Development of an In-vitro Organoid Assay to Study Engraftment of Photoreceptor Precursors

by

En Leh Samuel Tsai

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2017

Abstract

Labeled cells in the host retina following transplantation of reporter-labeled donor cells were thought to arise from donor cell migration and engraftment into the tissue, but more recently this interpretation has been challenged with new findings that material transfer occurs between host and donor photoreceptors. *In-vivo* studies are laborious and impractical to investigate the mechanisms of cell engraftment. As such, we have developed a simpler *in-vitro* assay that uses retinal organoids to monitor photoreceptor precursor engraftment. Dissociated retinal cells have the propensity to self-organize and form spherical laminated structures or organoids that have features in common with normal retinal organization. Here, we characterize the cellular composition and the cell type requirement for efficient organoid formation. We have further developed an engraftment assay, established a cell integration baseline and shown the application of this assay to models of retinal degeneration.
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# Table of Contents

Acknowledgments.................................................................................................................................................. iii
Table of Contents ................................................................................................................................................ iv
List of Figures ................................................................................................................................................... vii
List of Tables .................................................................................................................................................... viii
List of Abbreviations ........................................................................................................................................ ix
Chapter 1.......................................................................................................................................................... 1
  1 Background .................................................................................................................................................. 1
      1.1 General Overview .................................................................................................................................... 1
          1. Neurodegeneration .............................................................................................................................. 1
          2. Retinal Degeneration and Repair ....................................................................................................... 2
      1.2 The Retina ............................................................................................................................................. 3
          1. Overview ............................................................................................................................................. 3
          2. Anatomy and Function of the Retina ................................................................................................. 4
          3. Photoreceptors ................................................................................................................................... 6
          4. Phototransduction and the Pigment Epithelium ............................................................................... 9
          5. Photoreceptor Development ............................................................................................................ 12
          6. Disease and Degeneration ............................................................................................................. 15
      1.3 Photoreceptor Transplantation ........................................................................................................... 16
          1. Overview ............................................................................................................................................ 16
          2. Lineage Specific Reporters .............................................................................................................. 17
          3. Rod and Cone Photoreceptor Transplantation ............................................................................. 18
          4. Material Transfer ............................................................................................................................ 20
1.4 Organoids...........................................................................................................................20

1. Overview...............................................................................................................................20

2. Retinal Organoids..................................................................................................................22

Chapter 2.........................................................................................................................................26

2 Hypothesis and Rationale........................................................................................................26

2.1 Rationale................................................................................................................................26

2.2 Hypothesis............................................................................................................................26

2.3 Objectives.............................................................................................................................26

Chapter 3.........................................................................................................................................27

3 Materials and Methods.............................................................................................................27

3.1 Animals and Genotyping .....................................................................................................27

3.2 Preparation of Glass Surface.................................................................................................29

3.3 Organoid Formation.............................................................................................................29

3.4 Engraftment Assay...............................................................................................................30

3.5 Fixation and Immunostaining...............................................................................................30

3.6 Confocal Imaging, Quantification and Statistics..................................................................32

Chapter 4.........................................................................................................................................33

4 Results.........................................................................................................................................33

4.1 Optimization and Immunocytochemical Characterization of Mouse Retinal Organoid Culture................................................................................................................................33

4.2 Non-photoreceptor cells are required for efficient organoid assembly .........................39

4.3 Organoids are polarized structures that depend on junctional integrity .......................41

4.4 Establishment of Organoid Engraftment Assay...............................................................44

4.5 Rod-GFP donor cells exhibit a unique neurite outgrowth engraftment phenotype in Crx^-/- organoids....................................................................................................................47

Chapter 5.........................................................................................................................................50

5 Discussion..................................................................................................................................50

v
5.1 Establishment of an Organoid Culture.................................................................50
5.2 Utility of organoids in a novel engraftment assay ..................................................51
5.3 Comparisons to established in-vitro retinal models..............................................53
5.4 Photoreceptor motility .........................................................................................54

Chapter 6...................................................................................................................56

6 Conclusion .................................................................................................................56

References......................................................................................................................57
List of Figures

Figure 1.1 – Structure of the retina................................................................. 4

Figure 1.2 – Schematic of the phototransduction cascade.................................7

Figure 1.3 – Structure of rod and cone photoreceptors................................... 9

Figure 1.4 – Transcriptional control of photoreceptor development ...................12

Figure 1.5 – Timeline of retinal organoid research........................................ 21

Figure 4.1 – Schematic and localization of retinal organoid formation.............. 32

Figure 4.2 – 3-dimensional and post-mitotic nature of retinal organoids...............33

Figure 4.3 – Immunocytochemical characterization of retinal organoids............34

Figure 4.4 – Cell-type require for organoid formation..................................... 36

Figure 4.5 – Polarization markers and calcium sensitivity in retinal organoids..........39

Figure 4.6 – Organoid engraftment assay.....................................................42

Figure 4.7 – Donor cell engraftment into degenerate organoids........................ 45
List of Tables

Table 3.1: Summary of mouse lines used.......................................................... 25

Table 3.2: Primer sequences used for genotyping........................................... 25

Table 3.3: List of antibodies used for immunocytochemistry............................. 28
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARR</td>
<td>Cone arrestin</td>
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<tr>
<td>CRALBP</td>
<td>Retinaldehyde binding protein-1</td>
</tr>
<tr>
<td>CRX</td>
<td>Cone rod homeobox</td>
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<tr>
<td>DIV</td>
<td>Days in-vitro</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>EdU</td>
<td>5-Ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
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<td>Ganglion cell layer</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber’s congenital amaurosis</td>
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<td>LGN</td>
<td>Lateral geniculate nucleus</td>
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<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
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<tr>
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<td>Inner plexiform layer</td>
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<td>Neural cadherin</td>
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<td>OLM</td>
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<tr>
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<td>Peanut agglutinin</td>
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<td>Retina and anterior neural fold homeobox</td>
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<td>RNA-binding protein with multiple splicing</td>
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<td>Ribonucleic acid</td>
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<td>RORβ</td>
<td>RAR-related orphan receptor β</td>
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<td>RP</td>
<td>Retinitis Pigmentosa</td>
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<td>Retinal pigment epithelium</td>
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<td>Retinoid x receptor γ</td>
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Chapter 1

1 Background

1.1 General Overview

Neurodegeneration is possibly the cruelest forms of torture: it takes your memories, your ability to feel, your ability to sense and interact with the world and when you’re at your most vulnerable, your ability to be human.

1. Neurodegeneration

If one thinks of their body as a complex machine, neurons would be the wiring between all the different components. Sensory neurons detect information of the outside world, interneurons relay information from these sensors and motor neurons give commands for the different parts of the body to move. At the peak of it all is the brain, the master computer, a collection of intricately connected wires that processes all information and controls the machine. Every perception, thought and action anyone has ever experienced is mediated by neurons. This incredibly complicated network of wires and sensors is arguably the most important system in the body; the philosopher scientist, Gilbert Harman, once argued that it is probable that all of humanity could simply be reduced to brains in jars meaning that being human relies solely having a brain, a housing unit of over 100 billion neurons. This makes it all the more devastating when this system goes awry.
Neurodegeneration is a family of diseases that manifests itself in a variety of different symptoms with neuronal and/or synapse (connections between neurons) loss and death as an underlying cause. Neurodegeneration can manifest itself in various ways depending on where the dysfunction occurs: Alzheimer’s, Parkinson’s and Huntington’s disease arise from dysfunction in neurons in the central nervous system, amyotrophic lateral sclerosis (ALS) arises from neuronal dysfunction in motor neurons and age-related macular degeneration, retinitis pigmentosa and hearing loss arise from dysfunction in sensory neurons. These diseases are characterized by a general or subtype-specific death and loss of neurons. Neurodegenerative diseases strike hard, often rendering the victim incapable of controlling their movements, remembering their memories and sensing the world around them. Heartbreakingly, another common feature shared by neurodegenerative disorders is the lack of curative therapies.

2. Retinal Degeneration and Repair

Sensory neurons, as their name implies, are the neurons that sense cues from the environment and relay this information to the brain. The sense of smell and taste arises from chemoreceptors in the nose and tongue, the sense of hearing and touch from mechanoreceptors in the ear and skin, the sense of temperature from thermoreceptors all over the body. These sensory receptors take chemicals, mechanical stimuli and temperature and convert them into electrical signals to be processed by the brain, but perhaps the most interesting sensory receptor of all is the photoreceptor. Photoreceptors convert light into an electrical signal giving us the sense of sight. These fascinating cells have developed to make proteins that react to light causing a cascade of events that lead to a signal that can be interpreted by the brain. However, like the neurons in the brain, genetic factors and perturbations in the environment can cause these photoreceptors to degenerate.
Blindness caused by retinal degeneration is irreversible and is a leading cause of incurable vision loss. Retinal degeneration can have different underlying genetic or environmental causes that ultimately lead to blindness from the death of the sensory neurons in the eye, the photoreceptors. The most common forms of retinal degeneration are age-related macular degeneration (AMD) and retinitis pigmentosa (RP) (Hartong, D.T. et al., 2006, Hamel C.P., 2007, Wright, A.F., et al., 2010). AMD is a disease that is increasing in prevalence; AMD affects 1 in 4 people over the age of 75 and is characterized by a loss of daylight high acuity vision (Hamel, C.P., 2007, Wright, A.F., et al., 2010). RP is a rarer form of retinal degeneration that affects 1 in 2000 with genetic defects being the underlying cause (Hartong, D.T., et al., 2006). RP typically manifests itself as a loss in night vision followed by a progressive loss of daylight vision (Hartong, D.T., et al., 2006). Like all neurodegenerative diseases, there are no curative therapies for blindness caused by retinal degeneration. Current therapies manage symptoms and attempt to slow the progression of disease (Yonekawa et al., 2015); the lack of a curative therapy has researchers looking for novel ways to treat retinal degeneration.

