CHAPTER 1

GENERAL INTRODUCTION

Vision and the retina

Visual perception of the surrounding world is a complex and important physiological function that, in most instances, mediates our primary interaction with the environment. By sending approximately 30% of sensory inputs to the brain, visual stimuli greatly affect higher cognitive functions, including learning, memory and behavior (Rodieck, 1998). Inputs originate from neurons in the retina, the neurosensory structure in the eye and the most accessible portion of the nervous system. Light enters the eye after penetrating the cornea, passes through the pupillary aperture in the center of the iris and is more finely shifted by the lens to be focused on the retina at the back of the eye, where it stimulates retinal photoreceptors (Figure 1).

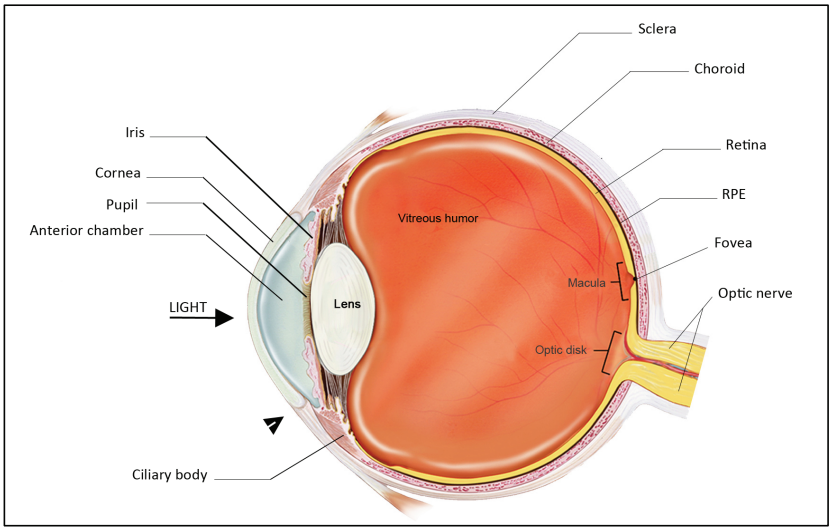

Figure 1. Anatomy of the eye. The eye is comprised of three tunics or layers. The outermost layer is the fibrous tunic, which gives shape to the eye. It consists of the sclera that extends into the cornea to the front, at the corneoscleral junction (arrowhead). The middle layer is the vascular tunic, which gives rise to the iris in the anterior eye, the ciliary body, and the choroid. The ciliary body is responsible for the production of aqueous humor, and the vasculature in the choroid supplies nourishment to the neural retina. The innermost layer is the photoreceptive tunic that comprises the retina. The space between the cornea and the iris, called the anterior chamber, is filled with aqueous humor, while the space between the lens and the retina contains vitreous humor. Light comes into the eye through the cornea, passes through the pupil aperture in the center of the iris, and is more finely shifted by the lens to focus on the retina at the back of the eye. Designed by: Lydia Kibiuk, NIH Division of Medical Arts.

The retina is a layered structure that includes six types of neurons, rod and cone photoreceptors, bipolar, horizontal, amacrine and retinal ganglion cells (RGCs) and one type of (Müller) glia (Figure 2). These cells are organized in three nuclear layers: the

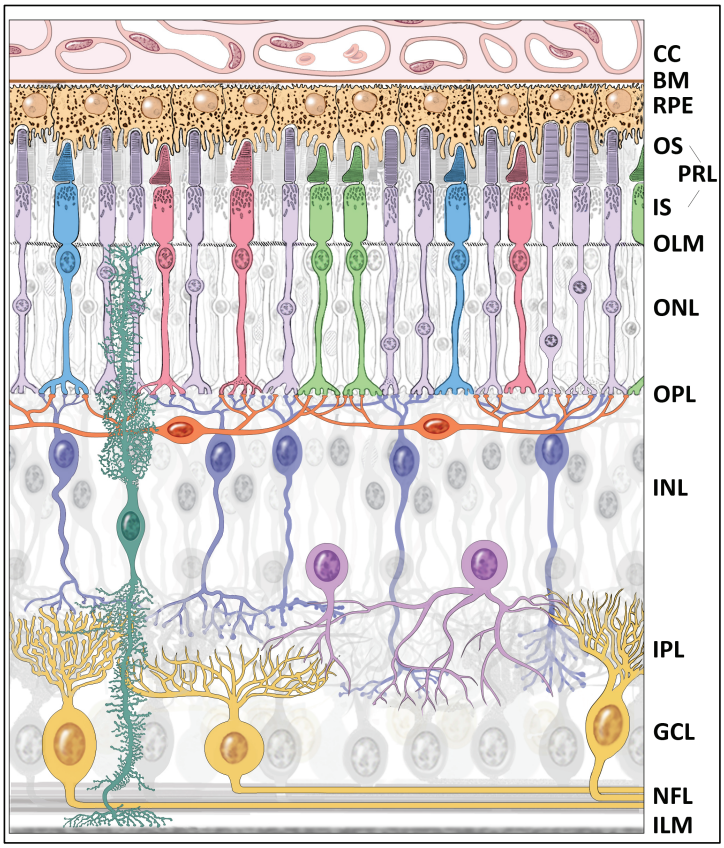

Figure 2. Retinal architecture and histology. The retina is composed of six types of neurons and one type of glia organized in three nuclear layers with synapses in two plexiform layers. The outer nuclear layer (ONL) contains rod (gray) and cone (blue, red and green) photoreceptor cell nuclei; the inner nuclear layer (INL) contains the cell bodies of bipolar (dark blue), horizontal (orange), amacrine (purple), and Müller glia (dark green) cells; and the ganglion cell layer (GCL) contains retinal ganglion cell (RGC) bodies (yellow) and displaced amacrine cells (not shown). Synapses of photoreceptors, bipolar and horizontal cells are localized in the outer plexiform layer (OPL), and synaptic connections of bipolar, RGCs, and amacrine cells in the inner plexiform layer (IPL). RGC axons extend along the inner surface of the retina to form the nerve fiber layer (NFL) and converge toward the optic disc to form the optic nerve. Rod and cone photoreceptors exist alongside each other, project their inner (IS) and outer segments (OS) toward the outer retina, and form the photoreceptor layer (PRL) in close relationship with the retinal pigment epithelium (RPE). The choriocapillaris (CC), separated from the RPE by Bruch's membrane (BM) provide nutrients to the outer retina. Outer (OLM) and inner limiting membranes (INL) are localized at the base of the IS and in the innermost retina, respectively. Designed by: Lydia Kibiuk, NIH Division of Medical Arts.

outer nuclear layer (ONL) containing rod and cone cell nuclei; the inner nuclear layer (INL) containing the cell bodies of bipolar, amacrine, horizontal and Müller glia cells; and the ganglion cell layer (GCL) containing RGCs and displaced amacrine cell bodies. Synapses between photoreceptor, bipolar, and horizontal cells are localized in the outer plexiform layer (OPL) and synapses between bipolar, RGCs and amacrine cells populate the inner plexiform layer (IPL). Müller glia cell bodies span the thickness of the retina, contributing with their end-feet to form the inner limiting membrane (ILM) and the outer limiting membrane (OLM). RGC axons extend along the inner surface of the retina to form the nerve fiber layer (NFL) and converge at the optic disc to form the optic nerve (Figure 1). Rod and cone photoreceptors (Figure 2) exist alongside each other and project their outer segments (OS) to form the photoreceptor layer (PRL).

