurine and retinoic acid, and significantly different (*P*=0.00052, *n*=3-5) compared to 5.82% ± 1.23% rhodopsin positive cells in pan-retinal differentiation control cultures. These results were similar to previous published data from our lab growing rod photoreceptos from adult derived mouse RSCs using retinoic acid and taurine (Ballios et al., 2012).

2.5 Conclusion

The present results provide an RSC expansion protocol using MCs in SSB to overcome problems associated with growing stem cells in static culture. We found that our bioreactor expansion protocol enhanced RSCs survival through optimizing culture conditions and using less aggressive MC detaching enzymes. Using FACTIII MCs in hypoxia increased the yield of RSCs 10 times compared to static normoxic culture conditions. We found that hypoxia (5% O2) was associated with better RSC expansion across all platforms; and this can be attributed to hypoxia-induced increase in survival and/or symmetric division of stem cells. RSCs within their niche *in vivo* reside in relatively hypoxic conditions (1–5% O2) (Panchision, 2009). A number of previous studies has shown that low oxygen tension is important for multiple stem cell types to maintain self-renewal and proliferation of the resident precursor cells (Cheung & Rando, 2013), including in the retina (Bae et al., 2011; DiStefano et al., 2018; Garita-HernÁndez et al., 2013). For example, earlier formation of neural rosettes was observed under hypoxia in addition to higher number of Pax6/ Chx10 positive retinal precursor cells in cultures derived from human ESCs and iPSCs embryoid bodies (EBs) in a static culture (Bae et al., 2011). Other studies also observed better growth of retinal organoids in hypoxia, both in stirring bioreactor (DiStefano et al., 2018) and static cultures (Chen, Kaya, Dong, & Swaroop, 2016). Several studies showed that hypoxia exerts this effect by stabilizing hypoxia-induced factor 1 alpha (HIF-1), which in turn activates genes that are implicated in many different cellular functions such as cell survival, proliferation, and selfrenewal (Ke & Costa, 2006). HIF up-regulates the Notch signaling pathway and its downstream cascade including Hes genes (Gustafsson et al., 2005; Irshad et al., 2015). Our previous demonstration that the

Notch ligand Delta 3 increased symmetric divisions by both adult and PND2 RSCs also has highlighted the critical role of Notch signaling in maintaining the precursor cell pool in the developing eye (Artavanis-Tsakonas et al., 1999; Balenci & van der Kooy, 2013; Jadhav, 2006). We suggest that hypoxia activated Notch signaling and its downstream Hes pathway through stabilizing HIF1 alpha, and this may explain why adding Dll3 to our hypoxic culture conditions did not produce any further expansion of the RSCs through symmetric divisions. In the future, it will be important to target the differentiation of RSCs and their progeny toward rod and cone photoreceptor phenotypes using FACTIII MCs inside bioreactors to expand their populations in order to produce large numbers of cells needed for transplantation. A similar expansion of human RSCs may allow us to differentiate large numbers of postmitotic cells (rods, cones, and RPE) that can be transplanted to patients with degenerative eye diseases.

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Chapter 3: A Subset of Clonal Retinal Stem Cell Spheres is Biased to RPE Differentiation

This chapter has been submitted for publication.

3.1 Abstract

Retinal stem cells (RSCs) are rare pigmented cells that reside in the pigmented ciliary epithelial layer of the peripheral retina of the eye in mouse as well as in adult human (Coles, Angenieux, et al., 2004; Tropepe et al., 2000). Previous studies have shown that RSCs are able to replicate to maintain the stem cell pool, in addition to producing retinal progenitors which can later differentiate into all neural retinal (NR) cell types and retinal pigment epithelial (RPE) cells (Ahmad et al., 2000; Ballios et al., 2012). *In vitro,* RSCs can proliferate to make floating clonal spheres of several thousand cells by 7 days of culture. Based on their levels and spatial distributions of pigment, RSC clonal spheres were classified into three types: heavily pigmented (HP), lightly pigmented (LP) and centrally pigmented (CP) spheres. We report here that the CP spheres (27% of all clonal spheres) are capable of generating large cobblestone lawns of adjacent cells, which can differentiate into RPE cells. The other two clonal sphere types (HP and LP) give rise to many cells with neural morphology but relatively few RPE cells. We find that the individual stem cells (cells that clonally formed the three sphere types) are homogeneous, but that their downstream progenitors are different. We suggest that the CP spheres contain highly proliferative RPE specific progenitors. Indeed, investigations of the intrinsic and extrinsic differences between the different sphere types revealed that only the CP spheres contained a population of early RPE progenitors that respond to proliferative signals from the surrounding non-pigmented retinal cells. The HP and LP spheres on the other hand contain late RPE progenitors which are not affected by these proliferative signals. Thus, early RPE progenitors from CP clonal spheres are intrinsically different, but their ability to make large numbers of differentiated RPE cells depends on extrinsic signals from the surrounding non-pigmented retinal progenitors.

3.2 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 RSC population expands and starts producing retinal progenitors, 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 & Wallace, 2012; Cepko, 2014; Dyer & Cepko, 2001; Prada et al., 1991; Wong & 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 & Cepko, 1988; Wetts & Fraser, 1988). This process was found to be governed by both intrinsic and extrinsic signals *in vivo* (Rompani & 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 at the periphery of the eye (Ahmad et al., 2000; Tropepe et al., 2000). This population can be distinguished from the surrounding pigmented ciliary epithelium by their size (large) and the level of pigmentation (heavily pigmented) (Ballios et al., 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% of pigmented CE cell cultures, either as a single cell per well or at low cell densities (< 20 cells/ µl), produced clonal spheres after 7 days containing both pigmented and non-pigmented cells (Tropepe et al., 2000). The same study showed that many non-pigmented cells within the RSC colony express *Chx10* and nestin before differention (Tropepe et al., 2000). *Chx10* is a specific marker for 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., 2018a), respectively. Larger numbers of RPE cells could be produced from adult RSCs by adjusting culture density and media conditions (De Marzo, Aruta, & Marigo, 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 stochasticity (Cayouette et al., 2003; Cepko, 2014; Fekete et al., 1994; Harris, 1997; He et al., 2012; Trimarchi et al., 2008; Turner & Cepko, 1988; Wetts & 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 müller glial cells born last (Bassett & Wallace, 2012; Cepko, 2014; Dyer & Cepko, 2001; Prada et al., 1991; Wong & 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 & 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: heavily pigmented (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 & LP spheres. RPE cells are important to maintain healthy and functioning photoreceptors. Loss of RPE cells will consequently result in loss of photoreceptors and subsequently blindness (Hollyfield & Witkovsky, 1974; Miller, Bagheri, & Vavvas, 2017; Mitrousis et al., 2020). We suggest that the highly proliferative population in the CP spheres will provide a pure and safe source to replace lost RPE in retinal degenerative diseases.

3.3 Methods

3.3.1 Mouse and human eyes source

RSCs were derived from the ciliary epithelium of adult (7 – 8 week-old) C57BL/6 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 were procured from the Eye Bank of Canada (Toronto, ON) within 24h postmortem (and approved of by the UofT Human Research Ethics Board).

3.3.2 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) and plated the cells 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.

3.3.3 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. Refer to Figure A-16, 17 and 18 for sphere type examples. 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).

3.3.4 Sphere Sections

Spheres were collected into 1.5mL microcentrifuge tube in minimal volume (under 20µl), fixed by adding 200uL of fresh 4% PFA for 10 minutes then centrifuged and supernatant was removed. Spheres were kept in 500uL of 20-30% sucrose at 4 ºC 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 ºC Freezer spheres until ready for sectioning. Sphere sections (10 µm) were prepared on a cryostat (-20 ºC).

3.3.5 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+).

3.3.6 RSC Differentiation and Immunostaining

Mouse RSC clonal spheres (CP, HP and LP) were picked individually and plated on laminin in a humidified 5% CO_2 incubator and differentiated using pan-retinal differentiation conditions that involved 1% FBS + FH for 21 days. 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.

3.3.7 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, US) were used: Claudin-1 (Mm00516701_m1), Occludin (Mm00500912_m1), Integrin 5 alpha (Mm00439797_m1) and Cdh1 (E- cadherin: Mm01247357_m1).

3.3.8 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.

3.3.9 Statistics

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 $p < 0.05$.

3.4 Results and discussion

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

Adult mouse clonal RSC spheres were classified based on the distributions and levels of pigmentation observed into heavily pigmented (HP), lightly pigmented (LP) and centrally pigmented (CP) spheres (Figure 3.1A and B). The same three clonal retinal sphere types also were grown from embryonic day 14 (E14) mouse presumptive CE with differentiation morphologies similar to those observed with adult clonal spheres (Figure 3.1C). 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 (Figure 3.1D).

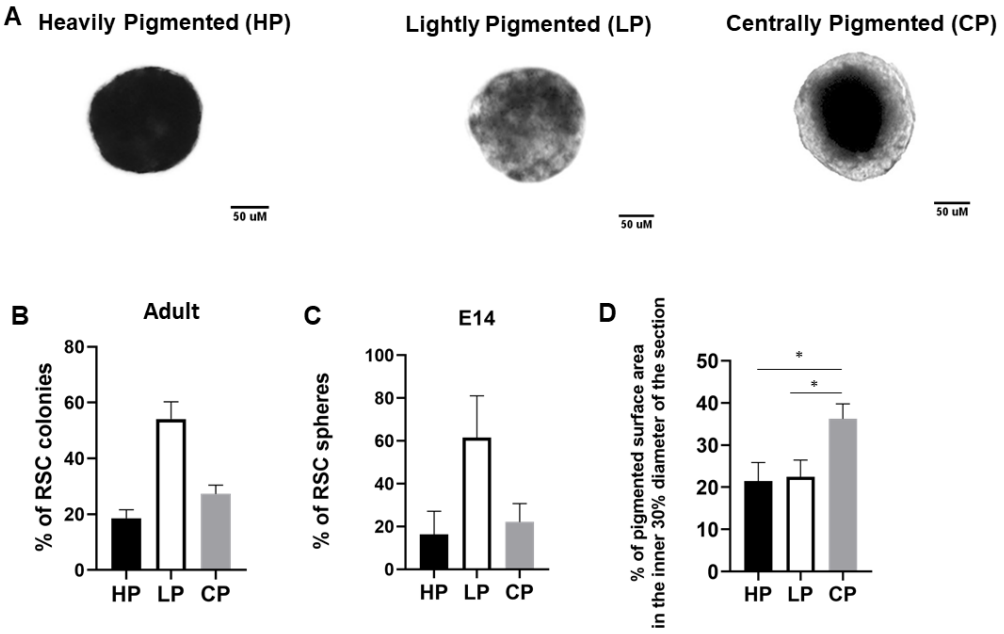

Figure 3.1: Three types of clonal RSC spheres can be isolated from adult and E14 pigmented ciliary epithelium.

(A) Types of retinal stem cell clonal spheres based on their pigmentation levels and distributions. 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) . Data represent means ± S.E.Ms.

CP spheres (27±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 neural retinal (NR) cells (Figure 3.2A). In comparison, HP (18±3% of all clonal spheres) and LP (54 ± 6% of all clonal spheres) colonies produced more NR cells and only scattered pigmented cells (Figure 3.2B and C). 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 3.2D).

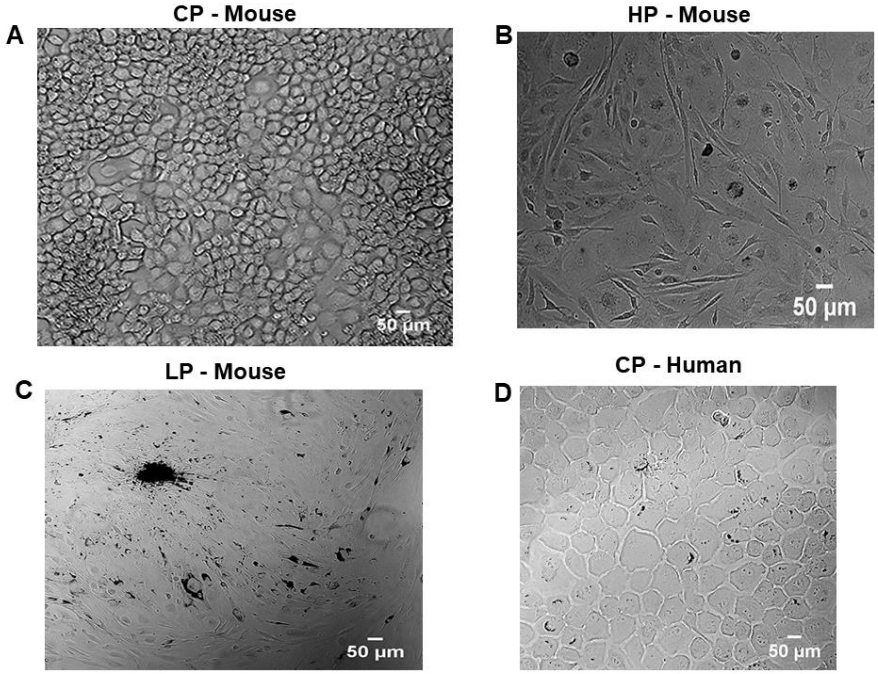

Figure 3.2: Centrally pigmented spheres produce large numbers of cobblestone cells resembling RPE morphologies.

(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.

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 (Figure 3.3A and B). 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.