1.2 The Retina

“The whole is greater than the sum of its parts.” - Aristotle

1. Overview

The retina is a neural laminar tissue that is located in the posterior region of the eye. Retinal cells, cells comprising the retina, are specialized in the conversion of light sensory stimuli into neural signals feeding into the optic nerve. These signals are then sent to the brain for further processing to produce vision. The structure of the retina was described as early as 1900 by Santiago Ramon y Cajal: a man revered as the father of modern neuroscience. His hand drawn
diagrams back in the 1900’s depict the structure of retina to an incredible degree of accuracy (Figure 1.1) (Ramon y Cajal, S., 1900, Wassle, H. and Boycott, B.B., 1991, Masland, R.H., 2001).

2. **Anatomy and Function of the Retina**

The retina consists of three cell layers: the outer nuclear layer (ONL), the inner nuclear layer (INL) and the retinal ganglion cell layer (GCL) (Figure 1.1). The ONL consists of the rod photoreceptors and cone photoreceptors (Masland, R.H., 2001). Photoreceptors are the light-sensitive cells of the retina. Rod photoreceptors and cone photoreceptors extend rod spherules and cone pedicles, respectively, to synapse with bipolar cell and horizontal cell dendrites of the INL (reviewed in Masland, R.H., 2001). This layer of neuronal synapses is known as the outer plexiform layer and lies between the ONL and INL (Masland, R.H., 2001). The INL lies basal to OPL and consists of bipolar cells, horizontal cells, amacrine cells and Müller glia. Bipolar cells then further extend their neural processes basally to synapse with retinal ganglion cell dendrites (Euler, T., *et al.*, 2014, Masland, R.H., 2001). This layer of neuronal synapses is called the inner plexiform layer (IPL). Basal to the IPL is the GCL, which consists mainly of retinal ganglion cells and displaced amacrine cells (Perry, V.H. and Walker, M., 1980). The retinal ganglion cells extend axons that bunch up to form the nerve fibrillary layer (NFL), which then exits the retina as the optic nerve (Masland, R.H., 2001). The ganglion cell axons synapse in the thalamus of the brain, specifically in the lateral geniculate nucleus (LGN) (Hubel, D.H. and Wiesel, T.N., 1968). From there, neurons carry the information further into the visual cortex of the brain where it is processed into what we perceive as vision (Hubel, D.H. and Wiesel, T.N., 1968).
Figure 1.1: Structure of the retina. Drawings by Ramon y Cajal from 1900 (reproduced under public domain license). In the outer nuclear layer (3): rod photoreceptors (a, c), cone photoreceptors (b, d). In the inner nuclear layer (5): horizontal cell (c), bipolar cell (f, g), amacrine cell (h) and Müller glia (B). In the ganglion cell layer: ganglion cells (i, j).
3. Photoreceptors

While rod photoreceptors are fairly homogenous between mammalian retinas, the distributions and subtypes of cone photoreceptors differ substantially between mammalian species (Carter, L.D. and Lavail, M.M., 1979). Humans have 3 types of cone photoreceptors that can be classified into S-cones, M-cones and L-cones (Jacobs, G.H., 1993, Boynton, R.M., 1988). Mice, however, only have S and M-cones (Jacobs, G.H., 1993). Additionally, the retina has melanopsin-positive ganglion cells, which are also sensitive to light; these cells are important in the maintenance of circadian rhythm and influence the release of melatonin in the pineal gland (Freedman, M.S., et al., 1999). However, these cells are very rare, are not the primary cells mediating vision loss in retinal degeneration and thus, will not be further discussed.

Rod and cone photoreceptors differ in their distribution in the retina. In humans, cone photoreceptors are most abundant in the central retina in an area known as the fovea, while rod photoreceptors are more abundant in the peripheral retina (Curcio, C.A., et al., 1987). Mice, however, do not have a fovea and instead cone photoreceptors in the murine retina are dispersed throughout rather than concentrated at a fovea (Jeon, C.J., et al., 1998, Ortin-Martinez, A., et al., 2014). In both mice and humans, cone photoreceptors comprise the minority of the photoreceptors of the eye: 5% of the photoreceptors in humans, and 3% of the photoreceptors in mice are cones (Curcio, C.A., et al., 1987, Jeon, C.J., et al., 1998). However, it should be noted that while being a small minority of the photoreceptor population, more than half of the sensory output of the human retina is from cone photoreceptors owing to the high ratio of cone photoreceptors to ganglion cells: often 5 cones:1 ganglion cell compared to 1000 rods:1 ganglion cell (Curcio, C.A. et al., 1990).
Photoreceptors are a highly specialized sensory neuron that has evolved to efficiently detect photons and this is reflected in the unique morphological features of this cell type. The photoreceptor consists of a cell body with an inner fiber, inner segment and outer segment. Starting with the nuclear morphology, rod photoreceptors in nocturnal animals, such as the mouse, are characterized by a spherical nucleus with a singular tightly packed heterochromatin core surrounded by a small layer of euchromatin (Figure 1.2) (Carter, D.H. and Lavail, M.M., 1979). Cone photoreceptors, however, have an elongated nucleus with several foci of heterochromatin (Figure 1.2) (Carter, D.H. and Lavail, M.M., 1979). Furthermore, the synaptic terminals of cone and rod photoreceptors also differ. Rod photoreceptors synapse on rod bipolar cells via a small spherule terminal, whereas cone photoreceptors synapse on cone bipolar cells via a comparatively large broom-shaped terminal called a pedicle (Figure 1.2) (Carter, D.H. and Lavail, M.M., 1979). Basal to the cell body, the inner fiber of the photoreceptor synapses with cone or rod specific bipolar cells. Cone photoreceptors and rod photoreceptors are named after the shape of their outer segments when they were first described in frogs; the morphological differences between cone and rods in mammalian retinas are more subtle (Figure 1.2) (Walls, G.L., 1942, Carter, D.H. and Lavail, M.M., 1979). These outer segments consist of densely packed discs that are contained within the cell membrane in rod photoreceptors and continuous with the cell membrane in cone photoreceptors (Carter, D.H. and Lavail, M.M., 1979). These discs contain opsin molecules as well as cation channels, proteins that are essential for the phototransduction cascade (Carter, D.H. and Lavail, M.M., 1979).
Figure 1.2: Schematic of murine photoreceptor morphology: rod photoreceptor on the left and cone photoreceptor on the right.
4. Phototransduction and the Pigment Epithelium

Phototransduction is the process by which light stimuli are converted into a change in membrane potential that subsequently causes a change in neurotransmitter release. Photoreceptors produce what is known as a dark current; whereas most neurons in the body depolarize when activated, photoreceptors, in the absence of light, are depolarized. In the absence of light, the opsins are bound to a light-sensitive chromophore, 11-cis-retinal (reviewed in Arshavsky, V.Y., et al., 2002). When light hits retinal, it undergoes an isomerization to all-trans-retinal (Arshavsky, V.Y., et al., 2002). All-trans retinal no longer fits within opsin and is ejected from its binding site causing a conformational change in opsin (Arshavsky, V.Y., et al., 2002). When not bound to retinal, opsin is able to activate G-coupled protein, transducin, which switches out its GDP for GTP allowing its Gα subunit to dissociate with its Gβ and Gγ subunits (Figure 1.3) (Arshavsky, V.Y., et al., 2002). The GTP bound Gα subunit of transducin then activates phosphodiesterase, which starts breaking down the signaling molecule, cGMP, into 5’-GMP (Figure 1.3) (Arshavsky, V.Y., et al., 2002). The lowering of cGMP levels causes sodium channels to close resulting in a hyperpolarization of the membrane potential due to the constant efflux of potassium ions (Figure 1.3) (Arshavsky, V.Y., et al., 2002). This hyperpolarization closes voltage-gated calcium channels, lowering the calcium levels in the cell (Figure 1.3) (Pugh, E.N. and Lamb, T.D., 1990, Arshavsky, V.Y. et al., 2002). Subsequently, less glutamate is released, as calcium is required for the fusion of glutamate vesicles to exit the cell (Figure 1.3) (Arshavsky, V.Y., et al., 2002). Lower glutamate levels disinhibit the bipolar cells, causing them to be activated and relay the information to ganglion cells and ultimately the brain.
An important cell layer that is not part of the retina, but is crucial for its function, is the retinal pigment epithelium (RPE), which lies apical to the photoreceptors (Masland, R.H., 2001, Strauss, O., 2015). The RPE is an integral player to the phototransduction process and recycles all-trans retinal to 11-cis retinal (Figure 1.3) (Arshavsky, V.Y., et al., 2002, Lamb, T.D. and Pugh, E.N., 2006, Strauss, O., 2005). Additionally, maintaining a dark current requires a large amount of energy and generates a high level of oxidative stress (Strauss, O., 2005). The RPE engulfs the damaged discs of the photoreceptors, as well as supplying glucose and oxygen to them (Strauss, O., 2005). Moreover, the pigment in the RPE absorbs any extra light to prevent light from causing additional oxidative stress (Strauss, O., 2005). As there are many players involved in the phototransduction cascade, problems with any of the players can have large deleterious effects on vision and the retinal cells.
Figure 1.3: Schematic of the phototransduction cascade. Light striking rhodopsin in the discs (1) causes 11-cis-retinal to undergo a conformational change becoming all-trans retinal (2). The conformational change causes retinal to dissociate from opsin (3). The unbound opsin activates transducin, which switches its GDP to GTP. Activated transducin now binds to phosphodiesterase, activating it (4). Phosphodiesterase catalyzes the conversion of cGMP to GMP. The decrease in intracellular levels of cGMP causes cGMP-gated cation channels to close (5). The membrane potential hyperpolarizes which decreases downstream glutamate release. All-trans retinal is transported to the RPE (A-B). All-trans retinal is then converted via several enzymatic steps back into 11-cis retinal (C) and is transported back into the photoreceptor to restart the cycle (D). (Adapted from Lamb, T.D. and Pugh, E.N., 2006)
5. Photoreceptor Development

