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Modeling 3D Retinogenesis in Mouse Embryonic Stem Cells Following CRISPR-mediated Crx Knockdown

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Modeling 3D Retinogenesis in Mouse Embryonic Stem Cells

Following CRISPR-mediated Crx Knockdown

A Thesis Submitted to the Faculty of

Dominican University of California

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Biology

By:

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San Rafael, California

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INTRODUCTION

Since their initial discovery in 1981, mouse embryonic stem cells have allowed research on regenerative medicine to progress greatly in a little over three decades (Evans, 1981; Martin, 1981). These embryo-derived pluripotent cells have the ability to retain a normal karyotype when cultured *in vitro*, and possess a highly differentiative potential (Bradley, 1984). In addition, mouse embryonic stem cells have the ability to develop into a wide range of adult tissues, providing a leeway for introducing genetic changes to its genome (Thomson, 1998).

In addition, an emerging technology known as three-dimensional (3D) tissue engineering has allowed scientists to mimic tissues found *in vivo*. Previous studies indicate that it is possible to differentiate dissociated mouse embryonic stem cells (mESCs) into 3D retinal tissues *in vitro* (Bertacchi, 2015; Eiraku, 2012). The newly differentiated retinal tissues are said to encompass all of the major components found in retinal tissues. Prior to this, it was only possible to differentiate mESCs into two-dimensional retinas in a culture dish; however, the differentiation into 3D retinas are more beneficial in research, as the 3D retinal culture allows us to visualize tissue morphology as well as cell-cell interactions (Ravi, 2014).

The generation of *in vitro* 3D tissues holds great potential in terms of patient-specific disease modeling. Although various diseases have been well-studied in animal models, there are limitations with regards to patient-specificity. For example, there are presently a total of 49 mutations in the human gene Cone-Rod Homeobox (Crx) that have been linked to various retinal degenerative diseases such as Leber Congenital Amaurosis,

Cone-Rod Dystrophy, and Retinitis Pigmentosa (Huang, 2000). The generation of animal models to study each specific mutation would be impractical and costly. With *in vitro* generation of tissues and organs, scientists have the capacity to generate disease models consisting of mutations specific to patients. The generation of 3D disease models would also allow scientists to pinpoint the molecular pathways associated with each mutation, which could potentially result in more personalized treatments for patients.

There are various gene editing techniques available to generate and recreate disease-specific 3D disease models using stem cells. The newest gene editing technique known as Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) has allowed scientists to edit the genome with greater precision than other genome editing technologies like Zinc Finger Nucleases (ZFNs) or Transcription Activator-like Effector Nucleases (TALENs). The CRISPR-Cas9 system was initially adapted from bacteria and archaea, in which it is used as a defense mechanism to fight foreign invaders such as phages (Segal, 2015). With genome engineering, the CRISPR-Cas9 complex consists of a helicase, nuclease, and a target-specific guide RNA (gRNA) which all work together to perform disease-specific gene editing.

In order to overcome current limitations on disease-specific model systems, we developed a protocol for differentiating mouse embryonic stem cells into 3D retinas *in vitro*. In addition, to confirm the utility of this 3D model for future disease-specific mutations, we utilized the CRISPR method as a means to knockdown the transcription factor CRX, which is known to regulate key developmental decisions during retinogenesis, and as such, mutations in CRX have resulted in various diseases such as Leber Congenital Amaurosis, Cone-Rod Dystrophy, and Retinitis Pigmentosa.

The specific aims for this study are as follows:

1. To develop a protocol for differentiating mouse embryonic stem cells into three-dimensional retinas *in vitro*.
2. To confirm the critical role of key transcription factor, Cone Rod Homeobox (Crx), during three-dimensional retinal development *in vitro* following Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) knockdown.

BACKGROUND

1. Anatomy and Physiology of the Retina

The retina lines the posterior portion of the eye. It is responsible for responding to incoming light and jump-starting the early stages of image processing. The retina is made up of four main layers: the ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and the retinal pigment epithelium (RPE) (Figure 1). In addition, there are eight main cell types present in the retina. These include retinal ganglion cells, Müller glia, bipolar and horizontal interneurons, amacrine, rod and cone photoreceptors, and retinal pigment epithelial cells. The intricate physiology of retinal components shows how they work together to allow us to visualize the world around us clearly and efficiently.