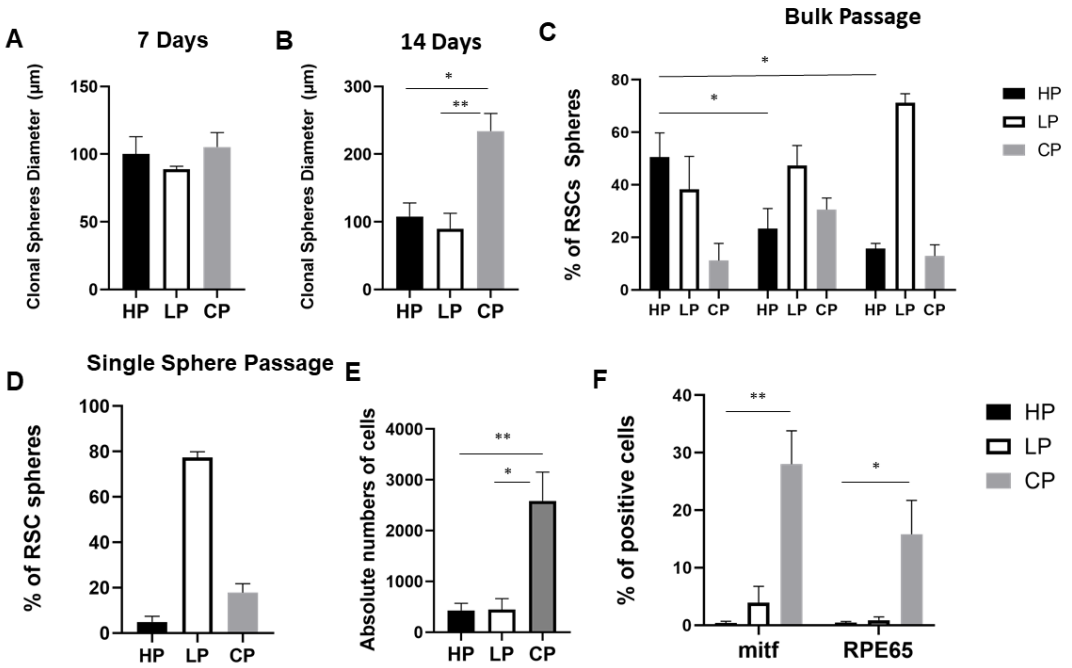

Figure 3.3: CP spheres express RPE markers and are more proliferative than HP and LP spheres.

(A), (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 that 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 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)**.** (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 nuclear staining, 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). Data represent means ± S.E.Ms.

3.4.2 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 sphere type could produce new clonal spheres of each of the three sphere types (Figure 3.3C), 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 3.3C). 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 Figure 3.1B and 3.3D) 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.

3.4.3 Cobblestone morphologies are observed only with differentiated CP spheres

Individual primary RSC spheres were differentiated in the presence of 1% FBS, 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 A-8). 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 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 A-9). 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.

3.4.4 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 ± 566, 446 ± 218 and 430 ± 140, respectively) (Figure 3.3E). We also found that more cells arose from CP spheres express the immature RPE marker MITF (28 ± 5%) compared to LP spheres (4 ± 3%) and HP spheres (0.388% ± 0.280). Differentiated CP spheres express RPE65 in 24 times more cells than LP and HP spheres (Figure 3.3F and Figure A-10A). The tight junction protein ZO-1 (zonula occludens-*1*), which is important for accelerating claudin polymerization/ assembly, binding to F-actin and initiating cell signalling (McNeil et al., 2006; Rizzolo, 2007; Shin & Margolis, 2006), was expressed in CP sphere cells but not in the HP sphere cells (Figure A-10A and B), 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 (Figure A-11A and B). 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. 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 favour RPE proliferation (Leschey, Hackett, Singer, & Campochiaro, 1990; Schönfeld, 2000; Spraul, Kaven, Lang, & Lang, 2004).

To investigate potential intrinsic differences between HP, LP and CP RPE progenitors, q-PCR 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 (Figure 3.4A and B).

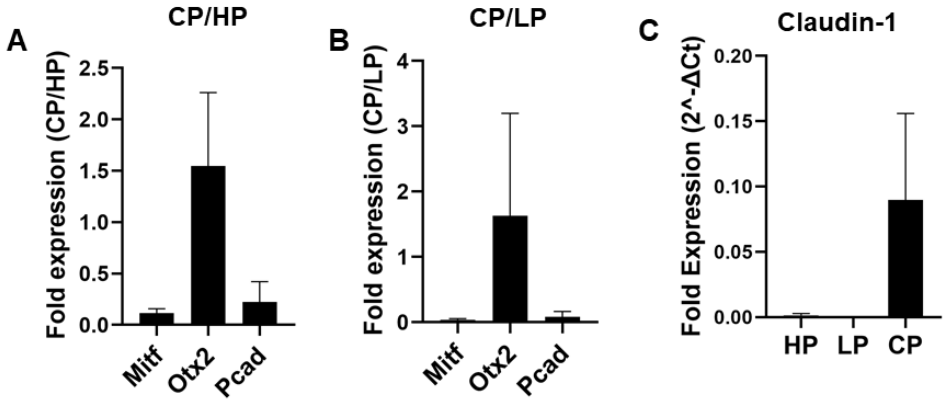

Figure 3.4: Pigmented cells in CP spheres express early markers of retinal differentiation.

(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.

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, *Otx*2 is initially expressed in the entire embryonic optic vesicle, but its expression soon becomes restricted to the presumptive RPE, where it is maintained throughout adulthood (B. M. Lane & 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 ECM 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 3.4C), but

knocking down claudin-1 did not result in a decrease in the types, numbers or sizes of the spheres compared to controls (Figure 3.5). q-PCR expression analysis confirmed that claudin-1 expression was 98% lower after our siRNA treatment compared to controls (Figure 3.5D).

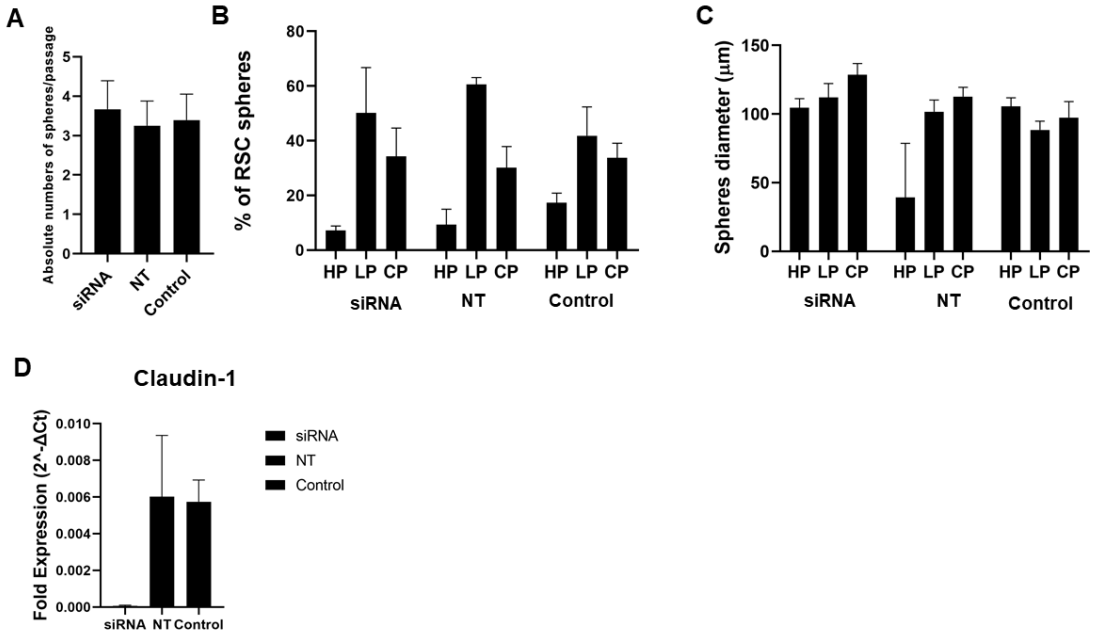

Figure 3.5: Knockdown of claudin-1 in CP spheres did not affect sphere diameters nor passaging.

(A) siRNA depletion of claudin-1 did not affect the numbers of passaged spheres compared to non-targeting (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-targeting siRNA controls.

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 knock-down experiment, the stem cell was capable of producing 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 A-12). 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.

3.4.5 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 neural retinal 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. 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 3.6A). 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 3.6B and Figure A-12). Further, these expansions of CP sphere pigmented cells were due to proliferation of the cells, as these cells were labelled with the proliferation marker Ki67 (Figure 3.6C)**.** To summarize, early RPE progenitors in CP spheres are responsive to signals received from non-pigmented cells derived from all sphere types.

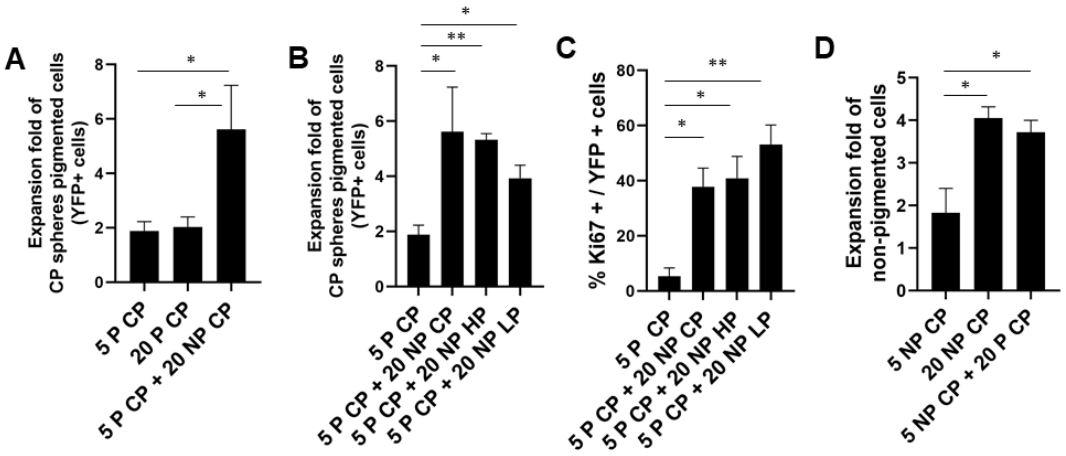

Figure 3.6: Early RPE progenitors in CP spheres proliferate in response to extrinsic signals received from the surrounding non-pigmented NR cells.

(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 labelled with the Ki67 proliferation marker. 5 pigmented CP cells co-cultured with C57BL/6 non-pigmented cells from CP, HP or LP spheres showed significant increases in Ki67 double labelling 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, $p < 0.05$). Data represent the means ± S.E.Ms. P = pigmented, NP = non-pigmented.

Non-pigmented neural retinal 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 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 3.4d), as reflected by higher cell expansion and positive Ki67 staining. (Figure A-13A and B). No changes in the Ki67 labelling 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 A-13C), HP (Figure A-14A) and LP (Figure A-15A), 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 neural retinal 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 (Figure A-14B and C and Figure A-15B and C). 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 neural retinal progenitors. Proliferation of adult RSC derived non-pigmented progenitors is independent of the pigmented RPE progenitors, and non-pigmented neural retinal progenitors appear to proliferate in response to factors secreted from other non-pigmented neural retinal cells in the cultures.

3.5 Conclusion

The present results reveal the heterogeneity of clones grown clonally from a homogeneous population of retinal stem cells. We were able to identify three types of clonal spheres that contain different populations of RPE progenitors both in mouse and human. Each sphere type was passaged into all sphere types indicating that the stem cells that formed the three sphere types are identical. We were able to identify two distinct RPE progenitor populations that express different levels of developmental genes and respond differently to extrinsic signals. Sphere clones of single RSCs that have their pigmented RPE cells segregated to the centers of spheres contain early RPE progenitors that selectively respond to proliferation signals from non-pigmented neural retinal progenitors. We suggest that these early RPE progenitors are caught in an adhesion network at the centers of the clonal spheres and have the ability to proliferate extensively and differentiate into RPE cells. Q-PCR analysis confirmed the expression of early genes responsible for RPE development and adhesion. Although early RPE progenitors in the CP spheres expressed high levels of claudin-1 gene, knockdown of claudin-1 using SiRNA did not affect the adhesion of pigmented cells at the centre of the sphere indicating that claudin-1 could be one part of a network of genes that can still function separately or that other tight/adherent junction genes are working alone to elicit this phenomenon. The other two sphere types (HP and LP) on the other hand, do not differentiate into cobblestone cells nor respond to proliferative signals from non-pigmented neural retinal cells. They also have low expression of adhesion genes and immature RPE markers. Further analysis of intrinsic gene expression and screening of proliferative factors will be needed to further charachterize the RPE progenitor populations.

Chapter 4: General discussion

4.1 Expansion of retinal stem cells

Adult RSCs are considered a safe source for retinal cells. The work in this thesis will promote better RSC expansion. To my knowledge and up to the time of writing, the protocol published in this thesis is the first and only protocol available to expand the number of RSCs from adult CE in a bioreactor system. Using microcarriers in bioreactor stirring suspension system, I designed an expansion protocol that was successful in enriching the numbers of RSCs 10 times the classic static culture. Expanded RSCs can be used to generate large numbers of retinal precursors that can be used for cellular transplantation.

4.1.1 Why do we need to expand RSCs?

Large number of photoreceptors and RPE are needed for cell replacement in degenerative eye diseases. Retinal degenerative diseases, including age-related macular degeneration and retinitis pigmentosa, are a major cause of blindness worldwide. In these neurodegenerative conditions, loss of photoreceptors leads to visual impairment. RSCs are considered a great source for cell transplantation, since they have the migratory capacity and cellular plasticity needed to integrate and differentiate in the diseased retina. Scientists faced many challenges in growing and transplanting retinal stem cells and their precursors. Limited availability of adult RSCs and their reduced proliferative potential is one of the major difficulties in translating such strategies into clinical applications.

4.1.2 Rational and challenges

The proliferation potential of CB-derived cells appears somehow limited, especially when compared with brain-derived stem cells (Moe et al., 2009; Yanagi et al., 2006) or retinal progenitor cells from neonatal retinae (Klassen et al., 2004; Merhi-Soussi et al., 2006). No more than 0.2% to 2% of CB-derived cells seem to be endowed with proliferative capacities (Ahmad et al., 2004; Tropepe et al., 2000). Also, their self-renewal and proliferation rates appear to gradually decrease with subsequent passages (Coles, Angenieux, et al., 2004; S. Xu et al., 2007).