In mice, the eye starts developing in the embryo at 8 days post conception. Evaginations of the ventral ectodermal neuroepithelial cells near the forebrain form optic vesicles, which will develop into the eye (Chow, R.L. and Lang, R.A., 2001). The optic vesicle will invaginate upon itself and form two layers: the outer layer and the inner layer separated by the intraretinal space (Chow, R.L. and Lang, R.A., 2001). The outer layer will differentiate into the RPE whereas the inner layer will differentiate into the neural retina (Chow, R.L. and Lang, R.A., 2001). The inner layer is home of the retinal precursor cells (RPCs). These RPCs are multipotent and will generate the seven subtypes of cells in the neural retina: cone photoreceptors, rod photoreceptors, bipolar cells, horizontal cells, amacrine cells, ganglion cells and Müller glia (Turner, D.L. and Cepko, C.L., 1987, Bassett, E.A. and Wallace, V.A., 2012). The timing of the birth and differentiation of the various cell types is depicted in the schematic shown in Figure 1.4. Cells fated to become ganglion cell drop out of cell cycle first and the majority of ganglion cells have been made by E15 (Young, R.W., 1985). Horizontal cells, cone photoreceptors and amacrine cells are also specified in the late embryonic days (Young, R.W., 1985). Most cone photoreceptors are specified and come out of cell cycle around E16.5. The peak production of rod photoreceptors, the largest proportion of cells within the neural retina, occurs at P0. The peak of bipolar cell and Müller glia specification occurs later at around P4 (Young, R.W., 1985).

RPCs exit cell cycle to become photoreceptor precursors and these precursors will then start to up-regulate photoreceptor specific genes, such as phototransduction genes. The maturation of photoreceptor precursors into fully functional photoreceptors takes place over weeks to months and occurs postnatal (Swaroop, A., et al., 2010).
Transcription factors play a key role in the regulation and direction of photoreceptor development. One such transcription factor is orthodenticle homeobox 2 (OTX2): an homeobox transcription factor that specifies a bipolar or photoreceptor cell fate. OTX2 can then upregulate the expression of cone-rod homeobox (CRX) to activate photoreceptor-specific genes or visual homeobox 2 (VSX2) to specify bipolar cell fate (Chen, S., et al., 1997, Furukawa, T., et al., 1997, Furukawa, T., et al., 1999, Koike, C., et al., 2007). Nuclear receptor subfamily 2 group e member 3 (NR2E3) and neural retina-specific leucine zipper (NRL) are both transcription factors that direct precursors towards rod fate and are essential for rod photoreceptor development (Mears, A.J., et al., 2001, Haider, N.B., et al., 2000). Cone progenitors will further preferentially differentiate into M cones (as opposed to S cones) by the expression of thyroid hormone receptor β 2 (Trβ2), onecut1/2 (Oc1/2) and neurod2 (Ng, L., et al., 2001, Hennig, A.K., et al., 2008, Brezinski, J.A. and Reh, T.A., 2015).

Mutations of these factors can lead to deleterious effects on the retina. A deletion of Nrl gene in mice results in an all-cone retina (Mears, A.J., et al., 2000). OTX2 and CRX deficiency in mice result in a severe degeneration of photoreceptors and profound congenital blindness (Furukawa, T., et al., 1999, Koike, C., et al., 2007). In humans, mutations in Otx2 have been related bilateral anophthalmia, mutations in Crx are correlated with Leber’s congenital amaurosis (LCA) and mutations in Nrl and Nr2e3 are associated with enhanced S-cone syndrome or Goldmann-Favre syndrome (Tajima, T., et al., 2009, Swaroop, A., et al., 1999, Wright, A.F., et al., 2004).
Figure 1.4: Transcriptional regulation of photoreceptor precursors showing the major transcription factors regulating the differentiation of rod and cone photoreceptors from mitotic multipotent precursors (Adapted from Hennig, A.K., et al., 2008)
6. Disease and Degeneration

The most common heritable form of retinal degeneration is RP (Hartong, D.T. et al., 2006). Although there are more than 44 genetic subtypes, there are very few phenotypic characteristics that can reliably distinguish between them (Wright, A.F., et al., 2010). The most common cause of RP is mutations in rhodopsin, though mutations in other components of the phototransduction cascade are also implicated in RP. (Wright, A.F., et al., 2010, Hartong, D.T., et al., 2006). Such mutations cause a disruption in the function and proper trafficking of this protein to the outer segments leading to death of rod photoreceptors (Wright A.F., et al., 2010). Patients with RP suffer from night-blindness followed by progressive loss of the peripheral visual field leading to tunnel vision (Hartong, D.T., et al., 2006). Unfortunately, in most cases the degeneration will not spare the cone photoreceptors, resulting in loss of central visual field and total blindness (Hartong, D.T., et al., 2006). Depending on the mutation, RP will affect the patient from early infancy to late adulthood (Hartong, D.T., et al., 2006). Leber’s congenital amaurosis (LCA), while similar to RP, is different in that the onset of blindness occurs at birth or a few months after birth (Wright, A.F., et al., 2010). There is a genetic overlap between RP and LCA; there are at least six genes that when mutated can cause either disorder (Wright A.F., et al., 2010).

Whereas RP usually affects patients before mid-adulthood, AMD usually affects patients during late adulthood, hence the name (Wright A.F., et al., 2010). AMD is also much more genetically complex than RP: 20% of patients with typical AMD have an affected relative, but monogenic cases of AMD are incredibly rare (Wright A.F., et al., 2010). The incidence of AMD is far greater than that of RP, being the cause of over one half of blindness and vision impairment in the industrialized world (Wright A.F., et al., 2010). AMD is phenotypically diagnosed by
presence drusen, yellow lipid deposits, in the macula (Yonekawa, Y., et al., 2015). AMD patients typically report a progressive loss in central vision. In severe cases of AMD, known as wet-AMD, neovascularization occurs in the subretinal space (Wright A.F., et al., 2010). These ectopic blood vessels leak proteins and blood under the macula and can cause significant damage if unmanaged (Wright A.F., et al., 2010). The pathogenesis of AMD, that is what the root cause of photoreceptor death, is unclear, though it is clear that the pathologies in the RPE play a role in the progression of the disease and there is an emerging hypothesis that inflammation may play a role as well (Yonekawa, Y., et al., 2015).

The pharmacological management for dry-AMD is limited to lutein and zeaxanthine supplements, both of these supplements have limited supporting clinical data for dry-AMD (Hobbs, R.P. and Berstein, P.S., 2014). Similarly, RP is managed using large doses of vitamin A; though, the use of vitamin A, like lutein and zeaxanthine supplements for dry-AMD, also has limited supporting clinical data (Berson, E.L., et al., 1993). Wet-exudative AMD, the more severe form of AMD, can be managed using laser coagulation and antibodies that prevent neovascularization or the formation of blood vessels (Yonekawa, Y., et al., 2015). In addition to being invasive and incredibly expensive, these treatments only temporarily slow the progression of AMD: there are no treatments that halt or reverse the progression of AMD.

1.3 Photoreceptor Transplantation

1. Overview

Although novel approaches to treat AMD and RP, including RPE transplantation, are at pre-clinical and early stage clinical trials, this approach, unfortunately, cannot reverse vision loss after cone degeneration (Schwartz, S.D., et al., 2012). Thus, researchers need to develop novel
strategies, such as cone transplantation, to restore vision to the AMD retina. In murine models of photoreceptor degeneration, photoreceptors are the only cell type affected leaving the downstream neural networks generally unscathed (Furukawa, T., et al., 1999). The observation of conserved retinal neural network led to the hypothesis that cell replacement therapy was possible. Thus, researchers investigated the feasibility of cell-mediated rescue of retinal degeneration.

2. Lineage Specific Reporters

One of the first hurdles that researchers needed to overcome is how to track the donor cells in the recipient retina. In 2006, researchers generated a mouse strain GFP reporter tagged to the Nrl promoter; as previously mentioned, Nrl is a rod-lineage specific transcription factor and is expressed in immature rod precursors and mature adult rods (Akimoto, M., et al., 2006). This mouse strain allowed researchers to track donor rod photoreceptors and also enabled the enrichment of rod photoreceptors via fluorescence activated cell sorting (FACS). With this new tool, researchers were able to investigate the feasibility of rod transplantation.