The highly polarized retinal pigment epithelium (RPE) has intimate relationship with photoreceptor OS and is essential for their functional maintenance. In particular, the RPE phagocytizes the membranous material shed from rod and cone OS, regenerates of 11-cis retinal (a vitamin A derivative) for re-entry into the visual cycle, and absorbs excess light. Disruption of the interaction between RPE and retina is detrimental to photoreceptor function and survival.

Photoreceptor types and distribution vary among species. Rod and cone photoreceptors are differentially distributed within the human retina. At the center of the retina (on the same axis as the pupil and lens and eccentric to the optic disc), a specialized region called the macula contains a higher density of cones compared with the rest of the retina (Figure 1). This area is responsible for central color vision and visual acuity. In the middle of the macula, the retina thins to form the fovea, which contains only cones and is avascular in its center (foveola). In humans and mice, rods represent over 95% of photoreceptors. The remaining 5% (human) and 3% (mouse) are cone photoreceptor subtypes. In the mouse retina, rods are relatively homogeneously distributed and there is no structure resembling the human fovea.

Rods and cones are highly specialized sensory neurons, characterized by distinct morphology, synaptic connections, and gene expression, that reflect their unique

functions in light detection and visual process: rod photoreceptors function in dim light, whereas cones are responsible for chromatic vision and visual acuity in bright light. These differences in light sensitivity are made possible by distinct visual pigments (opsins) that belong to a family of membrane-bound guanine nucleotide binding protein-coupled receptors, and are covalently linked to a vitamin A-derived retinaldehyde chromophore. Light travels through all layers of the neural retina to reach the photoreceptor OS where it is captured by the photosensitive pigments rhodopsin (Rho) in rods and cone opsins in cones. Humans and old world primates have three subtypes of cone photoreceptors: cones sensitive to blue light express short wavelength-sensitive (S)-opsin; cones sensitive to green light express medium wavelength-sensitive (M)-opsin; whereas those sensitive to red light express long wavelength-sensitive (L)-opsin. Only S-opsin and M-opsin are expressed in mouse cone photoreceptors. Absorption of a quantum of light by the opsin results in isomerization of the retinaldehyde and activation of the phototransduction cascade, leading to hyperpolarization of the photoreceptor membrane and decrease in glutamate neurotransmitter release at the photoreceptor-bipolar cell synapse. From bipolar cells, the visual stimulus is conveyed to RGCs, whose axons converge into the optic nerve and extend outside of the orbit to relay information to several processing areas in the brain where neuronal electrical stimuli are interpreted as visual sensation. Horizontal and amacrine cells integrate and modulate visual stimuli as they are transmitted from photoreceptors to RGCs.

Photoreceptor development in human and mouse

During development, the retinal neuroepithelium is populated by proliferating, undifferentiated, multipotent retinal progenitor cells (RPCs), from which all neural retinal cell types originate in a sequence initiating from the central retina and proceeding toward the periphery (Dyer & Cepko, 2001; Livesey & Cepko, 2001). Extrinsic and intrinsic factors regulate cell cycle progression, exit and differentiation. RPCs are maintained in a proliferative and undifferentiated state by several pathways, including

those activated by epidermal growth factor (EGF), fibroblast growth factor (FGF), sonic hedgehog (SHH), Wnt, and Delta/Notch. Master genes such as *Pax6* and *Six3* regulate the intrinsic transcriptional activity.

The SHH pathway comprises SHH, its receptor PTC and the effector protein smoothened (SMO). SHH de-represses SMO from PTC control, allowing the intracellular signaling cascade that leads to activation of cell proliferation genes (Jensen & Wallace, 1997; Oliver et al, 2003). Similarly, classic Wnt signaling through Frizzled and β-catenin activate c-*myc* and *cyclins*, sustaining cell proliferation (Ahmad et al, 2004; Sanchez-Sanchez et al, 2010). Interaction of Notch with its receptor Delta initiates a cascade leading to the expression of two basic helix-loop-helix (bHLH) transcriptional repressors (HES1 and HES5) that prevent transcription of proneural genes, required for differentiation (Hatakeyama et al, 2004; Henrique et al, 1997). This phenomenon is known as "lateral inhibition". Cells that escape lateral inhibition initiate differentiation. The competence model of retinal development explains the sequence of differentiation events whereby, over time, RPCs pass through stages of competence to produce a particular subset of retinal cells (Cepko et al, 1996). RGCs are generated first, followed in overlapping waves by cone photoreceptor, amacrine, horizontal, rod photoreceptor, bipolar and finally, Müller glia cells (Marquardt, 2003).

In the stereotypical progression of cell differentiation, cone genesis is initiated from common proliferating RPCs, in a sequential manner after RGCs and before horizontal and amacrine neurons. Rod birth is initiated later compared to cone genesis, but it precedes bipolar and Müller glia cells. However, the time intervals during which full complements of cone and rod photoreceptors are generated largely overlap (Marquardt, 2003). The last cells to exit the cell cycle are those at the retinal margin. Two key elements control the commitment and differentiation of photoreceptors: gene-regulatory networks that confer competence to the RPC and dictate differentiation events, and extrinsic factors that modulate transcriptional cascades in differentiating RPCs and later modulate cell–cell communication (Figure 3 and Belliveau & Cepko, 1999; Bradford et al, 2005; Livesey & Cepko, 2001). At the time of their last mitosis and