Attempts to replace lost photoreceptors have been made using embryonic, fetal and post-natal retinal stem/progenitor cells. These studies have generated variable data but as a whole demonstrated rather limited levels of integration and/or photoreceptor differentiation (Djojosubroto & Arsenijevic, 2008; Lamba, 2012; Pellegrini, De Luca, & Arsenijevic, 2007; West, Pearson, MacLaren, Sowden, & Ali, 2009). Successful Integration depends on several factors including the condition of transplantation

environment and the age of transplanted precursors. Previous work in our lab showed that injecting human adult RSCs into neonatal animal eyes (which provides a more appropriate host environment compared to adult retinae) resulted in cell migration, integration, and differentiation towards retinal cell fates (Coles, Angenieux, et al., 2004). In one other study, murine adult RSCs were shown to adopt retinal phenotypes when transplanted into diseased retinae, but not in healthy ones (James et al., 2003). The optimal developmental time point for isolation of transplantable photoreceptor precursors in the mouse is around postnatal day 4 (Bartsch et al., 2008; MacLaren et al., 2006), which corresponds to the second trimester in humans, therefore the use of such primary cells from humans is substantially limited by ethical concerns. Thus, the expansion of cells *in vitro* that have the capacity to generate mature photoreceptors after transplantation and therefore provide an unlimited amount of donor material represents a vital step for future therapeutic application.

Although pluripotent stem cells are considered a source for potentially infinite number of cells, RSCs derived from ESCs and iPSCs need to be assessed for long term safety due to the associated risk of tumorgenicity along with ethical concerns related to sourcing of ESC (Locker, El Yakoubi, Mazurier, Dullin, & Perron, 2010a). Urging the need to develop and establish protocols to expand RSCs of adult tissue origin.

Several methods have been used to expand the number of RSCs since their discovery in 2000. Most of the protocols were conducted in static (monolayer) culture. In this thesis, we wanted to grow RSCs in 3D culture and harness the benefits provided by bioreactor systems. I developed a protocol to expand the number of RSCs in a stirring suspension bioreactor using cell microcarriers. With this protocol, we achieved 10 times the number of stem cells grown in static culture. This method will help grow large number of RCSs that can be differentiated into different types of retinal cells which will be used to replace the cells lost due to disease or injury.

In the next section I will discus some of the methods that were used previously to expand retinal stem cells and progenitors and compare them to our results.

4.2 Methods of RSC expansion

4.2.1 Effect of mitogens on RSC expansion

Several protocols have been established to expand the number of RSCs *in vitro* using monolayer culture. These protocols depended on adding mitogens to the culture media, however, the expansion ranged only from 2 to 8 times the control. Of note, a number of studies showed that cells exposed to mitogens for several generations can undergo genetic changes. Djojosubroto et al. pointed out that adult CB-derived cell lines rapidly accumulated severe chromosomal aberrations upon prolonged cultivation and demonstrated tumorigenicity (Djojosubroto et al., 2009).

RSCs can proliferate as neurospheres in serum free media without exogenous growth factor addition (Tropepe et al., 2000). However, supplementation with EGF and/or FGF2 significantly increases the number of neurospheres formed from dissociated cells (Asami, Sun, Yamaguchi, & Kosaka, 2007; Coles, Angenieux, et al., 2004; Tropepe et al., 2000). Notably, the proliferative capacity of porcine, rodent and human RSCs maintained in monolayer was significantly increased compared to suspension sphere culture conditions (Asami et al., 2007; Coles, Angenieux, et al., 2004; Klassen et al., 2004; MacNeil et al., 2007). Self renewal was monitored through the extent of secondary sphere formation which could be significantly enhanced following stimulation with exogenous molecules such as Wnt3a (Yanagi et al., 2006), Stem Cell Factor (SCF) (Das, James, Zhao, Rahnenführer, & Ahmad, 2004), Pigment Epithelium Derived Factor (PEDF); (De Marzo et al., 2010) and dexamethasone (Grise et al., 2020).

4.2.2 Gene knockout

Knocking down certain genes like *Pax6* (S. Xu et al., 2007) and Notch (Balenci & van der Kooy, 2013) led to reduction in the numbers and sizes of RSCs. *Pax6* is enriched in RSCs. *In vivo* Inactivation of *Pax6* results in loss of competent RSCs as assayed by the failure to form clonal RSC spheres from the optic vesicles of conventional *Pax6* knockout embryos and from the CE cells of adult *Pax6* conditional knockout mice (S. Xu et al., 2007). *In vitro* clonal inactivation of *Pax6* in adult RSCs results in a serious proliferation defect, suggesting that *Pax6* is required for the proliferation and expansion of RSCs (S. Xu et al., 2007). NOTCH1 plays a selective role in RSC versus RPC in both developing and adult eye, overexpression of Notch1 in RSCs increases their symmetric divisions (Balenci & van der Kooy, 2013).

Coles et al., showed that blocking Chx10 and Mitf transcription factors resulted in 3-8 fold increase in the number of RSCs in both mutants (Coles et al., 2006). This increase reflects a presumed non–cell-autonomous feedback signal on the stem cell population from the neural retinal progenitor cells (Chx10 expressing cells) and the RPE progenitor cells (Mitf expressing cells). The loss of either specific progenitor cell type may decrease a signal that negatively regulates stem cell proliferation, and consequently increases the RSC population (Coles et al., 2006).

Combining the microfluidic platform with single-cell whole-transcriptome profiling Coles et al., 2020 (in progress), were able to identify 3 differentially expressed cell surface markers (FRIZZLED1, ABCG2 and NOTCH1) that when targeted simultaneously yield a 138-fold enrichment of RSCs.

4.2.3 Retinal organoids

Bioreactors were also used to expand retinal precursors (photoreceptors and RPE) from retinal organoids (Akhtar et al., 2019b; DiStefano et al., 2018; Ovando-Roche et al., 2018). Retinal organoids are grown from pluripotent stem cells (PSCs), such as ESCs and iPSCs (Fligor et al., 2018b; M. Völkner, Kurth, & Karl, 2019). Inside these organoids, cells assume organization similar to that found in the original organ they represent. This self-organization is the result of a multitude of instructive cues originating from the ECM and the surrounding culture media, as well as signals from other cell types within the organoid (Mazerik, Becker, & Sieving, 2018; Takebe & Wells, 2019). PSCs are grown on Matrigel matrix in 2D culture to produce EB, a 3-dimensional aggregation of cells that will later form the three embryonic layers: ectoderm, mesoderm and endoderm. EB bodies are then harvested and supplemented with neural and retinal induction media. When the optic vesicle become visible, usually by day 7 of EB differentiation, they get dissected and maintained in a culture flask or in a suspension stirring bioreactor. Then, they will be maintained in retinal differentiation media until mature differentiation is achieved. Bioreactors control culture conditions and support better cell survival within RSC spheres (Fligor et al., 2018b). The continued development of lab-produced retinal organoids has the potential to effectively alleviate many problems inherent to the current conventional techniques. Retinal organoid systems display cell–cell and cell–matrix interactions, cellular heterogeneity, and physiological responses reflective of human biology, thus, are much more accurate models for studying retinal pathology as well as normal physiology (Aparicio et al., 2017; Cora et al., 2019; Llonch, Carido, & Ader, 2018). The currently used development protocols, unfortunately, remain a major hurdle standing in

the way of fully harnessing the application of organoids as, using these protocols, organoids will pass through many developmental stages but are never able to progress to the full mature retina (Capowski et al., 2019; DiStefano et al., 2018; Fligor et al., 2018b; Llonch et al., 2018).

Advanced RNA-seq technologies and transcriptome analysis identified divergent regulatory dynamics between developing retinae *in vivo* and in organoids. Organoid-derived retinal cells displayed temporal dysregulation of specific signaling pathways and reduced expression of genes involved in photoreceptor function and survival, which can be contributed to the lack of natural instructive signals available *in vivo*. Brooks et al. showed that adding docosahexaenoic acid (DHA) or FGF1, the two components missing in organoid cultures, facilitated photoreceptor differentiation and maturation (Brooks et al., 2019). The advancement of new protocols is of paramount importance and is currently under way in different laboratories around the world.

I was unable to find any established protocol for expanding the number of adult derived RSCs and RPCs in stirring bioreactor systems. Using SSB, I designed an expansion protocol that was successful in enriching the numbers of RSCs 10 times the classic static (control) culture. This expansion was higher than what was achieved by adding any of the mitogens mentioned above. Next, work should be directed toward differentiating these RSCs to the required cell type on microcarriers in SSB (rods, cones or RPE cells) using or modifying the current available protocols (Ballios et al., 2012; Khalili et al., 2018b).

4.3 Stem cell niche and its effect on RSC expansion

In order to expand RSCs we need to understand their niche and the signals controlling their proliferation *in vivo*. Niche structure, cell interaction, oxygen level and factors secreted/diffused can affect the stem cell behaviour during development and in adulthood.

4.3.1 Definition of stem cells niche

The stem cell niche concept was first introduced in 1978 (Schofield R, 1978) and represents the natural microenvironment that surrounds stem cells; it can be defined as an anatomical and functional entity that plays a crucial role in maintaining tissue homeostasis and tissue repair and regeneration in case of injuries (Walker, Patel, & Stappenbeck, 2009). It is believed that the stem cell niche provides a complex array of physical signals, including cell-cell contacts and cell-matrix adhesions as well as and biochemical signals, such as growth factors, to stem cells in a temporal and spatial manner; the integration of both local and systemic cues in the niche guides these cells to proliferation and fate specification (Fuchs, Tumbar, & Guasch,

2004; K. A. Moore & Lemischka, 2006). This niche controls stem cell proliferation and fate through the action of several secreted factors and also through interactions with the integral membrane, ECM, and basal lamina proteins (Malicki, 2004)

4.3.2 Structure of the niche

The native stem cell niche consists of stem cells adhering to a group of supporting cells, soluble environment factors at specific sites embedded in the ECM serving as mechanical support and also as a reservoir for cellular signalling molecules (Daley, Peters, & Larsen, 2008). The ECM is composed of tissue-type-dependent fibrous structural proteins (collagens, fibronectin, laminin, elastin and vitronectin) and glycosaminoglycan (GAG) network. Collagen and elastin networks provide tissue with mechanical resistance to shear and tensile stress. ECM proteins such as heparan sulfate, chondroitin sulfate proteoglycans and collagens can modify the availability of signaling factors acting on the stem cells, either by sequestering them or by potentiating ligand binding (Fuhrmann, 2010b; Malicki, 2004). GAGs, thanks to the presence of hydrophilic groups in their backbone, provide compressive strength to the tissue. Cells interact with ECM components that contain various binding domains; these interactions occur through receptors known as integrins (Adam J. Engler, Humbert, Wehrle-Haller, & Weaver, 2009; Gersdorff et al., 2005; S. Li et al., 2002; Yurchenco & Wadsworth, 2004). Furthermore, cells interact with neighboring cells through receptors referred to as cadherins. The regulation of stem cell activities by their surrounding microenvironment is related to these receptors that act as physical anchors able to activate cell transduction pathways (Page-McCaw, Ewald, & Werb, 2007). A broader knowledge of these signaling pathways and about the role of ECM proteins can not only help to understand stem cell biology in the adult organism but will also give new hints for stem cell manipulation *in vivo* (Gonzalez-Nunez, Nocco, & Budd, 2010).

Oxygen concentration represents another important issue in the natural stem cell niche that is a critical component in the regulation of stem cell commitment (Mohyeldin, Garzón-Muvdi, & Quiñones-Hinojosa, 2010). This point will be discussed in a separate section below.

The well-studied niches for haematopoietic, intestinal and skin stem cells, as well as the examples of the hair follicle, mammary gland and neural stem cell niches, have shown that tissue-embedded adult stem cell states can include actively dividing cells as well as cells in a state of quiescence (Greggio et al., 2013; N. Li & Clevers, 2010).

4.3.3 Niche factors

Self renewal, proliferation status and fate of downstream progenitors of many stem cell types are finely regulated by changes in their surrounding *niche*. These changes are dictated by both, extrinsic factors (biophysical factors, like physical activity, stress, environmental enrichment and aging) and intrinsic factors (biochemical factors, like cytokines, growth factors, hormones and neurotrophins) (Ming & Song, 2011).

Biochemical cues are provided by reciprocal interactions between the cell, soluble bioactive agents and the ECM. Soluble factors include growth factors, morphogenetic factors, cytokines, enzymes and small cell-permeable molecules such as transforming growth factors (TGF), bone morphogenetic protein (BMP), vitamin C, sodium pyruvate, retinoic acids (RAs) and other small molecules (Kawaguchi, Mee, & Smith, 2005; H. Zhang, Dai, Bi, & Liu, 2011). These factors, when added to the cell culture, or secreted by stem cells or niche cells, diffuse and bind to cell membrane receptors and activate cellular signal pathways able to alter stem cell gene expression (Ding & Schultz, 2004; Z. J. Liu, Zhuge, & Velazquez, 2009).

There is increasing evidence that mechanical factors are potent enough to control stem cell fate *in vitro*. For instance, it has been demonstrated that the matrix elasticity can influence the lineage commitment of MSCs into neurons, osteoblasts, and myoblasts (A. J. Engler, Sen, Sweeney, & Discher, 2006). Other than the substrate stiffness, these mechanical factors include the surface topography of the biomaterial scaffold, its three-dimensional (3D) geometry and the external forces cells are subjected to (Discher, Janmey, & Wang, 2005; Guilak et al., 2009).