However, finding a similar way to enrich for cone photoreceptors proved to be a challenge. Generating a cone-specific reporter mouse line was a challenge since there were very few cone lineage markers known at the time. Further adding to the challenge, only 3% of murine photoreceptors are cone photoreceptors (Jeon, C.J., et al., 1998). Both the lack of a method to enrich and track cone populations, and the rarity of cones in the retina has caused a lag of cone transplantation studies behind rod studies. In 2001, though, researchers generated an Nrl knockout mouse strain (Nrl−/−) that was characterized by a cone-dominant retina (Mears, A.J., et al., 2001, Daniele, L.L., et al., 2005). RNAseq and other experiments to determine expressome were performed using this mouse line to identify cone lineage markers (Mears, A.J., et al., 2001,
Yoshida, S., et al., 2004). Researchers found that the \( Nrl^{-/-} \) mouse retina overexpresses many hallmark cone genes and that the mouse has an exaggerated b-wave in their electroretinogram (ERG) traces, indicating that the cone photoreceptors were functionally active (Mears, A.J., et al., 2001, Daniele, L.L., et al., 2005). By crossing the \( Nrl^{-/-} \) mouse with a ubiquitous GFP reporter mouse, researchers were able to enrich for reporter labeled cone photoreceptors using magnetic bead sorting using the CD73, a pan photoreceptor surface receptor (Koso, H., et al., 2009, Santos-Ferrera, T., et al., 2015). However, it was not until 2016, that researchers were able to enrich for endogenous cones using a cone-specific GFP reporter mouse line, using a GFP gene-trap into the locus Coiled-coil domain containing 136 (Ccdc136) gene (Yoshida, S., et al., 2004, Smiley, S., et al., 2016). The \( Ccdc136^{GFP/GFP} \) mouse labels cones as early as embryonic day 13.5, during peak cone production, making it a novel early cone-lineage marker (Smiley, S., et al., 2016). \( Ccdc136^{GFP/GFP} \) also labels bipolar cells in the retina at postnatal day 14; however, since cone and bipolar cell development is temporally distinct, researchers were able to enrich for cones without contamination from bipolar cells (Smiley, S., et al., 2016). Such tools set the stage for research studying cone transplantation.

3. Rod and Cone Photoreceptor Transplantation

Although researchers had been transplanting cells into the subretinal space of mice (Chacko, D.M., et al., 2000), one could argue the ignition of the field of rod photoreceptor transplantation occurred in 2006 when researchers published functional rescue following transplantation of rod photoreceptors that were enriched from the \( Nrl-GFP \) mouse (MacLaren, R.E., et al., 2006). Morphologically mature GFP-labeled rod photoreceptors were observed in the ONL of the recipient retina (MacLaren, R.E., et al., 2006). The number of these GFP-labeled cells was interpreted as being correlative to the amount of functional rescue achieved in the recipient

The first cone transplantation was performed in 2015 by Santos-Ferreira, T. et al. utilizing genetic and magnetic bead methods to enrich for cone-like (cod) photoreceptors in a Nrl<sup>-/-</sup> background. There was modest rescue using electrophysiological measures, in this case retinal ganglion cell patch clamping. Furthering the field, in 2016, Smiley, S., et al. transplanted cone photoreceptors using similar genetic manipulation and fluorescence activated cell sorting with the advantage of a cone-specific GFP reporter mouse line. It was then, that the researchers reported the interesting observation that the engrafted cells in the outer nuclear layer of wildtype recipients had rod-like morphology, even though cone cells were transplanted (Smiley, S., et al., 2016). This observation would call into question the nature of donor cell engraftment.
4. Material Transfer

Late in 2016, it was reported that GFP-labeled cells in the outer nuclear layer of recipient mice, that had been transplanted with donor GFP+ photoreceptors, were actually a result of material transfer rather than donor cell engraftment proper (Pearson, R.A., et al., 2016, Santos-Ferreira, T., et al., 2016, Singh, M.S., et al., 2016, Ortin-Martinez, A. et al., 2017). Though researchers were skeptical that there would be sufficient reporter transfer to fill an entire cell, evidence soon mounted against the traditional interpretation of transplantation: dual-reporter transplant experiments showed double labeled cells, indelible EdU-labeled donor cells failed to enter the outer nuclear layer and Y-chromosome labeling showed that male donor nuclei did not engraft into the recipient retina (Pearson, R.A., et al., 2016, Santos-Ferreira, T., et al., 2016, Singh, M.S., et al., 2016, Ortin-Martinez, A., et al., 2017).

The advent of this discovery required the careful re-interpretation of data generated over a decade as the factors that were previously interpreted as affecting engraftment may be actually affecting material transfer instead. With the increased scrutiny for future experiments and also the unresolved issue of low photoreceptor engraftment, a new approach is required to determine the factors at play in this incredibly complex system.

1.4 Organoids

“All things being equal, the simplest solution tends to be the best one” – William of Ockam

1. Overview

While in-vivo studies have the advantage of being more relevant and recapitulating of what happens in a fully functioning organism, in-vitro studies have the advantage of being easier and
quicker to perform. Due to the laborious nature of *in-vivo* experiments, *in-vitro* models are a useful tool to gain insight into the players and pathways present for a particular phenotype.

Organoids, as the name suggests, are an *in-vitro* structure that are a level of complexity above single cells, but not quite at the organizational level of a full organ (Lancaster, M.A. and Knoblich, J.A., 2014). Organoids are typically characterized as 3-dimensional miniature version of an organ that can be grown *in-vitro*. The idea of growing organs in a dish was proposed after the observation that sea sponge cells after being dissociated into a single cell suspension would re-aggregate to form the original organism. However, it would not be until after the discovery of stem cells that the potential of cells to grow into different organs was fully realized.

To classify an aggregate as an organoid, the following criterion was developed: One: organoids must be comprised of more than one organ-specific cell type formed by organ-specific stem or progenitor cells. Two: organoids must be capable of recapitulating some specific function of the original organ. Three: the cells must be spatially organized in a manner similar to the organ (Lancaster, M.A., Knoblich, J.A., 2014). Organoids have been described for many organs of the human body, including thyroid, lung, pancreas, liver, stomach, intestine, heart, skeletal muscle, bone, kidney, brain, retina, pituitary, mammary gland, inner ear and skin (Lancaster, M.A., Knoblich, J.A., 2014). These organoids allow researchers to ask mechanistic questions about phenotypes that include, but are not limited to cell development, motility, induction and lamination, in a simplified system where genetic manipulations can be performed with relative ease. In addition to providing to transplantable material that resembles the host tissues, researchers can use these organoids to quickly screen through small biological molecules, siRNA libraries and drugs to see if the organoid will produce a particular phenotype. Moreover, these organoids have been used extensively to study development and disease states.
2. Retinal Organoids

Embryonic retinal dissociates form rosette shaped organoids when cultured (Vollmer G. et al., 1984, Kelley, M.W., et al., 1994). Retinal organoids were first explored in chick embryonic dissociates in the early 1960’s (Steffanelli, A. et al., 1961, Moscona A. A., 1962). These studies showed that chick embryonic dissociates formed beautifully laminar structures, termed ‘spheroids’, that recapitulate the layers of the retina when grown in culture. In a later study, researchers showed that the orientation of these structures depended on the presence of RPE in the culture; presence of RPE caused the structure to be apical, photoreceptor layer, oriented on the outside of the spheroid, whereas in the absence RPE the layers adopted the opposite topology (Vollmer, G., et al., 1984, Vollmer, G. and Layer, P.G., 1986). This in-vitro model has been used to study retinal development and photoreceptor specification (Vollmer G. and Layer P.G., 1986, M.W. Kelley et al., 1999). Later studies using chick retinal dissociates implicated cholinesterases in the regulation of retinal cell proliferation and the requirement of Müller glia for the proper formation and lamination of the aggregates (Vollmer, G., and Layer, P.G., 1987, Willbold, E., et al., 2000). Similar experiments performed using embryonic rat retinal dissociates showed the effects of taurine and retinoic acid on photoreceptor differentiation (Kelley, M.W., et al, 1994, Kelley, M.W., et al., 1999). In a later study, using a rotational culture system, rat retinal aggregates increased in size and showed the additional presence of both amacrine and ganglion cells (Rothermel, A., et al., 2005). The structure and orientation of the rodent dissociates were similar to chick dissociates cultured in the absence of the RPE: photoreceptors oriented with their apical processes oriented towards the lumen with the basal side facing out. Research performed using these organoids or in-vitro retinae as the researchers termed them (IVR) were primarily performed to ask questions regarding the developmental cues that induce the differentiation of
the RPCs (Vollmer, G. and Layer, P.G., 1986). These experiments would provide insights for later work that unveiled the transcriptional regulation and other developmental cues that allow RPCs to differentiate. Most, if not all, experiments performed today utilize in-vivo models with knock-ins or knockouts of key genes to study development.