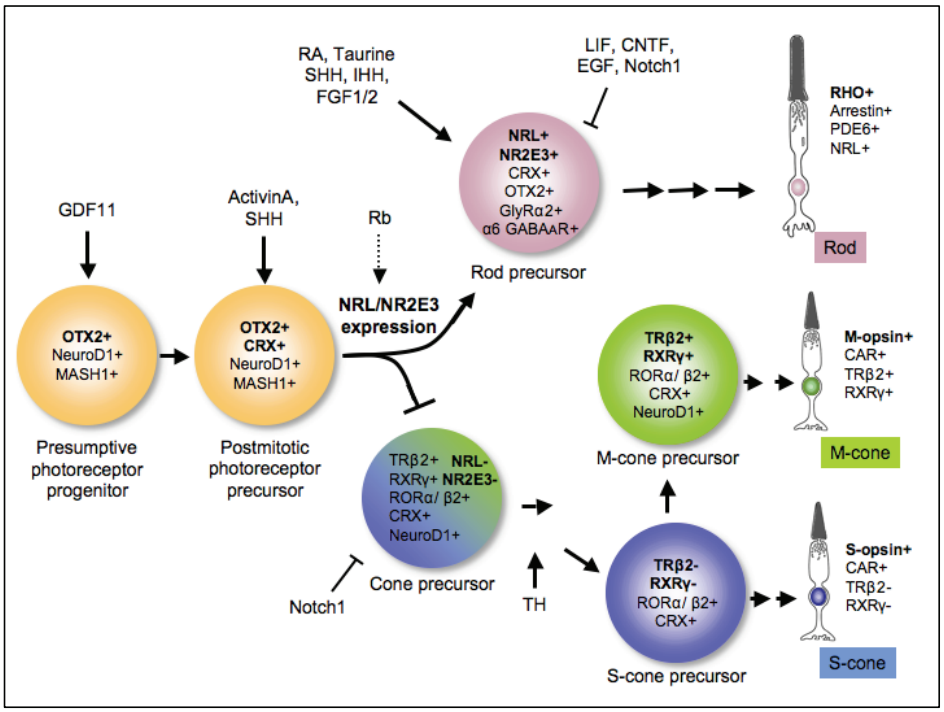

Figure 3. Model of photoreceptor development in the murine retina. Rods, S and M cones are generated from a common pool of retinal progenitor cells (RPCs), which exit the cell cycle at specific times during retinogenesis. A synergistic interaction between intrinsic and extrinsic factors progressively restricts cell fate, biasing individual cells towards differentiation into a unique mature cell type. Some of the known molecules involved are illustrated here. Proteins that appear to be major contributors to specification/determination of cell fate are shown in bold. Expression of Otx2 and Crx defines the postmitotic pool of cells fated to become photoreceptors (photoreceptor precursors). Expression of Nrl and its target Nr2e3 determines rod photoreceptor cell fate. Photoreceptor precursors that do not express Nrl/Nr2e3 progress towards the cone lineage. Upon downregulation of TRβ2 and RXRγ, cone precursors become S cone precursors and synthesize S-opsin. For M cone precursors to develop, S-opsin expression must then be repressed by the heterodimer TRβ2/RXRγ, and M-opsin synthesis initiated by another TRβ2-containing complex. Arrows indicate active promotion and truncated lines inhibition of a developmental stage. Dotted lines indicate tentative roles. Adapted from: I.O. Nasonkin et al. Photoreceptor development: Early steps/fate, in Encyclopedia of the Eye. Volume 3, pp. 332-339, 2010.

prior to exiting the cell cycle, RPCs fated to become photoreceptors upregulate the expression of paired-class homeodomain transcription factor orthodenticle protein homolog 2 (OTX2) (Nishida et al, 2003). OTX2 plays an important role as an early factor that specifies photoreceptor cell fate, and probably as a late factor that may promote terminal differentiation by participating in upregulation of photoreceptor-specific genes

(Koike et al, 2007). At the molecular level, OTX2 activates the promoter of cone–rod homeobox (*Crx*) transcription factor gene, encoding a closely related homeodomain transcription factor (Omori et al, 2011). OTX2 and CRX cooperate to complete photoreceptor differentiation programs (reviewed in Swaroop et al, 2010).

Postmitotic precursor cells become committed toward rod or cone fates once photoreceptor fate is specified (Figure 3). The Maf family basic motif-leucine zipper transcription factor NRL and its target, the photoreceptor-specific orphan nuclear receptor NR2E3, are the primary determinants of rod versus cone cell-fate (Cheng et al, 2006; Cheng et al, 2004; Mears et al, 2001). S cone cell fate is specified as a default pathway and inhibition of this pathway by NRL/NR2E3 is permissive to rod specification (Jia et al, 2009; Ng et al, 2011; Oh et al, 2008; Swaroop et al, 2010). Following this, NRL and NR2E3 – together with CRX – lead to the expression pattern typical of mature rod photoreceptors (Figure 3).

Cone precursors must go through an additional specification process that determines distinct cone sub-types (Figure 3). In mouse and human cone precursors, S-opsin expression is detected first, followed by M-opsin expression. In mice, onset of cone opsin expression is regulated by numerous factors; these include CRX, members of the nuclear receptor family, thyroid hormone nuclear receptor beta 2 isoform (TRβ2) and retinoid X nuclear receptor gamma (RXRγ), and of the retinoic acid (RA) receptor-related orphan receptor – RORα and RORβ2 (Ng et al, 2011).

Several extrinsic factors contribute to rod development (Figure 3). As mentioned, cell–cell interaction mediated by Notch1 is known to sustain the undifferentiated and proliferating state of RPCs, repressing neuronal fate in general (Hatakeyama et al, 2004; Henrique et al, 1997). Recently, Notch1 signaling has been shown to specifically inhibit photoreceptor fate. This function of Notch1 may allow differentiation of other neuronal cell types (Jadhav et al, 2006; Yaron et al, 2006). Other extrinsic factors inhibiting rod fate are leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and EGF (Ahmad et al, 1998; Ezzeddine et al, 1997; Graham et al, 2005). Furthermore, taurine,

secreted by RGCs, is suggested to stimulate rod photoreceptor production through glycine (Gly) and gamma aminobutyric acid (GABA(A)) receptors (Young & Cepko, 2004). Proper maturation and/or migration and integration of young rods into the ONL may require gradients of Indian Hedgehog (IHH) secreted by the RPE, and SHH secreted by RGCs. IHH may also have a role in the specification of photoreceptor fate, possibly inducing *Nrl* gene expression in photoreceptor precursors (Dakubo et al, 2008; Levine et al, 1997). Similarly, FGF family members – acidic FGF (FGF1) and basic FGF (FGF2) – are implicated in rod maturation and may induce *Nrl* expression (Bugra et al, 1994). The role of RA in promoting rod photoreceptor differentiation is still unclear, though it can induce *Nrl* expression through RA-responsive sites present in *Nrl* promoter (Khanna et al, 2006).

Maturation of committed precursors to differentiated functional photoreceptors is a lengthy process and involves expression of cell-type-specific phototransduction genes, biogenesis of OS, and formation of synapses with specific interneurons. Expression of most photoreceptor-enriched genes depends on the synergistic or antagonistic actions of NRL, NR2E3, and CRX and their interaction with other regulatory proteins. In most instances, these proteins co-occupy the promoter/enhancer regions of their target genes (Swaroop et al, 2010).