Cells have the ability to actively sense their microenvironment and react to the properties of their surroundings (Kress, Neumann, Weyand, & Kasper, 2012). Anchorage-dependent cells are able to anchor onto the underlying substrate through focal adhesions formed by clusters of proteins, including integrins that are trans-membrane cell adhesion proteins (Lutolf & Hubbell, 2005). The cell cytoskeleton is a network of filamentous proteins that is mechanically linked to the focal adhesions, extending throughout the cytoplasm of eukaryotic cells, it consists of actin, microtubules, and intermediate filaments (Geiger, Spatz, & Bershadsky, 2009). The mechanical connections between the matrix and the cytoskeleton allow cells to exert traction forces that are transmitted to the cell nucleus through intracellular pathways; the resulting force triggers signalling transduction into biochemical signals that affect stem cell response, for example, the synthesis of specific transcription factors in the nucleus (Nava, Raimondi, & Pietrabissa, 2012; N. Wang et al., 2009).

4.3.4 RSC niche

RSCs appear to be inhibited from contributing to homeostasis or regeneration by their CE niche. Isolated RSCs from the CE proliferate extensively to form colonies even in the absence of exogenous FGF (Coles, Angenieux, et al., 2004; Tropepe et al., 2000). Within the intact adult mammalian CE, proliferation is exceedingly rare and, when induced by prolonged growth factor administration, does not lead to migration into the neural retina (Abdouh & Bernier, 2006).

It has been proposed that *in vivo* activity of stem and progenitor cells is hampered by intrinsic limitations and/or lack of permissive factors in their microenvironment. However, these cells retain the ability to respond to growth factor stimulation *in vivo*. Intraocular injection of FGF2 and Insulin was indeed reported to induce proliferation of quiescent CB epithelial cells *in vivo* (Abdouh & Bernier, 2006; X. Zhao, Das, Soto-Leon, & Ahmad, 2005). Abdouh and Bernier showed that these reactivated cells exhibited neuroepithelial characteristics (expression of Chx10, Pax6, nestin) but did not differentiate into neurons (Abdouh & Bernier, 2006). In contrast, Zhao et al. described the expression of several differentiated retinal cell markers in BrdU labeled cells. In addition, they found that these progenitors were able to migrate centrally toward the neural retina (X. Zhao et al., 2005).

Close et al. investigated the nature of extrinsic factors that might account for the postnatal decline in proliferation within the postnatal retina. She found that exogenous transforming growth factor β1 or β2 (TGFβ1/2) inhibited proliferation in rat retinal explants. Conversely, postnatal inhibition of TGFβ signaling *in vivo* extended the period of proliferation (Close, Gumuscu, & Reh, 2005). Based on these data, they proposed that a cytostatic TGFβ signal produced by the retinal neurons is involved in the maintenance of mitotic quiescence in the postnatal retina.

Postembryonic downregulation of *Shh* signalling might also contribute to silence the proliferative and neurogenic potential of adult RSCs. Indeed, mice with a single functional allele of the *Shh* receptor Patched, in which *Shh* signaling is constitutively activated, exhibit persistent progenitor cells in the retinal margin, reminiscent of the CMZ of fish and amphibians. Importantly, those Patched+/- proliferative cells were shown to trigger limited but observable neuronal regeneration in mice with a retinal degeneration background (Moshiri & Reh, 2004). In zebrafish, few genes have been described as specifically expressed in the stem cell zone and absent in the surrounding progenitors. Among them are components of the Shh signaling pathway, Gli2, Gli3 and X-Smoothened (Locker, El Yakoubi, Mazurier, Dullin, & Perron, 2010b), as well as the collagen encoding gene DrCol15a1b (Gonzalez-Nunez et al.,

2010). This previously uncharacterized collagen prevents the diffusion of signaling factors (of the Shh and notch pathways) out of the stem cell niche, thus maintaining neural stem cells within their proliferative microenvironment. Shh protein was ound also to promote proliferation within the CMZ of post-hatched chicken eyes (Moshiri & Reh, 2004). It was demonstrated that *Shh* signalling exerts ambivalent effects on both cell cycle kinetics and cell cycle exit of retinal precursors, thereby simultaneously promoting proliferation and differentiation (Locker et al., 2006). *Shh* signalling may regulate the transition from slow cycling retinal stem cells to fast cycling neuronal progenitors, bringing them closer to cell cycle exit (Locker et al., 2006).

A third player in the process was found to be the BMP pathway, which also interacts with FGF signalling although in a stage dependent manner, first activating FGF-receptor expression and then downregulating it (Haynes, Gutierrez, Aycinena, Tsonis, & Del Rio-Tsonis, 2007). Both Wnt and BMP signalling participate during embryogenesis to the determination and patterning of anterior eye structures including the iris and CB (Cho & Cepko, 2006; Kubo & Nakagawa, 2009; H. Liu et al., 2007; S. Zhao, Chen, Hung, & Overbeek, 2002). The Wnt pathway might also be critical for the formation of the CMZ by keeping cells of this region in a proliferative and undifferentiated state (Kubo, Takeichi, & Nakagawa, 2003). Recently, it was found that this function is mediated by c-hairy1, a member of the hairy-enhancer of split genes (Kubo & Nakagawa, 2009). Activation of Wnt signaling in transgenic tadpole retinae increased the number of CMZ proliferative cells, while blocking the pathway almost completely abolished proliferation (Denayer et al., 2008). These results substantiate the hypothesis that, in lower vertebrates, the Wnt pathway plays a crucial role in adult retinal stem cell maintenance (Locker et al., 2010b). β-catenin/Wnt signaling is thus required for the maintenance of retinal progenitors during both initial development and lesion-induced regeneration and is sufficient to prevent differentiation of those progenitors and maintain them in a proliferative state. This suggests that the β-catenin/Wnt cascade is part of the shared molecular circuitry that maintains retinal stem cells for both homeostatic growth and epimorphic regeneration (Meyers et al., 2012a).

The function of Wnt2b in maintaining the undifferentiated state is mediated through suppression of genes required for neuronal differentiation, namely, Notch1 and proneural genes (Kubo, Takeichi, & Nakagawa, 2005). In addition, Wnt2b enables sustained proliferation of retinal progenitor cells, which is highly reminiscent of retinal stem cells. For instance, when Wnt2b is stably expressed in the central progenitor cells that normally proliferate for only a limited period of time, the cells continue to proliferate to form

dramatically large sheet-like structures mainly consisting of undifferentiated cells (Kubo et al., 2005). Similar hyperproliferation was also observed when stabilized β-catenin, which constitutively activates the canonical Wnt signaling pathway, is introduced in the central retina of zebrafish (Yamaguchi et al., 2005). Furthermore, these Wnt2b-treated cells subsequently differentiate into neurons and glia upon blockade of Wnt signaling (Kubo et al., 2003, 2005), indicating that Wnt signaling induces continuous cell proliferation of retinal progenitor cells without losing their multipotency. All these observations are consistent with the idea that Wnt signaling provides progenitor cells in the CMZ with a stem cell-like capacity. It should be noted that mRNA for Wnt2b, LEF1 and several members of the Frizzled family are detected in neurosphere cultures prepared from the pigmented ciliary margin (PCM) of adult rodent eyes (Das et al., 2006), activation of the canonical Wnt signaling pathway increases the number of spheres generated from the PCM (Das et al., 2006; Yanagi et al., 2006). These results suggest that Wnt signaling has a positive effect on the formation of mammalian RSCs *in vitro.*

Alteration in any of the genes and growth factors above can also affect the fate of the downstream progenitors *in vivo*. For example, deletion of *Shh* from the mouse retina resulted in a premature induction of photoreceptors and an overproduction of ganglion cells (Neumann & Nuesslein-Volhard, 2000; Y. Wang et al., 2005). Injection of the mutant FGF receptor into Xenopus resulted in a 50% loss of amacrine cells and photoreceptors with a corresponding increase in müller glia (McFarlane et al., 1998). In the chick retina, a decrease in Notch levels led to a corresponding increase in retinal ganglion cells produced (Austin et al., 1995; Silva et al., 2003). Inactivation of Wnt/β-catenin signaling results in transdifferentiation of RPE to neural retina as documented by downregulation of RPE-specific markers Mitf and Otx2 and ectopic expression of neural retina specific markers Chx10 and Rx (Fuhrmann, 2008b; Fujimura et al., 2009; Meyers et al., 2012b; Jie Zhu et al., 2014). Whereas BMP was reported to reprogram the neural retina into RPE at optic cup stages in chick (Steinfeld et al., 2017).

As discussed above, the niche of stem cells controls their proliferative status and cell fate decision. I think that modifying these niche factors *in vitro* can influence the proliferation, survival and expansion of RSCs. Measures like taking the stem cell away from the inhibitory natural environment into a controlled microenvironment (adjustable pH, temperature and oxygen level) and providing an anchoring ECM surface made RSCs more active *in vitro*. RSCs formed clonal spheres that were passaged and differentiated into different retinal cells in pan-retinal conditions. In my second data chapter, I reported that RSCs were capable of producing

three different sphere types that contain heterogenous populations of progenitors (early and late RPE progenitors). The proliferation of early progenitors was totally dependent on factors secreted from other neural retinal cells found in the niche. Late progenitors were not responsive to these factors and did not proliferate *in vitro*.

4.3.5 Effect of oxygen on stem cell expansion

Evidence suggests that other components of the niche, such as oxygen tension, play an important role in cell fate determination during the development of many tissues, including the nervous system and the retina (Arden, Sidman, Arap, & Schlingemann, 2005; De Gooyer et al., 2006). Mouse embryonic stem cells (mESC) allow us to recapitulate retinal development *in vitro*. These cells are derived from the early embryo and are characterized by their two unique features of pluripotency and self-renewal (Evans & Kaufman, 1981). In particular, during implantation and fetal development, stem cells live at oxygen tensions between 2% and 8% (Maltepe & Simon, 1998). Early embryonic formation during mammalian development occurs in a precise environment, where the O_2 tension plays a critical role (Dunwoodie, 2009). In comparison to the atmospheric oxygen tension (20%), the uterus environment is hypoxic. Mammals including rabbits (8.7% oxygen tension) and monkeys (1.5% oxygen tension) (B. Fischer & Bavister, 1993) as well as humans develop embryos under low oxygen tension. Up until the second trimester in humans this ranges from 2% to 3% (Burton & Caniggia, 2001; Burton & Jaunaiux, 2001). The retina is not an exception and recent studies have shown the important role that hypoxia may play in neuroprotection and development of the human retina (Grimm & Willmann, 2012). This relative hypoxia or tissular normoxia is relatively low compared with traditional *in vitro* culture conditions (20% O_2) (Malicki, 2004). Some studies showed that mimicking physiological O_2 is a favorable condition for the efficient generation of RPCs and photoreceptors from both hiPSCs and hESCs (Bae et al., 2011; Garita-HernÁndez et al., 2013). Chen et al., also reported the efficient generation of retinal organoids from mouse stem cells using hypoxia (5% O2) (Chen et al., 2016).

Based on this knowledge, I compared the growth of RSCs and their ability to form clonal spheres both in hypoxia (5%) and in normoxia (21%). RSCs formed more colonies in hypoxia across different platforms. My work in SSB showed that this phenomenon is due to increased cell survival and symmetrical division in the hypoxic culture. A further increase in the number of generated colonies was achieved when positively charged collagen microcarriers were used.

4.4 Conclusion

The central hypothesis of this work was to bypass some of the main obstacles standing in the way of effective expansion of RSCs and their progeny through providing a supportive *in vitro* environment that enhance their symmetrical division. The work in this thesis (chapter 2) has shown that using hypoxia in combination with SSB and MCs has led to 10 times increase in the yield of RSCs compared to the commonly used normoxic static culture. I showed that hypoxia increases the survival and the symmetrical division but not the proliferation of RSCs and demonstrated that activation of the Notch pathway is the mechanism that drives this phenomenon. Adding Dll3 (Notch ligand) caused a non-significant increase in the number of RSCs compared to hypoxic culture alone which suggests that hypoxia had already increased symmetric divisions of RSCs and that adding Dll3 had no further effect. Hypoxia increased the number of RSCs across platforms (static, SSB and MCs) by about two times compared to normoxia. The survival-promoting effect of hypoxia was combined with the use of SSB culture that allowed for better control of the microenvironment. Stirring at low rate (50 rpm) ensured that nutrients were equally distributed to all cells while keeping shear pressure at minimum. This system allowed to monitor pH, temperature and oxygen and maintain them fixed throughout the culture period. Culturing RSCs on MCs contributes to improving survival of cells, partly because it doesn't require the cell-damaging enzymatic dissociation steps, through providing an adhering surface that allowed the cells to attach, divide and expand. The combination of these factors resulted in 10 times expansion in the number of RSCs compared to static culture. To my knowledge, this is the first work focused on creating a protocol for expanding adult RSCs in a bioreactor system. The expansion reported in this thesis is higher than that achieved with mitogens in static culture without the mitogen associated risks.

The second objective of this thesis is to study the behaviour of RSCs and RPCs on clonal sphere level. In this thesis and for the first time, I reported that there are a three morphologically distinct groups of RSC clonal spheres. Further study of those groups revealed that their differentiation potential was different.

To test the second hypothesis, RSC clonal spheres were grouped into three categories based on their pigment distribution. The differentiation potential of one of the groups (CP spheres) clearly stood out from the other two groups (HP and LP spheres). Passaging of these clones proved that they all came from the same parent stem cell in the pigmented CE margin. Further study of CP spheres revealed a population of highly proliferative RPE progenitors. Sorting pigmented (RPE) and non-pigmented cells allowed me to study the proliferation potential of

each cell type alone or in combination with each other. I was able to study the gene expression of different RPE and tight junction genes to look for the intrinsic differences between HP, LP and CP pigmented cells. This experiment showed that the pigmented cells of CP spheres express immature RPE genes (MITF and Otx2) in addition to claudin-1 gene (tight junction gene). I have shown that these pigmented cells are not proliferative on their own and only unleash their proliferation potential upon reception of signals from the surrounding non-pigmented cells. Unlike CP spheres, pigmented cells from HP and LP spheres did not respond to these proliferative signals. Depending on the gene expression and response to external signals, I classified the pigmented cells in RSCs clonal spheres into early and late RPE progenitors. For the first time in this thesis, the differentiation potential of RPE progenitors was studied on clonal sphere level. I have also showed that RSCs derived from adult pigmented CE constitute a homogenous population that has the ability to self-renew and differentiate into both RPE and NR cells.