Pluripotent cell technology combined with the wealth of knowledge of molecular regulation of development prompted the next generation of experiments to differentiate eye and retinal tissues from pluripotent cells. Researchers used mouse embryonic stem cells to form embryoid bodies, culturing them in a 3-dimensional culture using Matrigel and media that encouraged neuroectoderm differentiation (Eiraku M. et al., 2011). The embryoid body will then form buds that resemble optic vesicles and have the capacity to form a bilayer; they separated the optic cups from the embryoid body and grew the optic cup in a media that supported retinal differentiation (Eiraku M. et al., 2011). More recently, researchers used human pluripotent stem cells to form these optic cups (Nakano, T. et al., 2012). While similar to murine eye cups, eye cups derived from human cells took longer to culture, were larger and showed apical nuclear positioning (Nakano, T. et al., 2012). The ES-derived eye cups are incredibly homologous to what is observed in-vivo: in addition to mimicking the transcriptional timing and patterning of the developmental program, the eye-cups are also bear striking morphological similarity to the developing eye (Eiraku, M., et al., 2011, Nakano, T., et al. 2012). There are advantages and disadvantages to using primary cells versus ES cells as a source for organoid formation. While rodent and chick retinal aggregates needed only a culture time of roughly one week, the eye cups formed from mouse ES cells and human ES cells required a much longer 20 days and 126 days in culture to fully form, respectively (Vollmer, G., et al., 1984, Kelley, M.W., et al., 1994, Eiraku, M., et al., 2011, Nakano, T., et al. 2012). Moreover, the culture conditions required to form the eyecups require various media changes and condition changes throughout the culture
period, whereas the rodent and chick aggregates use one medium throughout (Vollmer, G., et al., 1984, Kelley, M.W., et al., Eiraku, M., et al., 2011, Nakano, T., et al., 2012). As a result, though ES cell-derived organoids are more morphologically similar and relevant to the *in-vivo* retina, its reproducibility issues, extended culture time and requirement for suspension culture make it an impractical choice as an *in-vitro* model for engraftment.
Figure 1.5: Timeline of retinal organoid research (Adapted from Lancaster, M.A. and Knoblich, J.A., 2014)
Chapter 2

2 Hypothesis and Rationale

2.1 Rationale

The field of photoreceptor transplantation is constantly challenged by low engraftment rates. Compounding this issue is the material transfer phenotype, which is difficult to distinguish from proper engraftment. The identification of the molecular regulators of photoreceptor cell integration and differentiation would be facilitated by the development of an *in-vitro* system. Retinal dissociates self-organize into organoids and have been used to study differentiation of photoreceptors making them a good candidate for studying engraftment and the differentiation of exogenous photoreceptors.

2.2 Hypothesis

Self-organizing organoids formed from dissociated perinatal mouse retina are an *in-vitro* retina surrogate and encourage exogenous photoreceptors to differentiate. These organoids can be further used to model and study donor photoreceptor integration into the outer nuclear layer.

2.3 Objectives

To test this hypothesis, I had the following objectives:

1. Optimize and characterize mouse retinal organoid formation *in-vitro*

2. Characterize the cell-type requirement of retinal organoid formation

3. Establish a cell-engraftment assay using retinal organoids generated from wildtype and degenerate retinas.
Chapter 3

3 Materials and Methods

“The only difference between science and mucking about is writing it down” – Adam Savage

3.1 Animals and Genotyping

The use of animals in this study was in accordance to the guidelines set out in the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. All experiments performed were approved by the University Health Network Research Ethics Board, in accordance to the guidelines of the Canadian Council on Animal Care and in conformity with the University Health Network Care Committee (protocol 3499.10). Mouse strains used in this study as summarized in Table 3.1. Briefly, C57BL/6J (Charles River), Nrl-GFP (Rod-GFP) (Akimoto, M., et al., 2006), Nrl<sup>−/−</sup>;Ccdc136<sup>GFP/GFP</sup> (Cod-GFP) (Smiley, S., et al., 2016) and Crx<sup>−/−</sup> (mouse model of retinal degeneration) (Furukawa, T., et al., 1999) mice of both sexes were used in this study. Genotyping was performed by extracting genomic DNA from ear clips or tail snips using an alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA, pH 8.0) for one hour at 95°C. The samples were then neutralized with neutralization buffer (40 mM Tris-HCl) and PCR was performed using the primer sets summarized in Table 3.2.
Table 3.1: Mouse lines used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>C57BL/6J</td>
<td></td>
</tr>
<tr>
<td>Nrl-GFP</td>
<td>C57BL/6J</td>
<td>(Akimoto, M., et al., 2006)</td>
</tr>
<tr>
<td>Crx^-/-</td>
<td>Mixed</td>
<td>(Furukawa, T., et al., 1999)</td>
</tr>
<tr>
<td>Nrl^-/-;Cc136^GFP/+</td>
<td>C57BL/6J</td>
<td>(Smiley, S., et al., 2016)</td>
</tr>
</tbody>
</table>

Table 3.2: Primer sequences used for genotyping

<table>
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<th>Target</th>
<th>Primer (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crx forward</td>
<td>AGGAGTTTGGCGATTCCAGGG</td>
</tr>
<tr>
<td>Crx reverse</td>
<td>CGATGATCTCGTCTGCTGACCC</td>
</tr>
<tr>
<td>Ccdc136 wildtype, forward</td>
<td>CCGTGGTGGGTTGTTGAATCCAA</td>
</tr>
<tr>
<td>Ccdc136 wildtype, reverse</td>
<td>TGGGAAAGTATGAAAGGGCCACCA</td>
</tr>
<tr>
<td>Ccdc136 GFP, forward</td>
<td>CACATGAAGCAGACGACTT</td>
</tr>
<tr>
<td>Ccdc136 GFP, reverse</td>
<td>TGCTCAGGTAGTGTTGCTCG</td>
</tr>
<tr>
<td>Nrl wildtype, forward</td>
<td>GTGTTCCTTGGCTGAAAGA</td>
</tr>
<tr>
<td>Nrl wildtype, reverse</td>
<td>CTGTTCCATGTGGGTCTTTCA</td>
</tr>
<tr>
<td>Nrl knockout, forward</td>
<td>TGAATACAGGGACGACACCA</td>
</tr>
<tr>
<td>Nrl knockout, reverse</td>
<td>GTTCTAATTCCATCAGAAGCTGAC</td>
</tr>
<tr>
<td>mT/mG, common</td>
<td>CTCTGCTGCCTCCTGCTTCT</td>
</tr>
<tr>
<td>mT/mG, wildtype reverse</td>
<td>CGAGGGCGGATCACAAAGCAATA</td>
</tr>
<tr>
<td>mT/mG, mutant, reverse</td>
<td>TCAATGCGGCGGGGTCGTT</td>
</tr>
</tbody>
</table>
3.2 Preparation of Glass Surface

In preparation for retinal cell cultures, glass-bottomed 96 well plates (Mat-Tek) were coated with poly-D-lysine (PDL) (Sigma Aldrich) by incubating PDL in Baxter water for 3 hours or overnight at a concentration of 10 µg/mL at 37 °C. The plates were then washed 3 times with Baxter water and coated with laminin (Sigma Aldrich) in Baxter water for 1 hour at a concentration of 1 µg/mL at 37 °C.

3.3 Organoid Formation

To establish self-organizing cultures, retinas from C57BL/6J, or Crx−/− at postnatal day 1-2, were harvested in CO2 independent media (Fisher Scientific) and dissociated with papain (Worthington Biochemical, UK) in accordance to manufacturer’s directions. The cells were then washed in Ca2+/Mg2+ free phosphate buffered saline (PBS) and subsequently counted using a hemocytometer and 0.4% trypan blue viability stain. To enrich for photoreceptors, the cells were re-suspended in 0.5% bovine serum albumin (BSA) (Sigma Aldrich) 25 mM HEPES, 2 mM EDTA, 0.005% DNase (Sigma Aldrich) in Ca2+/Mg2+-free PBS and the GFP+ cells were isolated using fluorescence activated cell sorting (Aria III, BD Biosciences) into 10% BSA in Ca2+/Mg2+-free PBS. The sorted cells were then counted using 0.4% trypan blue. The cells were then re-suspended in explant culture medium (DMEM with GlutaMAX (Gibco, 0.5 mL/mL), Nutrient F12 Ham (Gibco, 0.5 mL/mL), Sato’s supplement (10 uL/mL), insulin (Sigma Aldrich, 10 ug/mL), N-acetyl-cysteine (Sigma Aldrich, 60 ug/mL), gentamycin (Gibco, 10ug/mL) and plated on pre-coated glass-bottomed 96-well plate described above, 250 000 cells in 100 uL of media per well. The cultures were topped off with 100 uL media after 3 days in-vitro (DIV) and cultured for an additional 3 days for a total of 6 DIV. In the pharmacological disruption
experiments, wildtype rosettes were cultured in the presence of varying concentrations of ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.01 mM, 0.1 mM and 1 mM, for the duration of the culture period. All experiments were performed a minimum of 3 times in technical triplicates.

3.4 Engraftment Assay

For the integration assay, rosettes were pre-formed as described above. Donor cells were prepared using retinas from *Nrl-GFP* or *Nrl<sup>−/−</sup>;Ccnd136<sup>GFP/GFP</sup> mice at postnatal day 3-6. The retinas were dissociated and sorted as described above. The purified donor cells were then added to preformed rosettes at a concentration of 25 000 cells per well. The cultures were incubated for another 3 DIV before fixation and immunostaining.