Retinal dystrophies, photoreceptor degeneration and therapeutic approaches

In 2004, the World Health Organization (WHO) estimated that 4.7% of the world population (> 500 million individuals) was visually impaired, of which, 0.7% (> 45 million) were blind (Resnikoff et al, 2008). In developing countries, the most common causes of blindness are cataract and uncorrected refractive error. However, in developed countries, progressive dysfunction and loss of retinal photoreceptors is the predominant cause of visual impairment and blindness, with age-related macular degeneration (AMD) accounting for > 50% of blindness cases.

Retinal dystrophy is a generic term that refers to pathological conditions associated with retinal photoreceptor dysfunction and loss, and includes retinitis pigmentosa (RP), AMD, Usher syndrome, Leber congenital amaurosis (LCA) and diabetic retinopathy (DR), that are for the most part incurable diseases (Wright et al, 2010). Despite the unifying feature of photoreceptor degeneration, the clinical characteristics, etiology, genetics, molecular, and cellular mechanism underlying retinal dystrophies are very diverse. Primary rod deficiencies leading to loss in early-mid life, followed by loss of cones (rod-cone dystrophies) are most common in RP (Sahel et al, 2010). However, cones also can be the primary (cone-rod dystrophy) or sole (cone dystrophy) dysfunctional cell type. Macular degeneration is distinguished from RP because degeneration is localized to the macula and it involves rod, cones, and the RPE (Swaroop et al, 2009). In particular, in AMD, progressive accumulation of deposits between the RPE and Bruch's membrane (Figure 2), render the former progressively dysfunctional with consequent effects on photoreceptor function and, ultimately, survival. Finally, in DR, photoreceptor loss has been considered for a long time secondary to defects of the retinal microcirculation caused by uncontrolled elevated glucose levels in the blood. However, more recently, the existence of primary photoreceptor deficiencies has become evident (Antonetti et al, 2006; Barber et al, 1998).

Photoreceptor death is comparable to that of any other neuron. Thus, approaches for photoreceptor regeneration share similarities to those applied to other areas of the nervous system. Like the rest of the nervous system, the mammalian retina lacks the capacity to regenerate following damage and there are at present no regenerative therapies available for degenerative retinal diseases (Comer et al, 2005; Hogg et al, 2007; Rattner & Nathans, 2006).

Current and tested treatments, aimed at slowing the degeneration process and improving photoreceptor function in RP, include laser photocoagulation (LPC), photodynamic therapy (PDT) with verteporfin (Visudyne®), transpupillary thermotherapy (TTT), and specialized surgery (Sahni et al, 2011). In DR, the standard of care is focal laser photocoagulation (FLP). Novel approaches include therapies aimed at

limiting disease progression with inhibitors of advanced glycosylation end-products (AGEs) and antioxidants (Comer & Ciulla, 2005), anti-vascular endothelial growth factor (VEGF) antibody therapies (Brown et al, 2011; Campochiaro et al, 2011a), and corticosteroid medications (Campochiaro et al, 2011b; Pearson et al, 2011) for diabetic macular edema (DME) and proliferative DR, alone or in combination (Witkin & Brown, 2011).

In AMD, the recent introduction of anti-VEGF antibody therapies has been successful in improving vision in 40% of patients treated and in stabilizing the condition of the remaining 60% (Martin et al, 2011 and E. Chu, personal communication). This therapy is classified as anti-angiogenic and targets choroidal outgrowth (abnormal angiogenesis of the choroidal circulation supplying the RPE on the basal side), which is a hallmark of late stage AMD. However, anti-VEGF treatments modify the permeability properties of the choroidal vasculature and possibly slow growth, but do not drive retraction of the newly formed vessels. In some trials, anti-platelet-derived growth factor (PDGF) antibodies have been injected together with anti-VEGF antibodies in the hope to facilitate regression of neovascularization (Mabry et al, 2010).

One successful approach is the recent gene therapy applied for LCA, a very early onset subtype of RP associated with mutations in the RPE-specific gene *RPE65*. *RPE65* mutations ultimately cause retinal degeneration by interfering with the availability of 11-cis-retinal for the phototransduction pathway (Redmond et al, 1998). Young patients treated with an AAV-based vector expressing the wild type form of RPE65 protein, permanently recovered a substantial part of their visual function (Bainbridge et al, 2008; Jacobson et al, 2011; Maguire et al, 2008; Simonelli et al, 2010). An alternative approach to gene therapy is replacement of 11-cis-retinal with a synthetic retinoid. A clinical trial (ClinicalTrials.Gov, Safety/Proof of Concept Study of Oral QLT091001 in Subjects With LCA or RP Due to RPE65 or LRAT Mutations

http://clinicaltrials.gov/ct2/show/NCT01014052?term=koenekoop+mcgill&rank=1; http://www.qltinc.com/development/products/QLT091001.htm) is currently recruiting patients in Canada (in US, Holland, Germany and the UK in the future) to evaluate the

efficacy and safety of oral administration of the drug QLT091001 in LCA and RP due to mutations in RPE65 and lecithin-retinyl acyltransferase (LRAT).

Despite current and potentially future successes, pharmacological and gene therapy approaches require a substrate of surviving photoreceptors to act upon. At mid and late stages of disease, the numbers of functional neurons might not be sufficient for rescuing visual function, making cell replacement a more promising therapeutic option.

Regeneration and cell replacement in the adult retina of lower vertebrates

In the last twenty years, our understanding of the nervous system and its adult regenerative capacity as well as insight into cell plasticity have set the ground for studies into neurogenesis in the retina and the possibility of cell replacement therapies. Adult neurogenesis is a known phenomenon in a restricted group of vertebrates. Unlike mammals, whose adult regenerative capacity is limited, some lower vertebrates including urodele amphibians (e.g., salamanders and newts) and teleost fish (e.g., zebrafish, goldfish and rainbow trout) show remarkable ability throughout their lifespan to generate and repair damaged tissues and organs, including the retina.