Growing RPE as a source of adult stem cells has always been a challenge (Aruta et al., 2010; Uygun et al., 2009). A proliferative population of RPE derived from adult RSCs has been identified and characterized. This population will provide a pure and safe source of RPE cells that can be sorted later and used to replace RPE in degenerative retinal diseases. The work presented in this thesis will add to the considerable wealth of information previously published in the field. It will expand the knowledge and deepen our understanding of the basic biology of RSCs and RPCs and open the door to new approaches related to their utilization in bioengineering and therapeutics.

4.5 Future direction

Throughout this thesis I have conducted a lot of experiments trying to learn, understand and explore the mechanisms controlling RSCs and RPCs. I was fortune to be able to answer some of these questions but, as always in science, every answer brings another question.

I believe that the bioreactor expansion protocol and my discovery of CP spheres will be crucial to take this promising project to the next level that will lead to more advancement in the field.

We now have the expertise to effectively expand the number of RSCs, achieving up to 10 times the number of cells produced using static culture techniques. The next step after expanding RSCs is to test if adult derived photoreceptors can be expanded in a bioreactor setting. Primary adult derived clonal RSC spheres as well as NR progenitors will be dissociated, the cells will be cultured on different types of MCs in different agitation rates and oxygen gradients. My lab recently published a protocol for cone photoreceptor enrichment (Khalili et al., 2018a) that can be used to expand cone cells inside a stirring suspension bioreactor. The hypothesis is that by using stirring suspension bioreactors and controlled microenvironment conditions (pH, temperature, agitation rate, nutrient levels and O_2 concentration), we can enhance cell survival and ensure greater cell yields. Furthermore, utilizing cell microcarriers does away with the injurious enzymatic dissociation step, therefore, eliminating cell loss associated with the use of strong dissociative enzymes.

Many labs have been successful in differentiating photoreceptors and RPE from ESCs and iPSCs (Akhtar et al., 2019a; Buchholz et al., 2013; Clarke et al., 2012; Gonzalez-Cordero et al., 2013; Schwartz et al., 2012, 2016; Sowden, 2014; Uygun et al., 2009; S. Zhou et al., 2015; J. Zhu et al., 2018), but issues related to ethics, immune rejection and risk of neoplasia remain problematic (Hyde & Reh, 2014; Kim et al., 2010; Messina, Casarosa, & Murenu, 2012; Noguchi et al., 2018; Ramsden et al., 2013; N. Wu et al., 2016). Particularly, the clinical application of ESCs carries a risk of teratoma formation. RSCs derived from iPCs are potential autologous sources that circumvent the ethical and immune rejection concerns; however, the risk of tumor formation cannot be entirely excluded. Clinical application of adult RSCs is free of ethical concerns, safe and not associated with risks of neoplasia and rejection (Ramsden et al., 2013). I propose that CP spheres can be enriched using the same bioreactor protocol which will provide large quantities of RPE cells in mouse and human. My work showed that *Mitf* and *Otx2* are highly expressed in CP pigmented cells. It will be important to further characterize early RPE progenitors in CP spheres through RNA sequencing to study possible genes that can be used to identify and isolate a pure population.

This will help in understanding RPE development and the variance between different RPE progenitors that built the RPE layer in the optic cup. Once RPE progenitors are isolated, their numbers can be expanded using bioreactors and MCs. Cells grown from adult stem cells are considered safer sources for transplantation compared to pluripotent stem cells. So, I believe this work will bring us closer to providing a safe allogenic source of RPE cells.

In order to apply this protocol to clinical research, It will be important to expand human RSCs in SSB. Human primary RSC clonal spheres will be dissociated and cultured in static, SSB and on MCs while testing how different agitation rate, oxygen concentration and MCs coatings will affect the stem cells and photoreceptors behaviour.

Most CP cobblestone cells are non-pigmented cells that also express immature RPE marker Mitf. Next step should be focused on accelerating the maturation of these cells through modifying culture conditions such as using more serum and/or adding exogenous factors to the culture media, like: Activin A and BMP4 (TGF-β superfamily) (Aruta et al., 2010; A. Lane et al., 2014; Uygun et al., 2009). RPE maturity can be detected using electron microscopy to evaluate presence of melanin granules/pigmentation, apical-basal polarity, and formation of tight junctions. Additionally, the cells should be evaluated for phagocytic function using, for example, fluorescent-labelled photoreceptor outer segments from porcine eyes or fluorescent-labelled latex beads. Finally, the transplantation of these cells into models of RPE degeneration should be performed to determine their potential to replace lost RPE cells and rescue ONL photoreceptor loss and retinal function.

HP and LP spheres contain populations of pigmented progenitors that do not respond to NR cell signals. It will be interesting to sort the pigmented cells and do q-PCR or RNA-seq gene analysis to further study the origin and the identity of these cells. Immunohistochemistry is also important to investigate the expression of pigmented CE genes as Aquaporin 1, 4 and MSX1.

In this thesis I focused of studying the difference between pigmented cells in the three clonal RCS spheres. More experiments are needed to determine the expression of NR cell markers in non-pigmented cells from all sphere types.

I have shown that pigmented cells in CP spheres are responsive to proliferative signals from NR cells from all sphere types. More work is needed to identify and particularize the pathways behind this phenomenon.

Claudin-1 was highly expressed in CP pigmented cells; claudin-1 being a tight junction protein has led us to hypothesize that the aggregation of pigmented cells at the centre of spheres was a function of the highly expressed claudin-1. However, blocking claudin-1 did not disrupt the formation of pigmented centres nor the proliferation of CP spheres. Thus, there must be other pathways that work separately or in conjugation with claudin-1 to exert its action. Other experiments are still needed to investigate how pigmented cells adhere to each other at the center of CP spheres.

I found that hypoxia had a positive effect on the survival of RSCs. However, RSCs are quite rare in our culture, because of that, differentiating selective survival versus symmetric proliferation effects on RSCs is difficult. Isolating a pure RSC population will help in studying its behaviour in hypoxic conditions.

Appendix A

List of supplementary figures.

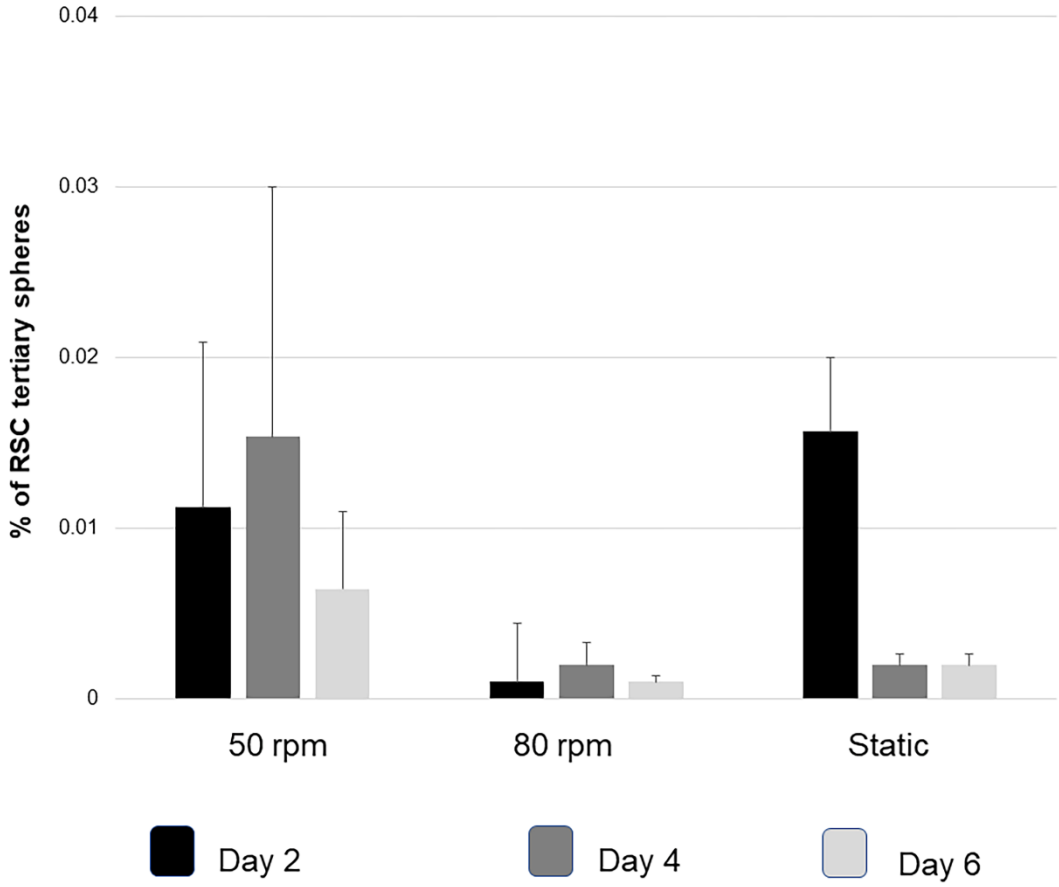

Figure A-1

Average tertiary clonal RSC spheres percentage per day (days 2, 4 and 6), high speed agitation (80 rpm) vs low speed agitation (50 rpm). Results represent the means of n = 2 biological replicates, n = 8-24 technical replicate per sample ± SEMs.

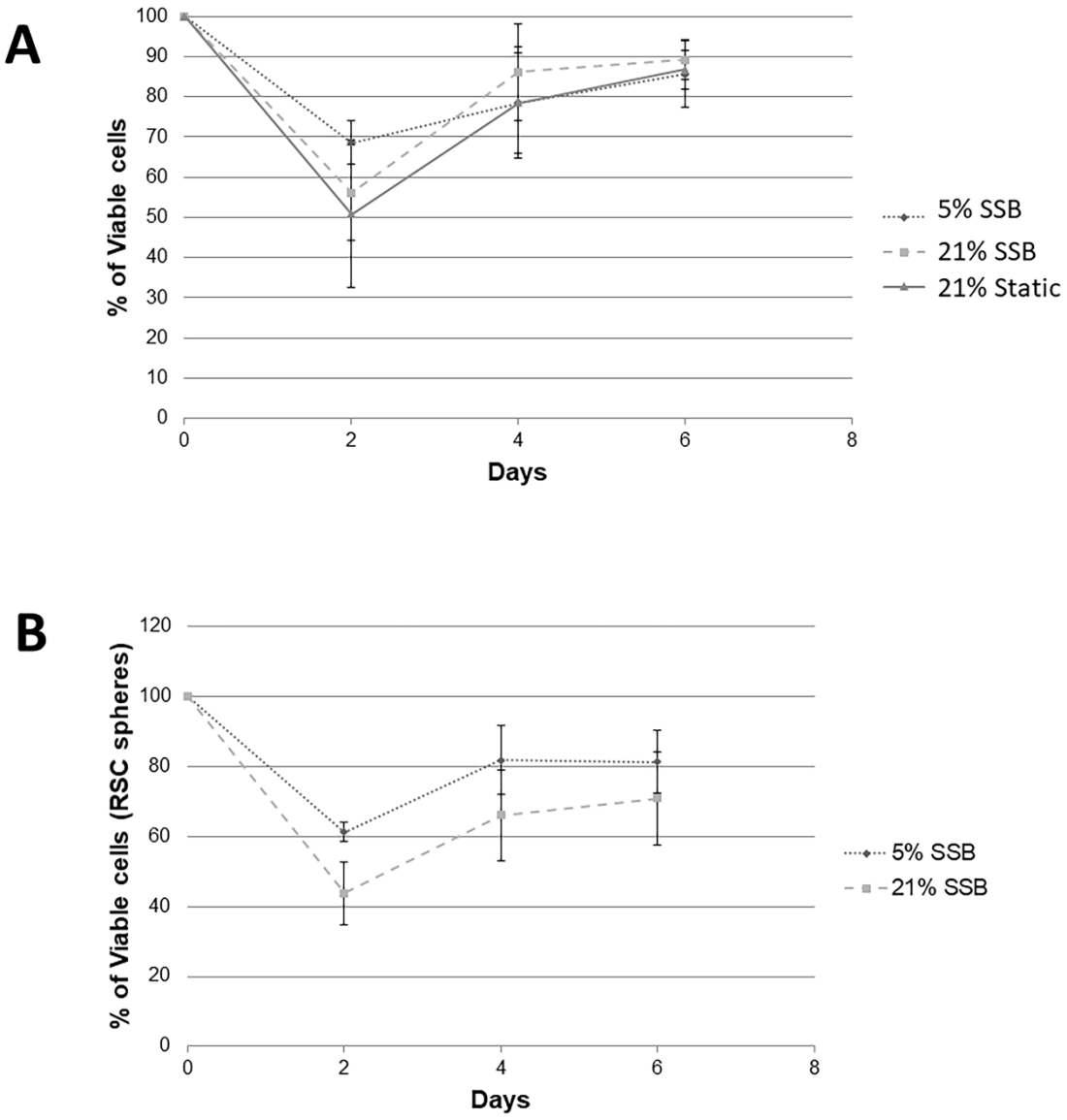

Figure A-2

Viability of RSCs in suspension (21% and 5% O_2) and stationary cultures. (A) Average cell viability (single cell + aggregate samples) was determined using Ethidium homodiamer-2 and Hoechst staining. The average 5% SSB viability (single cells + aggregates) over 6 days was similar to 21% SSB culture and 21% static culture. (B) Average cell viability of aggregate samples derived from 5% SSB and 21% SSB. There was 1.5-fold increase in viability of cells in the aggregate samples in hypoxia compared to normoxia in SSB cultures ($p < 0.0346$, $t = 2.77$, $df = 3$). Results represent the means of $n = 2\text{-}3 \pm$ SEMs.