3.5 Fixation and Immunostaining

To prepare the rosette cultures for immunostaining, the cultures were fixed by incubating with 4% paraformaldehyde for 10 minutes followed by 3 washes with PBS (0.14 M NaCl, 2.5 mM KCl, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.2 M KH<sub>2</sub>PO<sub>4</sub>). The cultures were then blocked in 10% donkey serum (DS) (Sigma Aldrich), 0.5% Triton-X in PBS for one hour at room temperature. The primary antibodies were diluted at various concentrations summarized in Table 3.3 in 5% DS, 0.25% Triton-X in PBS and were incubated overnight at 4°C. The cultures were then washed 3 times using PBS and then incubated with secondary antibodies at various concentrations summarized in Table S4 in PBS for 1.5 hours at room temperature in a light protected humidified box. Cell nuclei were counterstained with Hoechst 33342 (Life technologies) diluted in PBS at a concentration 1:15000 for 20 minutes at room temperature. The cultures were then washed one last time with PBS before being stored in a light protected container or imaging.
Table 3.3: List of antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
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<td></td>
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</tr>
<tr>
<td>Anti-Glutamine synthetase</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>Abcam (AB49873)</td>
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<tr>
<td>Anti-β-catenin</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Millipore (05-665)</td>
</tr>
<tr>
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<td>Rabbit</td>
<td>1:1000</td>
<td>Millipore (AB15282)</td>
</tr>
<tr>
<td>Anti-Vsx2</td>
<td>Sheep</td>
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<td>Exalpa (X1179P)</td>
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<td>Goat</td>
<td>1:500</td>
<td>Rockland Inc. (600-101-215)</td>
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<td>PNA</td>
<td>Lectin Biotinylated</td>
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<td>Vector (B-1975)</td>
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<td>Rabbit</td>
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<td>Rockland Inc (600401379)</td>
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<td>Anti Zo-1</td>
<td>Mouse</td>
<td>1:500</td>
<td>Life technologies (33-9100)</td>
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<td><strong>Secondary Antibodies</strong></td>
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<td>Streptavidin Cy3</td>
<td>Biotin</td>
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<td>Cedarlane (CLCSA1010)</td>
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<td>Donkey</td>
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<td>Thermo Fisher (A-11055)</td>
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<td>Thermo Fisher (A-21436)</td>
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<td>Anti-rabbit 647</td>
<td>Donkey</td>
<td>1:1000</td>
<td>Thermo Fisher (A-31573)</td>
</tr>
</tbody>
</table>
3.6 Confocal Imaging, Quantification and Statistics

Rosette cultures were imaged using a LSM 780 confocal microscope. Images for quantification were taken at a 20X magnification and representative images were taken at 40X magnification. Acquisition parameters were as follows: resolution was 2048 by 2048 pixels, laser intensity was set at 2%, digital gain at no more than 800, averaging was 4 and pinhole size was 1 airy unit (as determined by Zeiss). Comparative images were taken using identical parameters. Live imaging was performed using identical parameters at 20X magnification and 15 minute intervals in a CO₂-buffered environmental chamber (Zeiss). Both rosette size and number were quantified using ImageJ. Rosettes were counted if their lumen stained positive for peanut agglutinin (PNA) and if they had a significant up-regulation of β-catenin. A two-tailed student’s t-test or a one-way ANOVA was used to determine significance where appropriate. All data are presented as mean ± standard error of the mean (SEM).
Chapter 4

4 Results

4.1 Optimization and Immunocytochemical Characterization of Mouse Retinal Organoid Culture

We optimized the conditions required to generate reproducible organoid formation in serum free conditions from dissociates of perinatal mouse retina (Figure 4.1A schematic) by comparing tissue stage, cell density, well size, and coating substrate, all of which affect cellular organization in-vitro. We used defined, serum free conditions because of the reported inhibitory effects of serum on photoreceptor differentiation (Neophytou, C., et al., 1997). We determined that P1-2 retinas plated at 10^6 cells/0.785 cm^2 (24 well plate well) formed aggregates by 6 days in-vitro (6 DIV) more frequently than retinas from P4-P8 pups. Plating with multiple coating substrates determined that re-aggregate formation requires the presence of laminin. Although poly-D-lysine was not required for re-aggregate formation, it facilitated cell adherence to the glass surface to allow for easier handling and staining post-fixation. We characterized the landscape of cultures by quantifying the size and location of individual re-aggregates across multiple wells (Figure 4.1B-C). Re-aggregate structures formed at a 10-20-fold higher frequency in proximity to the wall of the well, having an average area of 2613 (± 454) μm^2 (Figure 4.1D). Orthogonal axis evaluation of re-aggregates via confocal Z-stack reconstruction revealed the 3-D organization of these structures (Figure 4.2A), which contain a central acellular lumen that extends throughout the full extent of the Z-axis (Figure 4.2A’-A’’’). Previous studies describe the transient persistence of cell cycling by rodent retinal progenitors (Lillien, L. and Cepko, C., 1992); and
continued proliferation of Müller glia (Nickerson, P.E., et al., 2008) when maintained in-vitro. To assay whether residual proliferation was associated with re-aggregate formation we pulsed cultures continuously with EdU starting from either day 0 (plating) or 24 hours after plating (Figure 4.2B-C). Cultures pulsed at day 0 and evaluated at 6 DIV contained EdU-positive re-aggregates (7.7 EdU-positive nuclei per organoid ± 1.1 – Figure 4.2D). In contrast, EdU labeling was nearly absent in cultures pulsed at 24h (0.6 EdU-positive nuclei per re-aggregate ± 0.2), indicating that aggregates grown in mitogen-free conditions are derived from post-mitotic cells and progenitors that completed their last S-phase in culture. To determine the cellular composition of retinal re-aggregates, we seeded cultures formed from either wildtype retinas, evaluation of immunocytochemical profiling with cell-type specific antibodies or lectins revealed that retinal re-aggregates contain multiple classes of retinal cells (Figure 4.3A-G). Specifically, cells in re-aggregates expressed the photoreceptor marker Otx2, cone markers PNA and cone arrestin (CArr), rod markers rhodopsin, Müller glia markers glutamine synthetase (GS) and retinaldehyde binding protein (CRALBP), and the bipolar cell marker visual system homeobox 2 (Vsx2). The absence of Vsx2/CRALBP co-localization (Figure 4.3H) indicated that Vsx2 labeling was specific to bipolar neurons, and that Müller glia do not exhibit features of de-differentiation in these conditions. Re-aggregates did not stain positive for RNA Binding Protein with Multiple Splicing (RBPMS), indicating the absence of surviving retinal ganglion cells by 6 DIV (Figure 4.3J). Quantification of the cell lineage composition of organoids based on our marker panel showed that >95% of re-aggregates stained positive for rhodopsin, GS and CArr, whereas only 20% of re-aggregates stained positive for Vsx2 (Figure 4.3K), indicating perinatal mouse retinal re-aggregates are composed of rod and cone photoreceptors, Müller glia, with variable levels of bipolar cell participation. We furthermore observed that the spatial distribution
of apical markers PNA and rhodopsin was restricted to the lumen of retinal organoids (Figure 4.3A, C), indicating that apical/basal polarity is a feature of these structures.
Figure 4.1: Spatial and size characterization of retinal organoids. Schematic diagram of the organoid culture protocol (A). Low magnification DIC image of retinal organoids (A’). Spatial distribution of organoids quantified and rendered as inner and outer quadrants (B-C). Organoids form at 10-20 fold higher frequency in proximity to the well wall (n = 4) (C). Size distribution of organoids (n = 4) (D).
Figure 4.2: 3-dimensional and post-mitotic nature of organoids. Z-stack confocal rendering of the 3-dimensional nature of retinal organoids (A). Visualization of the organoid central lumen at apical, central and basal optical sections in z-stack rendered image (A’-A’’’). EdU staining showing the loss of S-phase after 24 hours in culture. (B-C). Quantification of EdU+ cells in organoids (n = 3) (D). Scale bar = 20 µm
Figure 4.3: Immunocytochemical characterization of retinal organoids. Cone markers peanut agglutinin (PNA) and cone arrestin (CArr) (A-B), rod marker rhodopsin (C-D), Müller glial markers cellular retinaldehyde binding protein (CRALBP) and glutamine synthetase (GS) (E-F) bipolar cell marker Vsx2 (G) is not expressed in CRALBP-positive Müller glia (H). The intermediate filament protein GFAP is up-regulated in organoid Müller glia (I). Absence of RBPMS-positive retinal ganglion cells (J). Cell fate marker membership within organoids (n = 3) (K). Scale bar = 20 µm.
4.2 Non-photoreceptor cells are required for efficient organoid assembly

The major cell classes that make up organoids are photoreceptors and Müller glia, which is reminiscent of the close association between photoreceptor cell bodies and apical processes of Müller glia in the outer nuclear layer of the retina. To investigate the requirement for intercellular interactions in organoid formation we asked whether rod photoreceptors alone could self-organize to form these structures (Figure 4.3).

We enriched rod by cells by fluorescent activated cell sorting (Figure 4.4A-B) of GFP+ cells from P1-P2 retinas of Nrl-GFP mice, which express enhanced green fluorescent protein under the control of the rod-specific Nrl promoter (Akimoto, M., et al., 2006) and cultured these cells as described above. To control for effects of cell sorting, and genetic background effects of the Nrl-GFP mice, we cultured un-enriched Nrl-GFP cells that were run through the FACS as well as unsorted wildtype and Nrl-GFP retinal cells. Rod-enriched cultures generated on average 3-fold fewer organoids compared will all other control groups, indicating that non-rod cell types are required for efficient organoid assembly (Figure 4.4C). Immunostaining of the few organoids that did form in the rod-enriched cultures revealed that they contained Müller glia, which likely represents a minor contamination from the cell sorting (Figure 4.4D). The role of Müller glia in retina lamination and organization in-vivo and in-vitro is well-documented. Thus, although not causal, the positive correlation between Müller glial markers and successful organoid assembly is suggestive of their participation in this process.
Figure 4.4: Cell-type requirement of retinal organoids. FACS enrichment of NRL-GFP rods (A). Low magnification DIC and immunofluorescence image of enriched NRL-GFP rods at time of plating (B). Quantification of organoids in cultures generated from photoreceptor-enriched fractions as well as non-purified (FACS-treated), unsorted and wildtype controls (n = 3) (C). Immunocytochemical stains showing mixed composition of Müller glial (GS) and rod-GFP markers in photoreceptor-enriched cultures (D-D’’’). Yellow scale bar = 100 μm. White scale bar = 20 μm.
4.3 Organoids are polarized structures that depend on junctional integrity