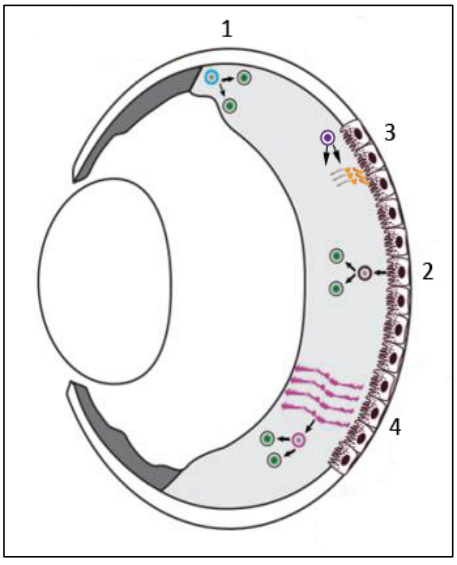

Figure 4. Stem cell niches in adult lower vertebrates. In salamanders and fish, new retinal cells are constantly added from a pool of specialized progenitor cells localized in the circumferential germinal zone/ciliary marginal zone (CGZ/CMZ) to sustain growth of the eye (1). Upon injury, salamander retinal pigment epithelial (RPE) cells de-differentiate, re-enter the cell cycle and replace lost cells by differentiating again into all retinal cell types (2). In fish, a sparse population of rod precursors (probably derived from Müller glia) proliferates at a very slow rate to add new rod photoreceptors in the healthy and damaged retina (3). Furthermore, upon injury, a subpopulation of Müller glia cells start proliferating and can differentiate to generate all types of lost retinal cells (4). Neurogenesis has also been observed from cells in the CGZ/CMZ in the post-hatched chick and from Müller glia in chicks and rodents. Adapted from: M.O. Karl & T.A. Reh, Trends in Molecular Medicine, 2010.

There are four mechanisms described for neurogenesis in the adult lower vertebrate neural retina (Figure 4): (1) differentiation of cells from the circumferential germinal zone, also known as ciliary marginal zone (CZG/CMZ), (2) transdifferentiation of the RPE, (3) migration and differentiation of rod precursors, and (4) proliferation followed by differentiation of Müller glia.

Differentiation of cells from the Circumferential Germinal Zone/Ciliary Marginal Zone

In association with continuous body growth, fish and amphibian eyes continue to enlarge throughout their entire lifetime under control of the growth hormone (GH)-insulin-like growth factor-I (IGF-I) axis (Boucher & Hitchcock, 1998; Otteson et al, 2002; Zygar et al, 2005). New retinal cells, including photoreceptors, are constantly added to the intact adult retina from at least two sources of cells (niches) and with different mechanisms (reviewed in Fleisch et al, 2011; Stenkamp, 2007): 1) cells from the CMZ/CGZ (Figure 4-1) serve as neurogenic pools from which all retinal cell types are generated (Wetts et al, 1989; Fausett & Goldman, 2006); 2) subsets of Müller glia (see below) give rise to rod photoreceptors and can respond to cell damage (Figure 4-4). CMZ/CGZ-derived newly differentiated retinal cells are constantly added to the most peripheral edge of the retina at a rate that parallels whole body growth. Neurogenic cells residing in the CMZ/CGZ have the appearance of neuroepithelial cells: they are polarized with elongated shapes, spanning the basal-apical axis, and are arranged in a single cell layer (Stenkamp, 2007). They were originally considered remnants of the embryonic retinal neuroepithelium (Johns, 1977). However, CMZ/CGZ adult neurogenesis appears to be regulated by distinct genetic pathways from those regulating developmental neurogenesis (Wehman et al, 2005), thus arguing for a distinct ontogenesis of this cell pool. CMZ/CGZ cells incorporate the S-phase marker bromodeoxyuridine (BrdU), express cell cycle markers, including proliferating cell nuclear antigen (PCNA), and key transcription factors that regulate retinal neurogenesis, such as retinal homeobox 1 (RX1), visual system homeobox 2 (VSX2/CHX10) and paired box gene 6a (*PAX6a*) (Raymond et al, 2006).

A zone of proliferating cells at the peripheral margin of the post-hatched chicken retina has also been identified, displaying features similar to those of the CMZ/CGZ of lower vertebrates and persisting up to three weeks post hatching (Fischer & Reh, 2000). Based on marker expression, these cells appear to share characteristics of embryonic multipotent progenitors. The size of a hypothetical marginal zone appears to be progressively restricted from lower to higher vertebrates, with opossums displaying only a few cells and no marginal zone visible in the mouse (Kubota et al, 2002; Moshiri & Reh, 2004). However, the human peripheral retina has been suggested to resemble the CMZ/CGZ, based on the absence of lamination and the presence of cells expressing stem cell markers, which have been shown to be Müller glia (Bhatia et al, 2009).

Transdifferentiation of the Retinal Pigment Epithelium

In adult amphibians, upon damage or removal, retinal cells and a new retina are generated predominantly from transdifferentiation of the RPE (Figure 4-2) (Keefe, 1973; Stone, 1949; Stone, 1950; Stone & Steinitz, 1957). RPE cells first de-differentiate, i.e., lose their pigmentation and detach from the basement membrane, then, proliferate and differentiate to produce a new pigmented and a non-pigmented layer (Klein et al, 1990; Mitashov, 1996; Reh, 1987; Stroeva & Mitashov, 1983; and reviewed in Del Rio-Tsonis & Tsonis, 2003; Moshiri et al, 2004). The non-pigmented layer initiates expression of retinal progenitor markers and follows a differentiation path that recapitulates developmental retinogenesis (Bugra et al, 1992; Cheon et al, 1998; Cheon et al, 2001; Cheon & Saito, 1999; Mitashov et al, 1995; Negishi et al, 1992; Sakakibara et al, 2002) and appears to be regulated by fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), and hedgehogs signaling, via MapK and Mitf transcription factors (Sakaguchi et al, 1997). Similar mechanisms underlie repair in the embryonic retina of amphibians (Mitashov, 1997) and chickens (Park & Hollenberg, 1989; Park & Hollenberg, 1991; Pittack et al, 1997). In the latter, however, the polarity of the newly formed retina is reversed. Rodent embryonic RPE cells under restricted conditions can regenerate

neural retina *in vitro* (Neill & Barnstable, 1990; Zhao et al, 1995), though the transdifferentiation potential of rodent RPE cells is limited.

Migration and differentiation of rod precursors, and proliferation followed by differentiation of Müller glia

The intact adult zebrafish INL maintains proliferating rod progenitors that migrate to the ONL (Figure 4-3), where they assume characteristics of rod precursors, and eventually differentiate to rod photoreceptors to accompany eye size expansion (Johns, 1982; Mack & Fernald, 1997). Recent data using transgenic reporter gene expression and morpholino knockdown suggest that INL proliferating rod progenitors originate from a subpopulation of Müller glia (Bernardos et al, 2007; Braisted et al, 1994; Fausett & Goldman, 2006; Raymond et al, 2006; Thummel et al, 2008b). In the intact retina, the neurogenic role of Müller glia is minimal, as indicated by the slow proliferation rate of the INL progenitors (Bernardos et al, 2007; Julian et al, 1998; Otteson et al, 2001). Yet, proliferation rate is higher toward the retinal periphery, in proximity of the CMZ/CGZ (Johns, 1982; Julian et al, 1998).