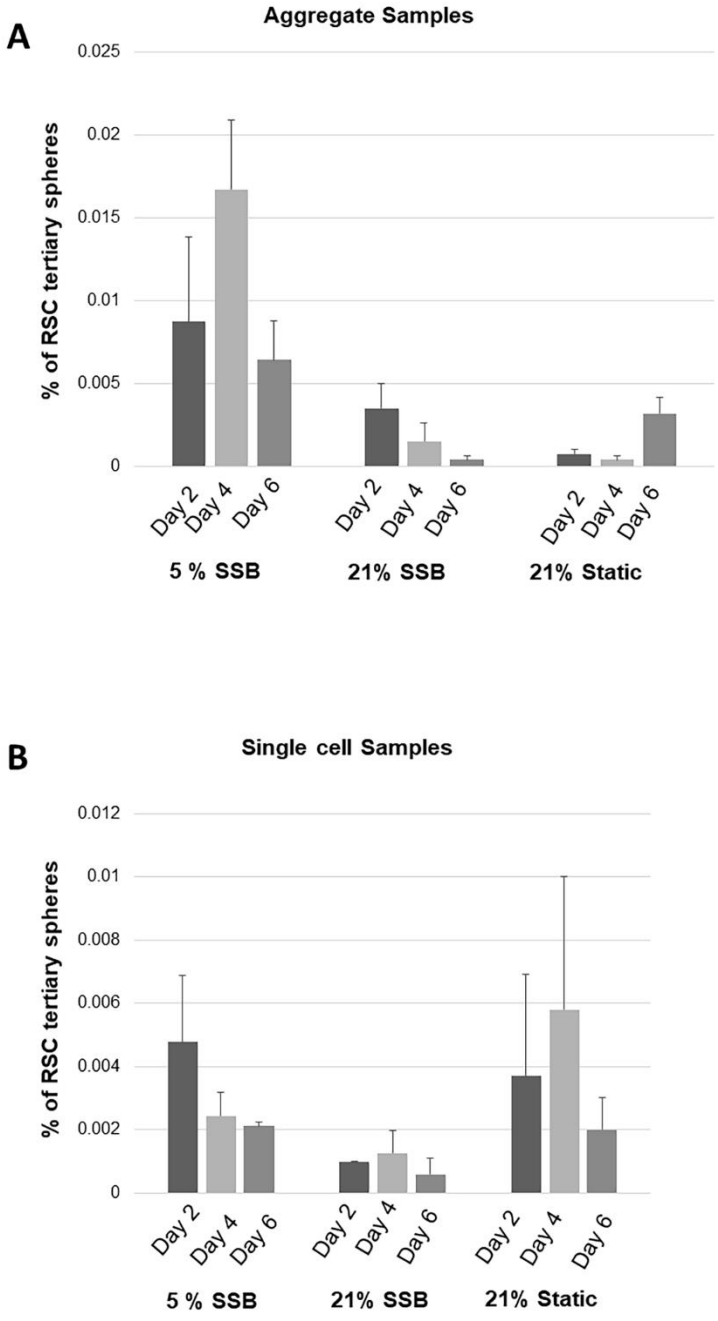

Figure A-3

Hypoxia (5%) vs normoxia (21%): Average tertiary sphere forming cell percentage per day (days 2, 4 and 6). (A) Aggregate samples. (B) Single cell samples. Results represent the means of *n* = 2-3 biological replicates, *n* = 8-24 technical replicate per sample ± SEMs.

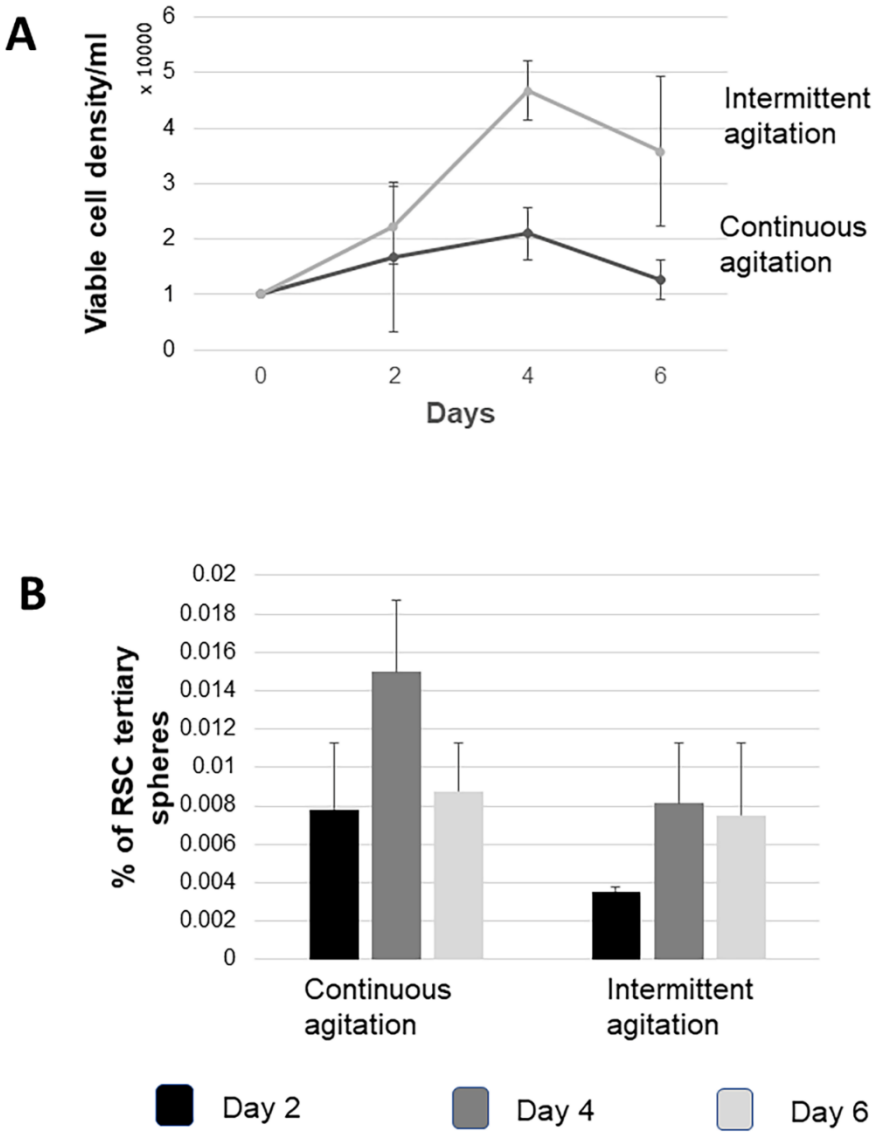

Figure A-4

Effect of agitation mood (continuous vs intermittent) on RSC expansion per day. (A) Viable cell density per ml. Using the 3-minute intermittent agitation protocol, we saw 3 times the expansion in overall cell density compared to continuous agitation. (B) Percentage of clonal tertiary sphere forming cells per day (days 2, 4 and 6). More clonal RSC spheres were observed in the continuous agitation culture on day 4, which was significantly higher than day 2 ($p = 0.0126$, $t = 25.140$, $df = 1$) but not to day 6 ($p = 0.0618$, $t = 5.08$, $df = 1$). Results represent the means of $n = 2$ biological replicates, $n = 8$-24 technical replicate per sample ± SEMs.

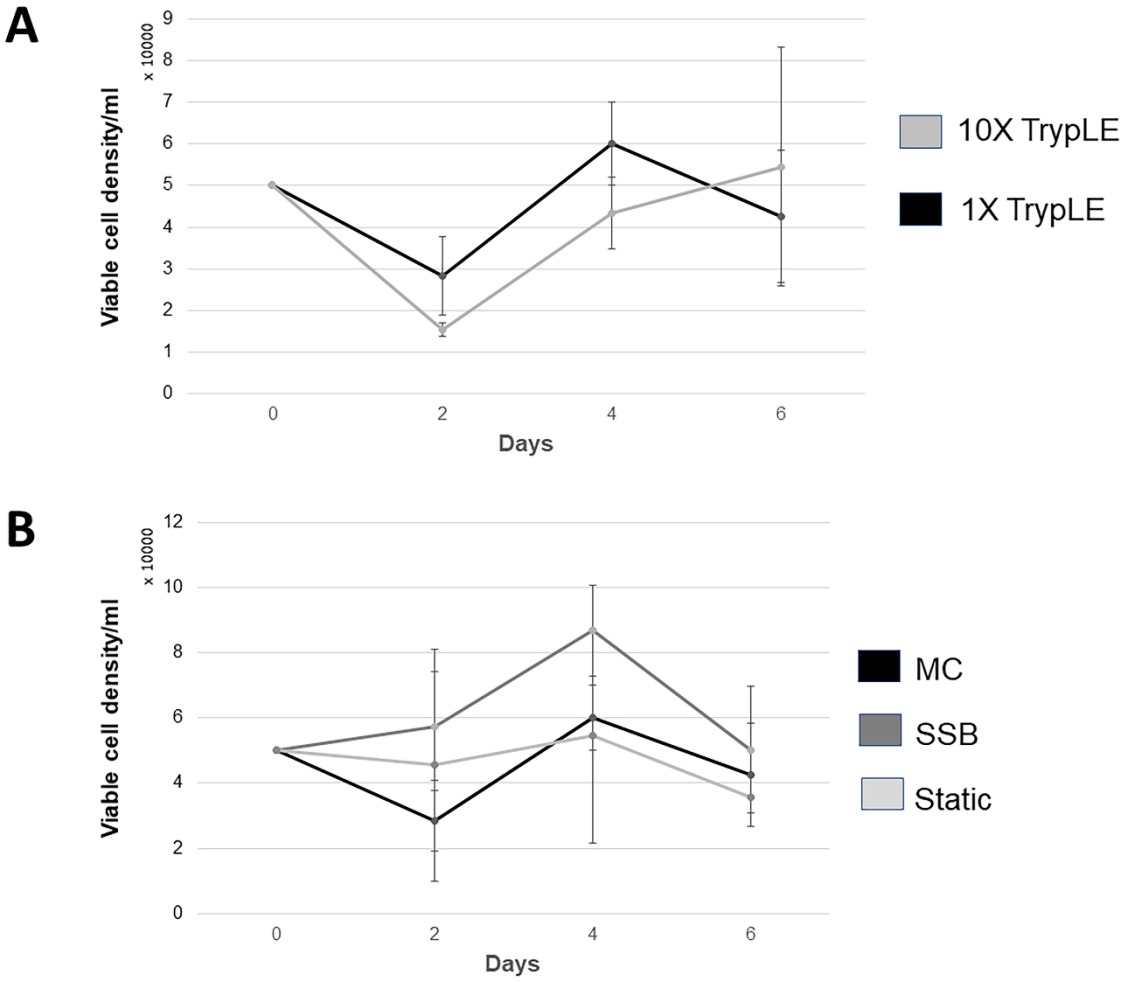

Figure A-5

Growth chart for RSCs in hypoxia under different culture conditions. (A) Comparison of 1X TrypLE and 10X TrypLE dissociation protocols on the growth of RSCs on FACTIII microcarriers under hypoxia. (B) Comparison of microcarriers in SSB (10X TrypLE), SSB alone and static culture under hypoxia. Results represent the means of n = 2 biological replicates, n = 8-24 technical replicate per sample ± SEMs.

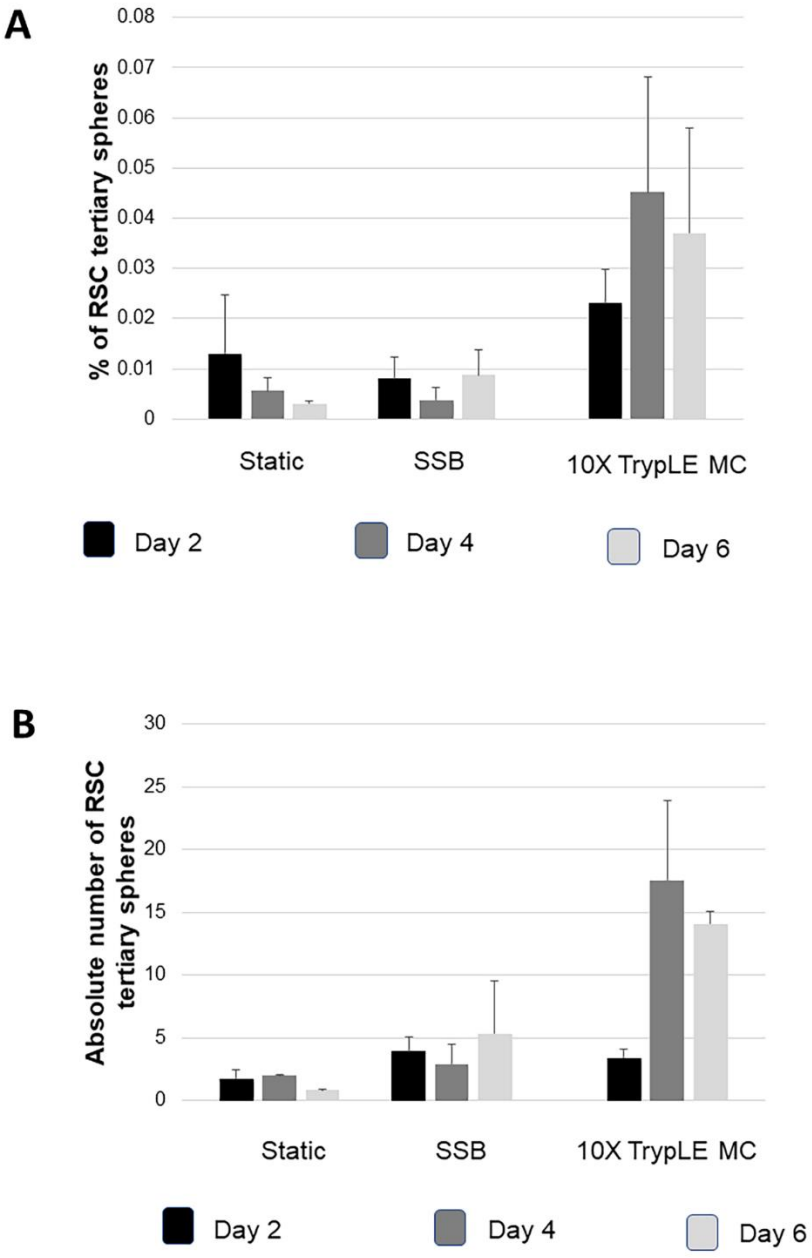

Figure A-6

Daily tertiary RSC clonal spheres assay. (A) Tertiary RSC percentages per day (days 2, 4 and 6) observed in MC-SSB, SSB and static culture, all in hypoxia. (B) The percentage of clonal RSC spheres per day. More spheres were observed on day 4 in MC experiment but the increase was not significant compared to SSB ($p = 0.133$, $t = 2.24$, $df = 1$) and static culture ($p = 0.122$, $t = 2.46$, $df = 1$). Results represent the means of $n = 2$ biological replicates,, $n = 8$-24 technical replicate per sample ± SEMs.