Cytochemical analysis (above) revealed that apical (inner/outer segment) markers such as PNA and rhodopsin localize to the lumen of the retinal organoids, illustrating the propensity of these structures to polarize. *In-vivo*, Müller glia form adherens and tight junctions along the inner segments of photoreceptors to form an OLM. To determine whether organoids establish analogous OLM characteristics, we stained for markers associated with a selection of junctional proteins (Figure 4.5A-H). We observed expression of β-catenin, N-cadherin, ZO-1 and the cell polarity regulator Par-3 in the organoids (Figure 4.5A, C, G, E). However, instead of them forming a compact line at the apical side of the organoid, analogous to the OLM in the retina, these markers were enriched in a diffuse pattern in the organoid lumen (β-cat, Zo-1 and N-cad) or distributed throughout the organoid (Par3) (Figure 4.5A, C, G, E). With the exception of Par3, the expression of the other junctional markers was nearly undetectable in basal regions of the organoid and in single cells in the cultures (Figure 4.5B, D, F, H), suggesting that the luminal accumulation was an indication of the presence of functionally relevant junctions. *In-vivo*, the absence or disruption of junctional complexes results in loss of apical/basal integrity and the formation of rosettes and dysplasic foci at the OLM (Masai, I., *et al.*, 2003, Wong, G.K., *et al.*, 2012). Junctional complexes have been shown to be dependent on the presence of calcium, and depletion of calcium has been shown to disrupt their ability to form (Bleich, M., *et al.*, 2012). To test whether calcium-dependent organoid junctions are necessary for assembly and polarity, we cultured retinal dissociates in the presence of varying concentrations of the calcium chelating agent, EGTA, and quantified the number of organoids at 6 DIV. Organoid assembly was
significantly disrupted in a dose-dependent manner, with a concomitant dose-dependent decrease in the level of β-catenin staining (Figure 4.5I-L’, M). To rule out the possibility that this effect was secondary to EGTA-induced toxicity, we quantified cell density in the central region of the well where organoids typically do not form, and verified that the density was not significantly different across treatment groups (Figure 4.5N). Thus, organoids exhibit apical enrichment of junction proteins and their formation is dependent on Ca\textsuperscript{2+} signaling, which together suggests that Ca\textsuperscript{2+}-dependent junction formation is required for self-organization.
Figure 4.5: Upregulation of junctional markers in organoids. Immunocytochemical stains showing presence of β-catenin (A-B), N-cadherin (C-D), Par-3 (E-F) and ZO-1 (G-H) in retinal organoids and single cells. Cadherin junctions (I-N) in organoids are sensitive to presence of calcium. A dose-dependent decrease in β-catenin is observed when EGTA added (I-L). Although no loss in cell density is observed in organoid-free regions of the well with EGTA-treatment (n = 3) (N), organoid assembly decreases in a dose-dependent manner (n = 3) (M). Scale bar = 20 μm.
4.4 Establishment of Organoid Engraftment Assay

We sought to determine whether organoids can be used to investigate photoreceptor cell motility and engraftment potential. To investigate both rod and cone dynamics, we FACS enriched populations of Nrl-GFP (Rod-GFP), as well as GFP-expressing photoreceptors from Nrl<sup>−/−</sup>;Ccdc-136<sup>GFP/GFP</sup> mice. Nrl<sup>−/−</sup>;Ccdc-136<sup>GFP/GFP</sup> mice contain a cone-rod (“Cod”) hybrid cell type that expresses a gene-trap GFP at the Ccdc136 locus (Smiley, S., et al., 2016), and are referred to as Cod-GFP cells. To test the engraftment potential of “donor” photoreceptors into organoids, Rod-GFP and Cod-GFP cells were added to previously-established (6 DIV) organoid cultures. Live-imaging over 5 days revealed the migration of single GFP-expressing photoreceptors toward organoids (Figure 4.6A). Furthermore, motile cells engage with the perimeter of organoids, and exhibit what appeared to be bona fide engraftment into the organoids (Figure 4.6A). Comparison of Cod-GFP and Rod-GFP apparent engraftment rate indicated differential integration capacities, with 64.8 ± 3.4% and 91.2 ± 3.7% of organoids containing at least one Cod or Rod-GFP+ cell, respectively (Figure 4.6H). To further confirm cell engraftment, and to exclude GFP material exchange-mediated false-positives, we pulsed Rod-GFP mice with three injections of EdU at P1 to indelibly label rod progenitors completing their last S-phase. At P4, EdU labeled retinas were harvested, FACS enriched, and added to established organoid cultures. Initial flow cytometric quantification of EdU coverage within the GFP+ population indicated that 15% of GFP donor cells were EdU labeled. Analysis of recipient organoids revealed that 18% of engrafted GFP+ cells derived from organoid cultures were also EdU-positive, confirming that GFP+ cells within organoids are of donor origin. Immunolabeling for β-catenin was performed to determine whether engrafted donors exhibit markers expression of junctional proteins, and thus participate in the polarized organoid structure. Staining revealed the β-catenin localization in GFP-positive
engrafted cells, indicating that donor cells upregulate junctional proteins (Figure 4.6B-F’). Collectively, these data indicate that donor photoreceptors engraft into established organoids, and participate with the polarization that is present in these structures.
Figure 4.6: Donor cells have capacity to engraft into organoids. Time-lapse series of Cod-GFP donor cell motility and engraftment into wildtype organoids (A). Presence of β-catenin surrounding engrafted donor cells (B-F). Pre-labeled EdU/Nrl-GFP co-labeled donor cell within an organoid, indicating that engraftment is not a function of donor/host material exchange (G). Quantification of the proportion of organoids that contain either Rod-GFP or Cod-GFP donor cells (n = 3) (H). Frequency distribution histogram of Rod-GFP or Cod-GFP donor cell engraftment into wildtype organoids (n = 3) (I). Scale bar = 20 µm
4.5 Rod-GFP donor cells exhibit a unique neurite outgrowth engraftment phenotype in Crx-/- organoids

To investigate how photoreceptor maturation affects organoid formation and cell engraftment, we assessed the ability of Crx-/- dissociates to form retinal organoids, and further to this, their ability to permit the integration of Rod-GFP donors. As mentioned in the introduction, CRX is a homeobox transcription factor that is required for photoreceptor development and in its absence photoreceptors are still specified, but they fail to differentiate and ultimately, they undergo degeneration (Furukawa, T., et al., 1997). Crx-/- cultures were able to form intact organoids, however, the immunocytochemical features of these organoids differed from wildtype counterparts. Specifically, although glutamine synthetase staining in Crx-/- cultures (Figure 4.7A) was similar to controls (Figure 4.3F - above), the mature photoreceptor markers CArr, PNA and rhodopsin were not detected (data not shown), indicating either an impairment of these cells to participate in assembly, or a failure of participating photoreceptors to mature. Staining of Crx-/- organoids for the early marker OTX2, a transcription factor that functions upstream of CRX and is expressed in photoreceptors and bipolar cells (Bovolenta, P., et al., 1997) showed that the majority of cells present were either a photoreceptor or bipolar cell fate (Figure 4.7B). Vsx2 staining revealed that much like wildtype organoids (Figure 4.3G), few bipolar cells are present in Crx-/- cultures indicating that the majority of Crx-/- organoid cells remain as immature photoreceptors (Figure 6C), which is consistent with the in-vivo defects in the Crx-/- retina [furukawa]. To test the integration capacity of Crx-/- organoids, we added P4 wildtype Rod-GFP photoreceptors to established Crx-/- organoids and quantified the number of engraftment events. No significant differences in total integration events were observed when comparing Crx-/- organoids to wildtype, despite a slight downward trend in the Crx-/- cultures (% of engrafted
organoids wildtype 91.2 +/- 3.7% vs Crx<sup>−/−</sup> 80.2% +/- 4.2%) (Figure 4.6H, 4.7D). Morphological analysis revealed that a subset of integrated Rod-GFP donor cells exhibited an increase in neurite extension in Crx<sup>−/−</sup> compared with wildtype organoids (Figure 4.7E-F). Quantification of the proportion of integrated Rod-GFP cells with neurites revealed that 40% (+/- 0.4%) of these cells had processes compared to 18% (+/- 4.3%) of wildtype counterparts (Figure 4.7G). Although not statistically significant, Rod-GFP donors in Crx<sup>−/−</sup> cultures had longer mean neurite length compared to those in wildtype recipient organoids (Figure 4.7H). Collectively, these data indicate that CRX is not required for organoid formation but that CRX-deficiency exert a unique outgrowth phenotype in wildtype donor rods, indicating that there are unique, non-cell autonomous factors in mutant environment that influence wildtype photoreceptor behaviour.
Figure 4.7: Immunocytochemical characterization of Crx⁻/⁻ organoids. Otx2 (A), Vsx2 (B) and glutamine synthetase (C) stains showing presence of photoreceptors, bipolar neurons and Müller glia respectively, in Crx⁻/⁻ organoids. Engraftment of donor cells and donor cell processes in both Crx⁻/⁻ (G-I) and wildtype organoids (J-L). Quantification of number of cells with neural processes (D) and length of neural processes (E) of donor cells in Crx⁻/⁻ and wildtype cultures (n = 3). Quantification of donor cell engraftment in Crx⁻/⁻ organoids (F). Scale bar = 10 µm
Chapter 5

5 Discussion

“Part of what it is to be scientifically-literate, it's not simply, 'Do you know what DNA is? Or what the Big Bang is?' That's an aspect of science literacy. The biggest part of it is do you know how to think about information that's presented in front of you.” – Neil DeGrasse Tyson

5.1 Establishment of an Organoid Culture

Previous work has shown the capacity of embryonic and perinatal retinal dissociates to re-aggregate into laminar structures (Vollmer, G., and Layer, P.G., 1987, Willbold, E., et al., 2000, Kelley, M.W., et al., 1994). Here we have characterized in more detail the self-organization property of murine retinal dissociates. Similar to experiments performed using chick and rat dissociates in a rotating culture, murine re-aggregates form polarized 3-dimensional structures organized around an apical acellular lumen. The cellular composition of these retina re-aggregates is consistent with previous studies revealing the presence of rod and cone photoreceptors, bipolar cells and Müller glia (Kelley, M.W., et al., 1994, Rothermel, A., et al., 2005).