In the damaged zebrafish retina, sparse photoreceptor loss is compensated for by proliferation and terminal differentiation of the photoreceptor precursor population (Figure 4-3). However, when acute and massive retinal degeneration occurs, a subpopulation of Müller glia (Figure 4-4) re-enter the cell cycle, proliferate, and ultimately, undergo asymmetric division to generate new retinal progenitors (Bernardos et al, 2007; Montgomery et al, 2010). This process involves downregulation of the post-mitotic cell specific genes glial fibrillary acidic protein (*GFAP*) and glutamine synthetase (*GS*) and upregulation of Notch-Delta signaling pathway genes, together with brain lipid binding protein (BLBP) (Thummel et al, 2008a). At the same time, N-cadherin (Cdh2) is upregulated and relocates to the basolateral plasma membrane. De-differentiated Müller glia proliferate to form small neurogenic clusters and differentiate into all retinal

cell types to restore the integrity of all layers (Raymond et al, 2006), after which, Müller glia cells return to quiescence.

A similar response is elicited in the retina of post-hatch chicks (Fischer & Reh, 2001). However, Müller glia cell proliferation and neurogenesis are limited in chicken compared to fish (Fischer & Reh, 2002) and although markers of differentiation can be detected, there is not evidence yet of functional integration of differentiated retinal cells in the damaged retina.

Müller glia- and Ciliary Epithelium-derived cells in mammals

Generation of all retinal cell types in mammals occurs during embryonic development and extends to early postnatal development in some species. After that, no further substantial neurogenesis or gliogenesis occur in the intact or injured retina. As mentioned, in the developing mammalian retina, all cell types originate from a common RPC that undergoes transitory competence states to generate, at stereotyped times and locations, retinal neuronal and glial phenotypes (Cepko et al, 1996; Livesey & Cepko, 2001; Turner & Cepko, 1987). RPCs are no longer present in the fully laminated, functional retina upon completion of development.

Greater understanding of adult neurogenesis in lower vertebrate eyes has encouraged investigations into the existence of potentially similar, yet dormant, cell types and mechanisms in mammals. The focus in recent years has been on Müller glia- and ciliary epithelium (CE)-derived cells.

Müller glia and retinal repair in mammals

Müller glia cells generally respond to degeneration and injury in mammals by initiating a reactive response resulting in upregulation of filamentous proteins such as GFAP and vimentin, a phenomenon known as reactive gliosis (Grosche et al, 1995; Lewis & Fisher, 2003; Sarthy & Egal, 1995). Similar responses can be elicited by treatment with growth factors such as FGF2 (Lewis et al, 1992) and interleukin 6 (IL-6) family members

such as CNTF and LIF (Wahlin et al, 2000; Xue et al, 2011). Insights into adult neurogenesis in the brain, showing that neural stem cells have characteristics similar to developmental radial glia (Ming & Song, 2011), and into regenerative processes in zebrafish (Bernardos et al, 2007) and in non-mammalian vertebrates such as the chicken (Fischer & Reh, 2001; Karl et al, 2008) suggest the hypothesis that Müller glia cells are the stem cell of origin in the adult mammalian retina (Ming & Song, 2011). The similarity of Müller glia gene expression profiles with those of retinal progenitors (Blackshaw et al, 2004) and neural stem cells (Das et al, 2006a) further support that hypothesis.

In the adult rat, N-Methyl-D-aspartic acid (NMDA) neurotoxic damage elicits proliferation of a small subpopulation of Müller glia that subsequently differentiate to express mature retinal cell markers, predominantly Rho (rod) and recoverin (photoreceptor and bipolar cell) (Ooto et al, 2004; Wan et al, 2008). The proportion of Müller glia re-entering the cell cycle and differentiating to mature retinal phenotypes can be increased by stimulation with growth factors, EGF, FGF, glial cell-derived neurotrophic factor (GDNF), and insulin, or of the SHH/Wnt/β-catenin pathway (Close et al, 2006; Insua et al, 2008; Osakada et al, 2007; Wan et al, 2007). Stimulation of Notch and Wnt signaling, in fact, may be sufficient to promote Müller glia transdifferentiation to photoreceptors even in the absence of retinal damage (Del Debbio et al, 2010). Furthermore, the retinal cell phenotypes generated and their proportions can be manipulated in rat either by RA treatment, to increase bipolar cells, or by overexpression of specific transcription factors genes such as *Math3*, *NeuroD1*, *Pax6* and *Crx* (Ooto et al, 2004) to generate virtually all cell types in the retina. In mouse, injection of either EGF, FGF1, or FGF1 and insulin biases differentiation toward amacrine cells (Karl et al, 2008). Notably, light damage induces upregulation of EGF receptor (EGFR) in rat Müller glia cells, restoring the mitogenic response to EGF stimulation observed during development (Close et al, 2006). De-differentiation to retinal progenitor and differentiation to retinal cell phenotypes can also be induced in mouse Müller glia by administration of sub-toxic doses of glutamate or its analogue alpha-aminoadipate (α-AA), suggesting not only that injury-induced Müller glia

proliferation/differentiation might be mediated by glutamate but also offering a potential pharmacological approach to retinal repair (Takeda et al, 2008). Finally, Müller glia cells proliferate and migrate towards the site of injury following laser photocoagulation damage in mouse, although they do not seem to undergo transdifferentiation (Tackenberg et al, 2009).

Cells with Müller glia characteristics that proliferate in response to EFG treatment and co-express markers of differentiated glia such as GFAP, cellular retinaldehyde-binding protein (CRALBP) and vimentin, and of neural progenitors such as Nestin, CHX10, SOX2, and Notch1, were recently identified at the non-laminated margin of the intact human retina, re-proposing the existence in mammals of an area comparable to the CMZ/CGZ in fish and amphibians (Bhatia et al, 2009). Nonetheless, the degree of cell proliferation and differentiation remains limited in mammals compared to lower vertebrates. It has been suggested that adult Müller glia quiescence is maintained by at least two molecular mechanisms: transforming growth factor (TGF)β2 release from retinal neurons and downregulation of EGFR in mature Müller glia (Close et al, 2005).

Spontaneously immortalized human Müller glia cells were first cultured *in vitro* (Limb et al, 2002). Subsequently, several cultures of human Müller glia cells (MIO cell lines) have been derived from the neural retina of postmortem healthy donors spanning a wide range of ages from infancy through aging (Lawrence et al, 2007). Besides displaying features of mature Müller glia such as CRALBP, glutamine synthetase, and vimentin expression, and depolarization in response to glutamate without change in membrane resistance, MIO cells express EGFR and retinal (e.g., CHX10) and neural (e.g., SOX2) progenitor markers to different degrees, depending on culture conditions (Lawrence et al, 2007). Upon exposure to differentiating conditions *in vitro*, Müller glia cell lines can be induced to express markers of differentiated retinal neurons, including recoverin, S-opsin, peripherin, calretinin, and Brn3. Similarly, when grafted in the subretinal space of retinal degeneration rat models, MIO cells migrate and acquire markers of mature retinal cells (Lawrence et al, 2007).