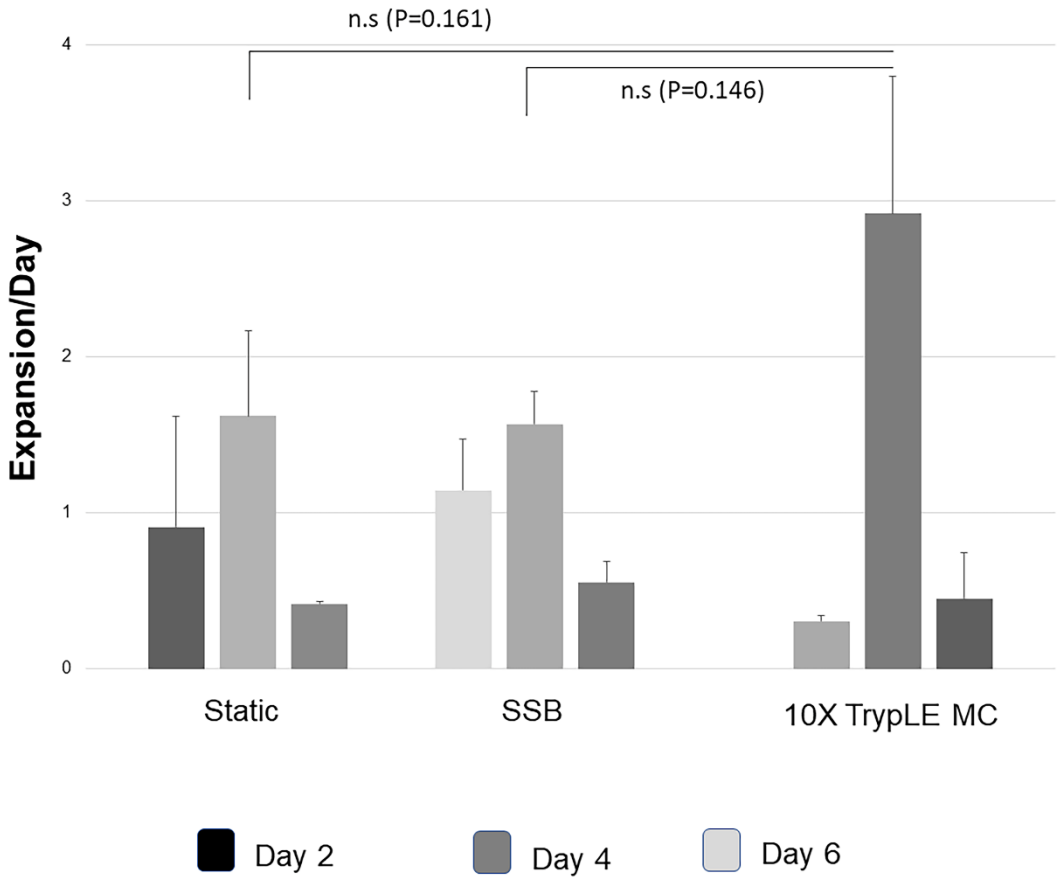

Figure A-7

Daily cell expansion obtained from MC-SSB, SSB and static experiments. A nonsignificant increase in cell expansion between MC and SSB experiment or MC and static experiment was noticed on day 4 ($p = 0.146$ and 0.161, respectively). Results represent the means of $n = 2$ biological replicates, $n = 8$-24 technical replicate per sample ± SEMs.

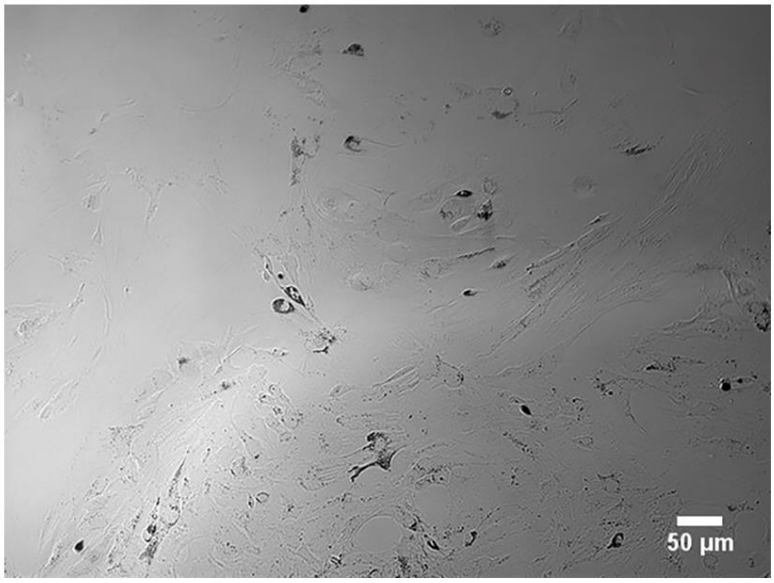

Figure A-8: Cobblestone morphology cannot be induced by differentiating 6 mouse HP spheres together. Differentiation of HP retinal spheres at high density (6 spheres/well) in a 6-well plate to test if RPE proliferation and maturation can be induced at these higher density cultures. We could not observe cobblestone morphologies in these cultures (n = 3).

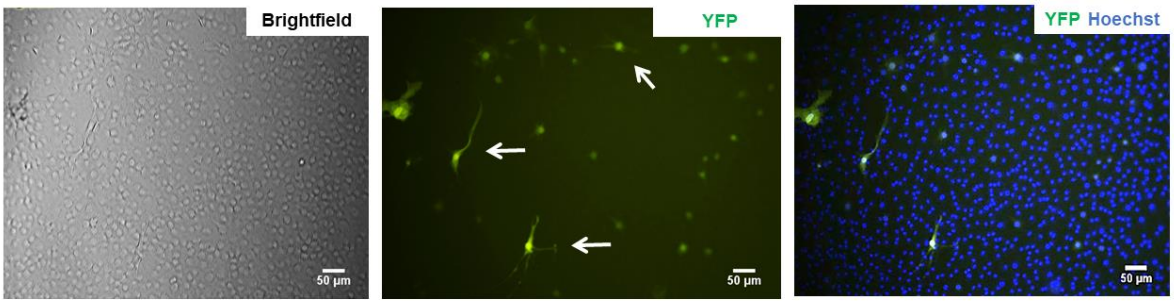

Figure A-9: NR cells in HP spheres maintain their identity when cultured with early progenitors in CP spheres. Culturing one HP YFP+ sphere with one CP sphere derived from one YFP- wildtype mouse on laminin for 21 days. We found that the YFP+ cells maintained their NR identities (arrows) without induction of cobblestone fates (n = 3).

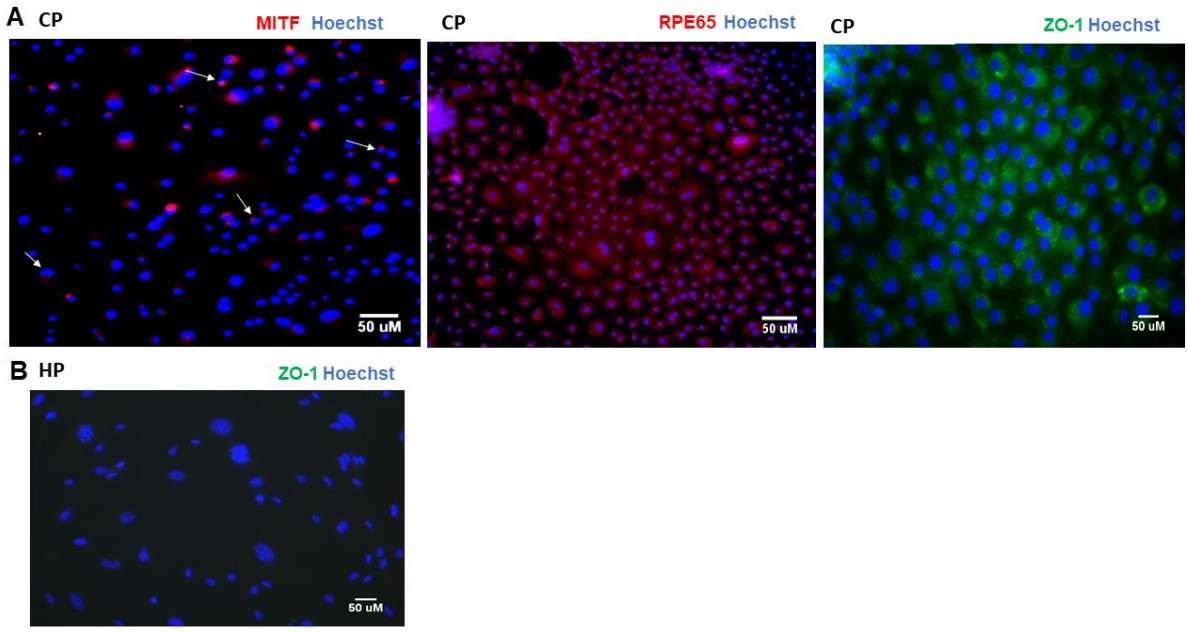

Figure A-10: Adult mouse derived clonal CP sphere progeny express RPE differentiation markers. (A) MITF (arrows), RPE65 and ZO-1 expression in CP clonal sphere differentiation culture. Spheres were plated on laminin as single spheres per well in pan-retinal conditions for 21 days. Cells were fixed with 4% PFA and stained for immature RPE marker (MITF) and mature markers (RPE65) and ZO-1 (n = 3). (B) ZO-1 expression was not seen in differentiated in HP sphere.

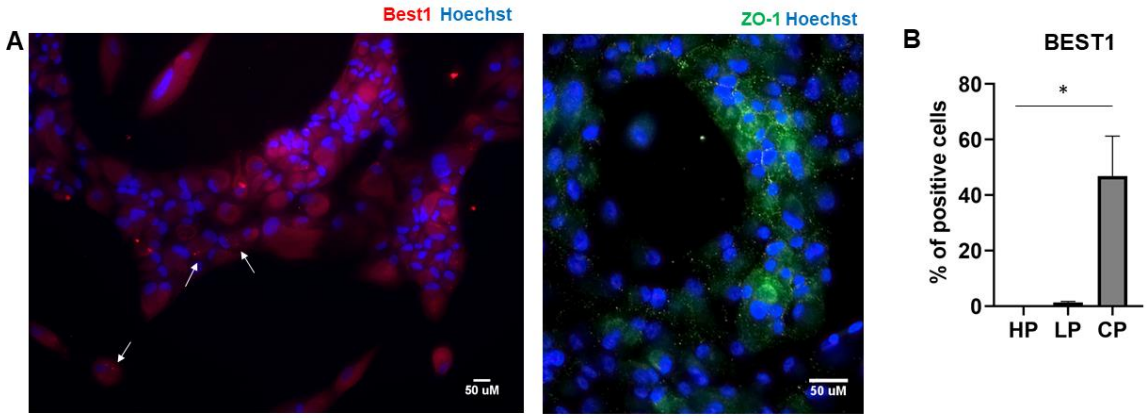

Figure A-11: Adult human derived clonal CP spheres differentiate into cobblestone cells and express RPE markers. (A) Bestrophin1 (BEST1, arrows) and ZO-1 expression in CP sphere differentiation culture after 60 days of pan-retinal differentiation. (B) Percentages of BEST1 positive cells were higher in cells from human CP than HP ($t = 3.28$, $p = 0.03$) and LP spheres ($t = 3.192$, $p = 0.03$) after 60 days of differentiation culture. Data represent means ± S.E.Ms ($n = 2$).