The cellular composition of our aggregates mimics that of the ONL of a retina \textit{in-vivo}. An integral component of the ONL is the OLM, which is formed from the interaction between photoreceptors and Müller glia. Although not as organized and well-defined as the OLM \textit{in-vivo}, our re-aggregates display up-regulation of relevant OLM junctional proteins. Consistent with the hypothesis that the up-regulation and formation of this OLM-like structure is required for re-
aggregation, we show that disruption of calcium ion concentrations inhibit the re-aggregation of retinal cells. Our aggregates show self-organization, the presence of multiple post-mitotic cell types in our aggregates and the up-regulation of relevant junctional proteins allow us to classify these structures as organoids.

We also wanted to determine whether organoid formation required specific cell types. Interestingly, we found that there was a requirement for a non-photoreceptor cell type for aggregation to occur. Re-aggregation in purified photoreceptor cultures was associated with contaminating Müller glial cells and together with our analysis showing that Müller glia, as opposed to bipolar cells, are represented in nearly all regular aggregates, strongly suggests a role for Müller glia in the self-organization. This idea is consistent with the integral role of Müller glia in the aggregation process in both chick and zebrafish dissociates (Willbold, E., et al., 2000, Eldred, M.K., et al., 2017). Thus, although we did not test the requirement for Müller glia directly, our findings strongly suggest that Müller glia play an essential role in the ability of cells to aggregate.

5.2 Utility of organoids in a novel engraftment assay

Organoids are being exploited to model disease states, as a platform for drug screening and as an efficient way of generating specific cell types for transplantation therapy (Lancaster, M.A., and Knoblich, J.A., 2014). Indeed, with respect to the retina, previous studies have shown the potential for ES and retinal stem cells to generate transplantable photoreceptors in a 3-dimensional culture system (Eiraku, M., et al., 2010, Inoue, T., et al., 2010). Here, in contrast, we investigated whether organoids could be used to model photoreceptor cell engraftment. Donor cells transplanted in the subretinal space need to overcome many obstacles to engraft,
primary of which is to migrate and interact with host tissue in a heterologous environment. The retinal organoids described in this study recapitulate aspects of the cellular composition and intercellular interactions in the endogenous ONL, including presence of photoreceptors and Müller glia making it a suitable platform for modeling the interaction between donor cells and host photoreceptors and Müller glia in-vivo.

Indeed, we show that donor photoreceptors added to established organoids can migrate in the culture and integrate into these structures. Additionally, we observe no evidence that donor cells disassociate from organoids after integration prompting us to speculate that donor cells are interacting with cells in the organoid. Furthermore, we confirm that reporter positive cells within organoids are a result of engraftment proper and not a result of material transfer. There is donor to recipient specificity to the engraftment: engraftment rates differ between rods and hybrid cone donor cells and mutant recipients affect the maturation of donor cells.

Although our organoid system recapitulates some of the interactions in an in-vivo transplant scenario, there are differences between our system and the in-vivo equivalent: these limitations include, partial maturity of the organoid cells, the inverted topology where in our system, the donor cell would encounter the basal surface instead of the apical surface as they would in a transplant scenario lack of RPE and immune response. However, while the developmental timing may not fully mimic the in-vivo environment, regulators of engraftment in this context could still be relevant and exploited in the in-vivo adult retina. Furthermore, our data validate the utility of donor-recipient modeling through the use of organoid cultures, and provide a baseline metric for follow-up studies that screen for mechanisms that impact this process.
5.3 Comparisons to established *in-vitro* retinal models

The topology exhibited by self-organizing retinal cultures differs between species and culture conditions. Classical work from Layer and Willbold demonstrated the capacity of dissociated chick retinal cells to self-organize and assemble into the various retinal layers (Layer, P.G., *et al.*, 2002, Layer, P.G., *et al.*, 2001). Recent work in zebrafish has established a similar potential of ocular organoids to organize into multi-laminar retinal structures (Eldred, M.K., *et al.*, 2017). Notably, these fish cultures, like avian organoids, require both retinal and RPE cells, and use of conical-bottom culture dishes that encourage the physical aggregation of cells into a pellet-like mass. Although these retina/RPE co-cultures provide an organoid preparation that exhibits similar architecture to the *in-vivo* retina and can be used to characterize the impact of neurochemical and cellular regulation of retinal development, these studies were performed in model systems that are not relevant for studying engraftment in the context of photoreceptor transplantation.

In mammalian systems, organoids formed using embryonic or perinatal tissues were unable to recapitulate the 3-dimensional structures with retinal layers observed in avian studies. Early studies using rat dissociates showed the presence of a 2-dimensional organized structure with multiple cell types. It was not until 2005 that Rothermel, A., *et al.* used a rotational culture system that 3-dimensional structure were generated using a mammalian dissociates. In contrast with the study performed by Rothermel, A. *et al.* in 2005 using rat dissociates, there was no presence of retinal ganglion cells. This difference could be attributed to both the species differences and the differences in culturing media: the presence of serum in their culture system versus serum-free condition in ours. The presence of serum could also explain the differences in the number of mitotic cells between the two cultures (Rothermel, A., *et al.*, 2005). As serum has
been shown to have inhibitory effects on photoreceptor development (Neophytou, C., et al., 1997), it seems that the presence of serum pushes organoids into an IPL-like spheroid and the lack thereof encourages the formation of an ONL-like organoid (Rothermel, A., et al., 2005). Additionally, due to the increasing number of genetic tools at their disposal, there is a shift in the scientific community to use mice over rats.

Another in-vitro retinal system researchers have utilized is the embryonic stem cells eye cups that bear striking resemblance to a developing eye (Eiraku, et al., 2011, Nakano, et al., 2012) and while this model may be more physiologically relevant, it has the caveat of requiring long incubation times and a suspension cultures system. The suspension culture system prevents donor cells from being added into a uniform field. These caveats coupled with reproducibility issues make this model impractical for modeling engraftment and potential high-throughput screens. Caveats aside, the ES-derived eye cup system could be modified to an engraftment assay for validating a refined list of candidate targets.

5.4 Photoreceptor motility

In a developmental context, photoreceptors and their precursors, unlike other neurons in the CNS, are not required to migrate at any point during their life cycle (Chow, R.L. and Lang, R.A., 2001). Photoreceptors are born in the apical region and do not exhibit movement as they are anchored both apically and basally (Baye, L.M. and Link, B.A., 2008). Although, it has been observed that during development, photoreceptors engage in interkinetic nuclear migration, this movement is limited to the nucleus rather than the cell itself (Baye, L.M. and Link, B.A., 2008). Thus, it is a surprising observation that donor cells are not only capable of movement, but also capable of engraftment into an established organoid. A potential mechanism underpinning the
observed motility could be the absence of tissue interactions that inhibit motility. Previous work studying neuronal migration has shown the motility of dissociated neural precursor cells in-vitro (Flanagan, L.A., et al., 2006). Interestingly, a major mediator of neural precursor cell motility in-vitro was laminin (Flanagan, L.A., et al., 2006): the same substrate used to induce aggregation in this study. Similarly, laminin enhanced migration of post-mitotic olfactory neurons as well (Calof, A.L. and Lander, A.D., 1991). Thus, the observed motility may be an inherent property of photoreceptors, but this phenotype is never observed due to the cell-cell interactions within the tissue and the presence of inhibitory cues. Further studies will be required to conclude which molecular pathways are involved in this process. Another potential factor underlying photoreceptor motility is the aforementioned interkinetic nuclear migration. The developmental timing of this process coincides with the dissociation of the donor cells used in this study (Baye, L.M. and Link, B.A., 2008). Further studies will be required to determine the effect of developmental age on cell motility.
Chapter 6

6 Conclusion

Retinal degeneration is slated to affect more than 200 million people worldwide by 2020 (Wong, W.L., et al., 2014). As there is no curative therapy currently, researchers have studied the feasibility of photoreceptor transplantation; however, with recent findings in photoreceptor transplantation confounding a decade’s worth of research, a validated tool is required to determine factors that influence engraftment and transfer specifically. Self-organizing primary and stem-cell derived cultures provide valuable insight into the collective interaction of heterogeneous cell populations that result in complex tissue structures. The procurement of organ and tissue primordia from a diversity of animal systems allows us to model the kinetics of cell motility, contact and aggregation \textit{in-vitro}, with the further benefit of allowing us considerable control over the culture microenvironment. Building on decades of \textit{in-vitro} retinal culture modeling, we have established highly reproducible organoid protocol that can be used to test the engraftment of donor photoreceptors.
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