In summary, Müller glia spontaneously respond to retinal damage and can be manipulated *in situ* to display retinal phenotypes. Cultures, transplantation and differentiation of human Müller glia cell lines suggest that Müller glia could be a viable source of cells for retinal replacement.

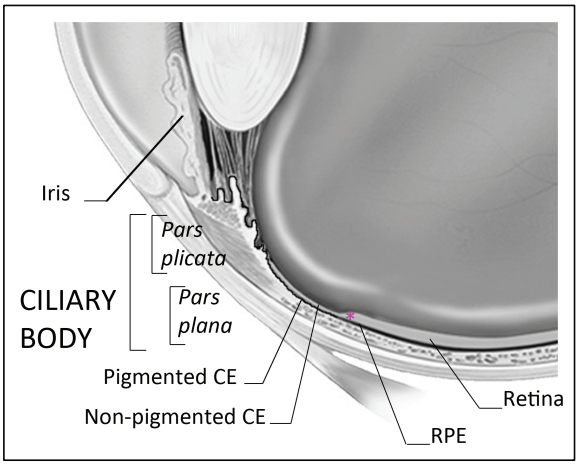

Figure 5. Ciliary body anatomy. The ciliary body is located between the peripheral margin of the retina and the iris. The neural retina extension of the ciliary body comprises two layers of epithelium that share with the retina their neuroectodermal origin, the pigmented and non pigmented ciliary epithelia (CE). Their architecture is flattened on the retinal side (*pars plana*) and folded on the iris side (*pars plicata*). The asterisk labels the peripheral retina, a non-layered portion of the retina in rodents that has been suggested to correspond to the CMZ in lower vertebrates. Designed by: Lydia Kibiuk, NIH Division of Medical Arts.

Retinal Stem Cells from Ciliary Epithelium of mammals

Retinal stem cells (RSCs) from the CE (Figure 5) were first isolated from mouse (Tropepe et al, 2000) and rat (Ahmad et al, 2000). Subsequently, reports were published on the isolation from human (Coles et al, 2004), rabbit (Inoue et al, 2005), and pig (MacNeil et al, 2007). RSCs express retinal progenitor transcription factors such as CHX10, PAX6, SIX6, SIX3, and RX (Ahmad et al, 2004; Lord-Grignon et al, 2006; Xu et al, 2007b; Yanagi et al, 2006) and share approximately 80% of their expressed genes with developmental RPCs (Das et al, 2005), including FGF, Notch, and SHH signaling pathway genes (Ahmad et al, 2004; Moshiri & Reh, 2004). Despite the definition as "stem cells", all reports concur that RSC have a limited capacity for *in vitro* self-renewal (Engelhardt et al, 2004; Inoue et al, 2005; MacNeil et al, 2007; Xu et al, 2007a). Neurosphere cultures of RSCs can be maintained *in vitro* for a relatively small number of passages (8-

10) and undergo senescence within two months from isolation (Inoue et al, 2005). Gene expression profiling of rat RSCs indicates that initial expression of the self-renewal genes *Notch1*, *Delta1*, *Hes*1 and *Hes*5 is downregulated upon passaging and that only a limited number of RPC markers continue to be expressed (James et al, 2004).

In vitro cell population expansion and number of passages can be increased by treatment with growth factors, e.g., EGF and FGF (Ahmad et al, 2000; Coles et al, 2004; Inoue et al, 2005; Mayer et al, 2005; Tropepe et al, 2000), pigment epithelium derived factor (PEDF) (De Marzo et al, 2010), as well as by activation of mitogenic pathways, such as the canonical Wnt pathway (Das et al, 2006b; Inoue et al, 2006). Since, RSCs do not self-renew indefinitely, they fail to meet the requirement to be considered *bona fide* stem cells. Notably, the origin, phenotype and "stemness" of RSCs have recently been challenged (Cicero et al, 2009; Gualdoni et al, 2010). The terms of the controversy will be illustrated in the discussion. For convenience, CE-derived neurosphere cultured cells will still be referred to as RSCs hereafter, mostly to distinguish them from RPCs present in the developing retina.

RSCs cultured *in vitro* can be induced to express genes characteristic of differentiated retinal neurons and glia (Ahmad et al, 2000; Coles et al, 2004; Inoue et al, 2005; Mayer et al, 2005; Tropepe et al, 2000). Temporally controlled activation of the canonical Wnt pathway affects the neurogenic potential of RSCs *in vitro* such that pre-induction activation promotes neurogenesis but activation of the Wnt pathway during induction represses neurogenesis (Das et al, 2006b). Similarly, inclusion of EGF and FGF during the RSC expansion period induces changes in growth factor receptor expression that result in biases in their differentiation potential (Giordano et al, 2007). Furthermore, EGF in the differentiation medium promotes neurogenesis and the appearance of bipolar cell phenotypes, whereas FGF biases differentiation towards cells expressing photoreceptor markers (Giordano et al, 2007). Notably, both mouse and human RSCs infected with a viral vector exogenously expressing the cone-rod transcription factor CRX, upregulate the photoreceptor-specific genes β-6 phosphodiesterase (*β-6PDE*), Rho, and blue opsin (*CNG3*), and hydrolyze cGMP upon

light stimulation, strongly suggestive of their acquired sensitivity to light stimulation (Jomary & Jones, 2008; Jomary et al, 2010).

However, in the absence of RPE *in vitro*, photoreceptor maturation cannot be completed and no OS are observed which would be the hallmark of fully mature functional photoreceptors. To overcome this limitation and also to test their potential for cell replacement in the retina, RSCs have been injected *in vivo*, either in the subretinal space or the vitreous. In general, RSCs survive in allogeneic and xenogeneic hosts after transplantation, showing variable degrees of integration into the intact and degenerating retina, and differentiate into cells expressing markers characteristic of retinal cells that display rudimentary morphological features of neurons (Coles et al, 2004; Kinouchi et al, 2003; Warfvinge et al, 2005). Although the number of integrated cells remains limited, recent data show more efficient generation of functional photoreceptors from human RSCs *in vitro* and *in vivo* after gene transfer and modulation of transcription factors *OTX2*, *CRX*, and *CHX10* (Inoue et al, 2010; Jomary et al, 2010). Furthermore, visual function improved upon transplantation of the transfected cells into the vitreous cavity of transducin mutant mice, a model in which photoreceptors do not degenerate but are not functional.