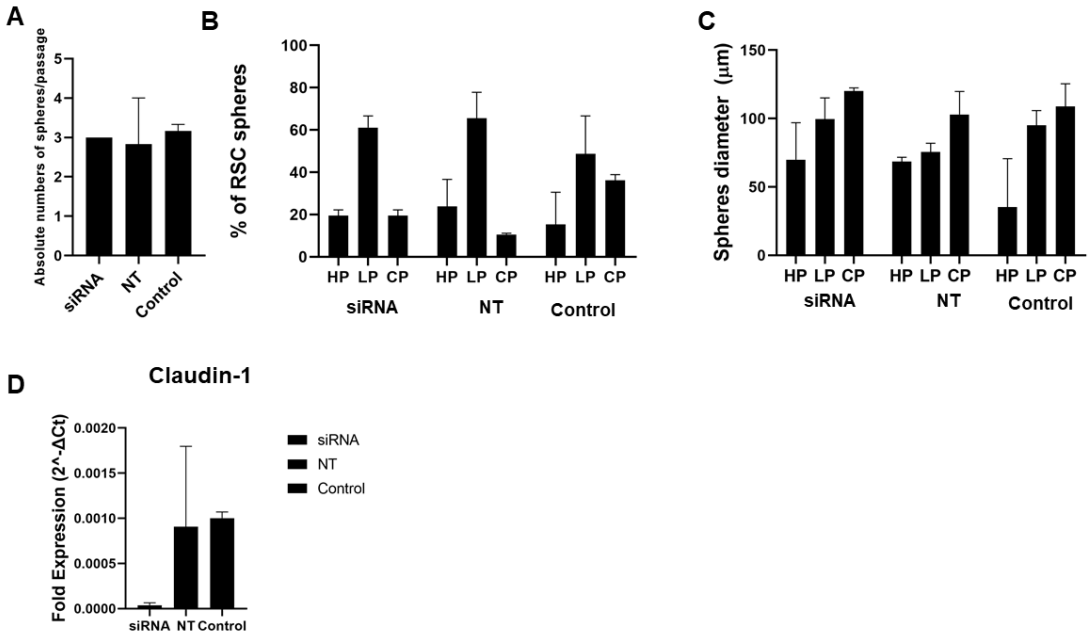

Figure A-12: No effects of claudin-1 knockdown on LP sphere diameters nor passaging.
(A) siRNA blocking of claudin-1 did not affect the number of passaged spheres compared to non-target (NT) siRNA and no siRNA treatment (Control) (*n* = 2, One-way ANOVA, F (2,3) = 0.060, p = 0.942). (B) The percentages of RSC spheres were similar among siRNA conditions and two-way ANOVA revealed a non-significant interaction effects on sphere type and siRNA condition (*n* = 2, Two-way ANOVA, F (4,9) = 1.28, p =0.345) and a significant main effect of sphere type (F (2,9) = 13.81, p = 0.0018,Tukey-Kramer post-hoc, p< 0.05). (C) No significant differences in the diameters of spheres observed among siRNA and control conditions (*n* = 2, Two-way ANOVA revealed no interaction effect between the spheres size in different conditions (F (4,9) = 0.577, p = 0.686) and only a significant main effect of sphere type (F (2,9) = 6.44, p = 0.018). (D) Low levels of claudin-1 were observed in siRNA experiment compared to the no treatment and non targeting siRNA controls.

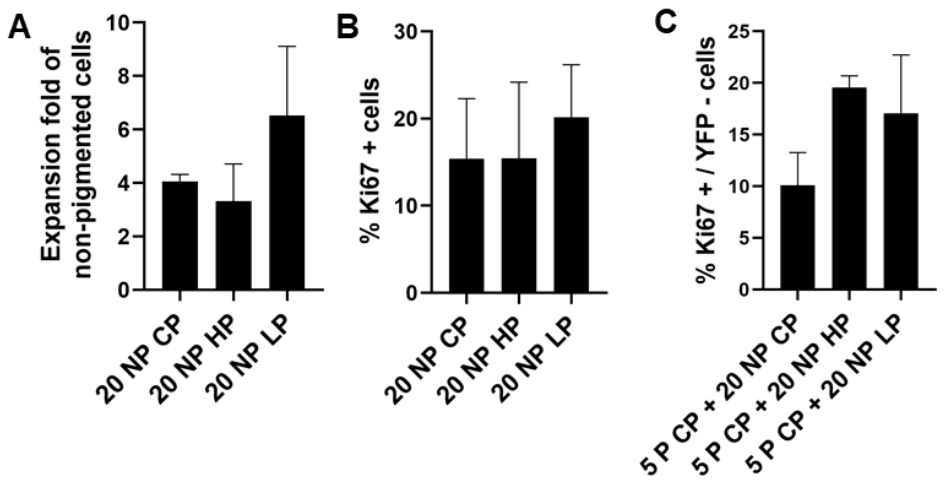

Figure A-13: Proliferation of non-pigmented NR cells is not dependent on signals from pigmented RPE cells**. (**A) Fold expansions of non-pigmented cells derived from CP, HP and LP spheres were not significantly different (one-way ANOVA, F (2,4) = 1.30, p =0.366). (B) Percentages of Ki67+ cells in non-pigmented YFP- cells derived from HP, LP and CP spheres after co-culture with pigmented cells from CP spheres show no significant differences in proliferation (one-way ANOVA, F (2,3) = 0.140, p = 0.874). (C) Non-significant differences among the percentages of Ki67+ cells in non-pigmented cells from the different sphere types (one-way ANOVA, F (2,6) = 1.67, p = 0.264). Data represent the means ± S.E.M (*n* = 2-3).

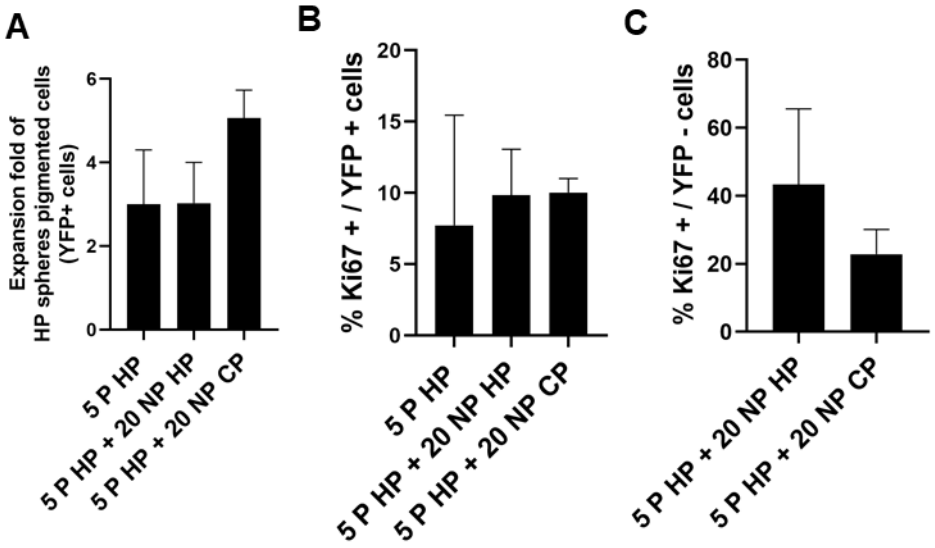

Figure A-14: Pigmented cells in HP spheres do not respond to proliferative signals from non-pigmented NR cells. (A) There was no difference in the fold expansions of HP YFP+ pigmented cells when combined with non-pigmented cells from HP or CP spheres (one-way ANOVA, $F(2,5) = 1.34$, $p = 0.340$). (B) No differences were observed in the percentages of YFP+ cells positive for the Ki67 proliferation marker (one-way ANOVA, $F(2,3) = 0.068$, $p = 0.935$). (C) Culturing YFP- cells (HP and CP NR cells) with pigmented HP cells did not affect NR cell proliferation ($t = 0.880$, $p = 0.471$). Error bars represent the means ± S.E.Ms (n = 2-3, 4-12 technical replicates per biological replicate).

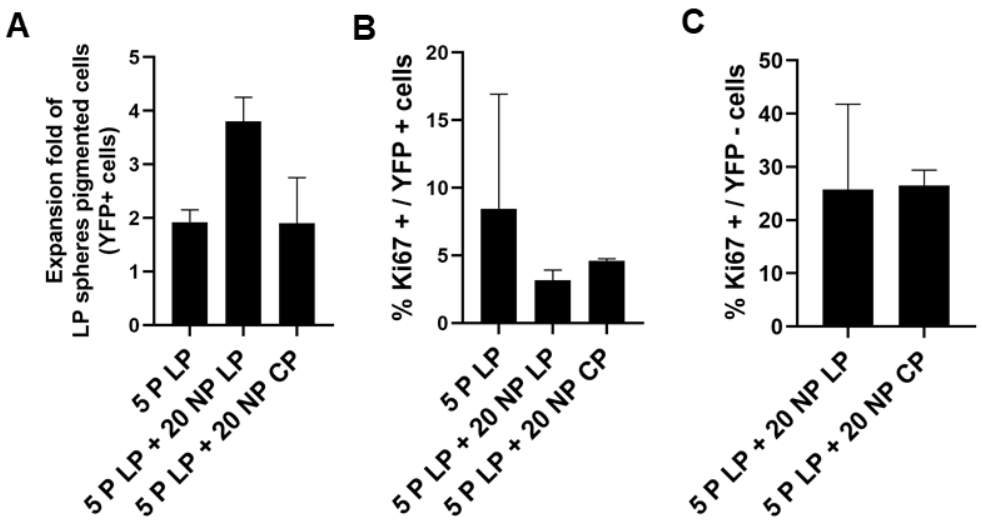

Figure A-15: Late RPE cells in LP spheres are not responsive to extrinsic signals from non-pigmented NR cells. (A) Non-significant differences in the fold expansions of the LP pigmented cells when combined with non-pigmented cells from LP or CP spheres (one-way ANOVA, $F(2,3) = 3.65$, $p = 0.157$). (B) Percentages of YFP+ cells from LP spheres that also are positive for the Ki67 proliferation marker were not different when cultured with non-pigmented NR cells from LP versus CP spheres (one-way ANOVA, $F(2,3) = 0.311$, $p = 0.753$). (C) No changes observed in the percentages of Ki67 positive cells in YFP- cells (NR derived from LP and CP spheres) when co-cultured with pigmented LP cells ($t = 0.047$, $p = 0.966$). Data represent means ± S.E.Ms (n = 2-3).

Figure A-16: Examples of heavily pigmented RSC spheres (HP). Images showing the pigment distribution in clonal HP spheres. Pigment covers most of the surface areas of the spheres.

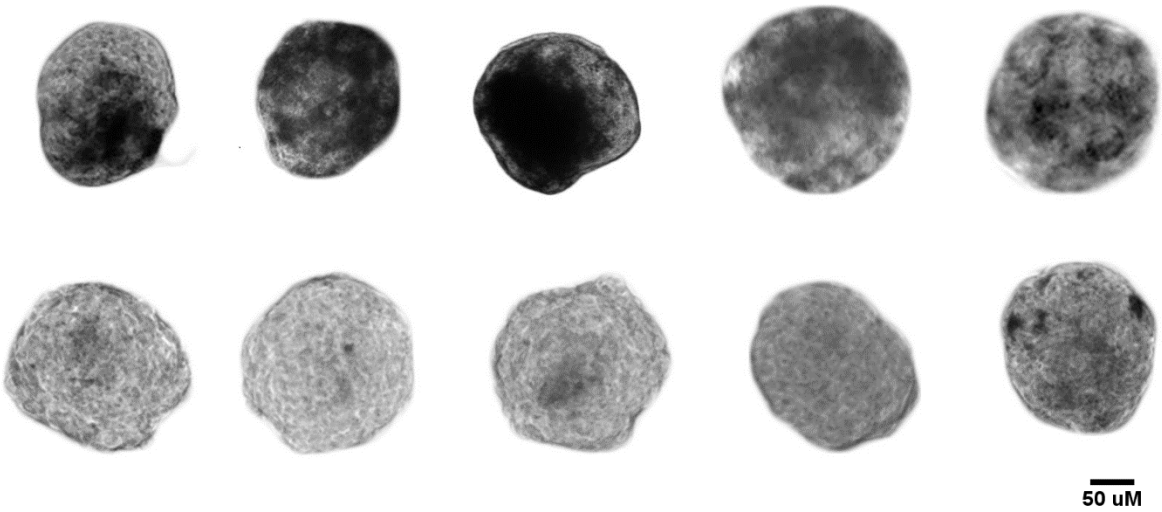

Figure A-17: Examples of lightly pigmented RSC spheres (LP). Images showing pigment distribution in clonal LP spheres. Variable levels of pigmentation with sporadic distributions on the surface areas of spheres.

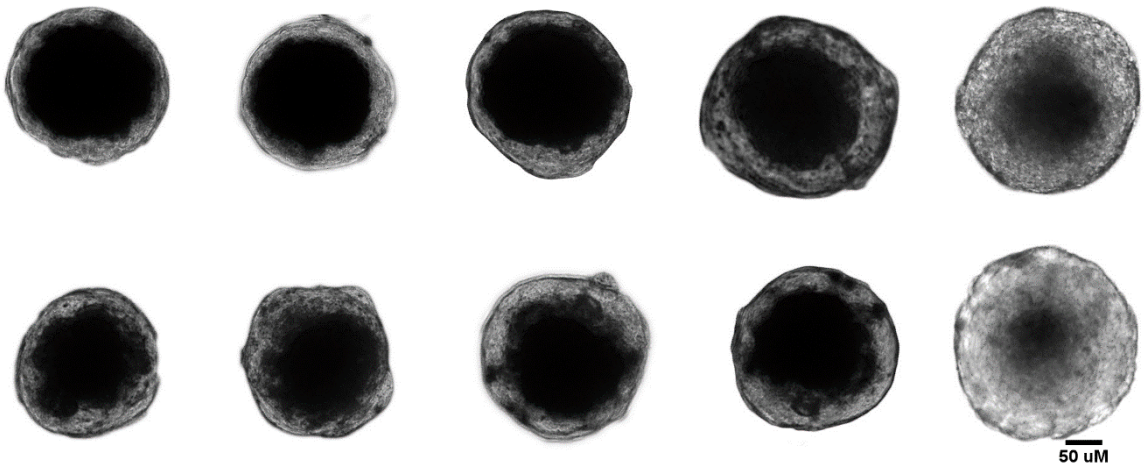

Figure A-18: Centrally pigmented RSC spheres (CP) are characterized by central pigment distributions. Examples showing pigment distributions in clonal CP spheres. The pigment is heavily concentrated in the centers of the spheres surrounded by less pigmented rims.

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Akhtar, T., Xie, H., Khan, M. I., Zhao, H., Bao, J., Zhang, M., & Xue, T. (2019b). Accelerated photoreceptor differentiation of hiPSC-derived retinal organoids by contact co-culture with retinal pigment epithelium. *Stem Cell Research*, *39*, 101491. https://doi.org/10.1016/j.scr.2019.101491

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