The evidences above support the hypothesis that cells in neurosphere cultures derived from the CE have partial stem/progenitor cell characteristics. Whether these characteristics are intrinsic or acquired in culture remains to be established. In wild type mice, rare nestin-positive cells can be detected in the non-pigmented layer of the CE up to P18 (Nishiguchi et al, 2008). Furthermore, cells in the P7-21 mouse CE proliferate in response to bFGF and insulin administration *in vivo* and express the retinal progenitor markers PAX6 and CHX10 (Abdouh & Bernier, 2006; Zhao et al, 2005).

Data obtained from *in vivo* studies of retinas undergoing pathological or injury-induced degeneration are more convincing. Proliferating cells labeled with BrdU are found up to P30 in the most peripheral retina of the *rd1* genetic mouse model of retinal degeneration (Nishiguchi et al, 2007). Furthermore, single intravitreal injection of VEGF at P9 dramatically increases the number of BrdU-positive cells in the peripheral retina

and CE. Cells expressing the photoreceptor and bipolar cell marker recoverin can be detected in the *pars plana* of the adult *rd1* mouse up to four months of age, at a time in which the ONL is absent and retinal function is completely lost in untreated animals (Nishiguchi et al, 2009). The majority of these cells appear to be of the cone lineage as shown by peanut agglutinin (PNA) double-staining, with only a small proportion belonging to the rod lineage (Rho double-positive). Some of them appear to have matured to develop cone pedicles (Nishiguchi et al, 2008; Nishiguchi et al, 2009). Nonetheless, VEGF administration does not prevent the normal course of retinal degeneration in *rd1* mice, and photoreceptors are lost by one month of age (Nishiguchi et al, 2007). Similar results can be obtained by induction of photoreceptor degeneration with N-methyl N-nitrosourea (NMU) (Nishiguchi et al, 2009), or following RGC degeneration induced by optic nerve axotomy (Nickerson et al, 2007). In another model of retinal degeneration, the Royal College of Surgeon's (RCS) rat, higher numbers of BrdU-labeled cells compared to controls were detected during the degenerating window (P15-P60) in the peripheral retina and CE (Jian et al, 2009). Cell proliferation is associated with upregulation of *Shh* and *Smo* expression and an increasing number of cells labeled with the retinal progenitor cell marker CHX10 are found in the same timeframe. However, by P90, when degeneration is terminal, both BrdU- and CHX10-labeled cells decrease to levels comparable to controls. In addition, these cells do not seem to have neurogenic potential as none of the BrdU-positive cells could be double-stained with retinal cell markers.

A recent histological/immunohistological study of three adult human pathological post-mortem eyes (from patients who underwent multiple surgeries for retinal detachment and vitreoretinopathy) reports proliferation in the CE, overexpression of EGFR, apparent migration of neurosphere structures into the adjacent vitreous, and expression of early and late photoreceptor markers, potentially indicating reactive neurogenesis in the adult human CE (DuCournau Y 2011). All together these data suggest that a subpopulation of cells in the adult mammalian CE maintains the capacity to respond to cues generated by injury or death of retinal neurons.

Final Remarks

This thesis focuses on CE-derived cells and their applications for transplantation. Cultures derived from mammalian Müller glia and CE constitute a potential source for cell therapies in retinal degenerative diseases. However, both Müller glia and CE-derived cultures require further investigations to validate their potential: a) transplantable cells need be a homogeneous, well characterized population; b) cell numbers should be expandable in the order of millions; c) differentiation should be controlled to generate the desired cell type(s); and d) efficiency of transplantation should be improved. Both cell populations have the advantage over other pluripotent cell sources, such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), to possess phenotypic characteristics and to be developmentally closer to the differentiated retinal cells they are to replace. Although Müller glia cells are more easily expandable, they would be derived from heterologous sources. On the other hand, CE-derived cells could allow autologous transplantations after being obtained from the patient upon a relatively simple vitrectomy procedure. Considerations have also been given to harnessing the regenerative potential of these cells *in vivo* for replacing lost photoreceptors. This approach might be feasible at the very early stages of degeneration, when the numbers of photoreceptors to replace are small and comparable to the number of Müller glia and CE cells in the eye and provided that further degeneration is also prevented. At later stages of degeneration, it is unlikely that larger numbers of progenitor cells be generated by proliferation of Müller glia or CE without increasing the risk of tumor formation, making this approach non-viable at the present status of the research.

Hypothesis

We hypothesize that cells derived from the CE of the porcine eye could be propagated in culture and differentiated to photoreceptors *in vitro* and upon transplantation in the subretinal space of allorecipient pigs.

Aims

- To study the chronological development of the pig retina as a model for retinal differentiation in human.
- To isolate and characterize cell cultures derived from the porcine CE.
- To graft porcine CE-derived cells in the normal porcine retina and evaluate their integration and differentiation potential.

Animal Model

The pig is an attractive non-primate animal model to study retinal biology for several reasons:

- The porcine eye is comparable to the human eye in size and shape;

- Fundus imaging reveals a radial (holangiotic) vasculature and an oval-shaped optic nerve head (Middleton, 2010);

- The thickness and appearance of porcine retinal layers are comparable to human (Johansson et al, 2010);

- The photoreceptor mosaic in pig and human shares a high degree of similarity (Chandler et al, 1999; Curcio et al, 1990). Although the average retinal rod:cone ratio in human is 20:1, compared to 8:1 in pig, cone density in pig is increased in a broad region dorsal to the optic disk, extending both nasally and temporally (*area centralis*), where

the rod:cone ratio decreases to 6:1 and is comparable to the average human macular rod:cone ratio of 10 (Chandler et al, 1999; Hendrickson & Hicks, 2002; Sanchez et al, 2011);

- Both human and pig retina are well developed at birth (Chandler et al, 1999; De Schaepdrijver et al, 1990; Hendrickson et al, 2008), although the gestation period for pig (112–115 days) is significantly shorter compared to human;

- Pig models of retinitis pigmentosa, glaucoma, and retinal detachment have been developed (Iandiev et al, 2006; Petters et al, 1997). Transgenic pigs with systemic expression of green (Park et al, 2001) and red fluorescent proteins (Matsunari et al, 2008) have also been produced;

- The pig eye has been useful for modeling human ocular surgery due to its size, anatomy, and vasculature (Del Priore et al, 1996; Ghosh et al, 2004).

- Pigs have been used for isolation of RPCs, and subretinal allotransplantation of these cells demonstrates their ability to migrate, morphologically differentiate, and express retinal cell markers (Klassen et al, 2007; Klassen et al, 2008).

Using the pig as an animal model was advantageous to us in that the protocols we were planning to develop for isolating CE-derived cells from the porcine eye could easily be applied to human donor tissue. Furthermore, a larger size of CE yields a higher number of cells to work with *in vitro*. Finally, we could more accurately target transplanted cells to selected sites in the subretinal space and test immune response in large animals, besides performing surgical procedures to test conditions for cell transplantation in humans.