Figure 1: Overview of the Retina. The retina lines the posterior of the eye. A zoomed in region of the retina shows the various cellular bodies. The retina consists of four major layers: Ganglion Cell Layer (GCL), the Inner Nuclear Layer (INL), Outer Nuclear Layer (ONL), and Retinal Pigment Epithelium Layer (RPE). The GCL consists of ganglion cells as well as the optic nerve, which travels to the visual cortex of the forebrain. The INL consists of amacrine, bipolar and horizontal cells. The ONL consist of rod and cone photoreceptor cells. The RPE layer consists of RPE cells. The choroid, located directly behind the RPE, is the main source of blood supply to the retina.

The ganglion cell layer (GCL) is where cells collect visual information from

amacrine and bipolar cells. The GCL is where all of the chemical signals are transformed into electrical signals, which are then transmitted into the optic nerve and the brain (Tanaka, 2015). The nerve fiber layer (NFL) is made up of ganglion cells with fibers that connect to the brain (Kaplan, 2001). All information from the GCL travels through the NFL and into the visual cortex of the brain, which is the final visual assembly point.

The inner nuclear layer (INL) consists of horizontal, bipolar, amacrine, and interplexiform cells. Horizontal cells are flatter with dendrites dividing into several branches in the outer plexiform layer. What distinguishes horizontal cells from the rest of the cells in the retina is that their axons run horizontally. The job for bipolar cells is to receive input from photoreceptor cells (Bloomfield, 2001). Amacrine cells contain neurotransmitters and receive input from the bipolar cells (Farsaii, 2011).

The outer nuclear layer (ONL) contains the cell bodies of rod and cone photoreceptor cells. Rod cell bodies are spherical, more abundant, and dispersed to different layers throughout the ONL (Bloomfield, 2001). The cone cell bodies, however, are stem-like and are mostly located closer to the center of the retina (Purves, 2001). The layer of neuronal synapses is embedded in the outer plexiform layer, where the axons of horizontal and bipolar cells meet the axons of photoreceptor cells (Yau, 1994). Here, the visual signal is split depending on information received from rod and cone cells. The outer plexiform layer detects and focuses objects either lighter than the background, or darker than the background.

The photoreceptor cells are collectively responsible for absorbing and responding to any light that reaches the eye. Cone cells are responsible for responding to higher wavelengths of light and processing colors (Bowmaker, 1979). They are mostly found in

the central fovea. Rod cells are more abundant than cone cells, accounting for 95% of all photoreceptor cells in the retina (Kaewkhaw, 2015). Rod cells respond to changes in light intensities and absorb black, gray, and white light (Bowmaker, 1979). The loss of rod cells light absorption and therefore results in blindness.

The retinal pigmented epithelium (RPE) layer is involved in light absorption, epithelial transport, spatial ion buffering, the visual cycle, phagocytosis, and immune modulation (Strauss, 2005). Any light not absorbed by photoreceptor cells gets absorbed by the formation of a dark pigmented wall in the inner bulbs of the RPE for further image processing (Beatty, 2000). This process also helps to prevent the scattering of light, which can result in the obstruction of clear vision. Water is produced in large quantities in the retina due to an abundance of neurons and photoreceptors and intraocular pressure (Strauss, 2005). While water in the inner retina is removed via Müller glia (MG), all water present in the sub retinal space is removed through epithelial transport in the RPE (Alm, 1973). The RPE is also involved in the transportation and distribution of glucose and other nutrients from the blood stream, and retinol to photoreceptors. Spatial ion buffering compensates for rapid changes in ion concentration in the RPE. Because photoreceptors are constantly exposed to intense levels of light, they need to constantly replenish themselves. All waste accumulated through the replenishment process is phagocytosed in the RPE (Strauss, 2005). Finally, the RPE is in charge of secreting a variety of growth factors and other essential nutrients that are useful in the maintenance and structure of the retina.

The choroid layer lines the outermost segment of the retina. This layer consists of a series of blood vessels and connective tissue. Traditionally, the main function of the

choroid was to provide the outer retina with oxygen and nutrients. However, recent studies suggest the choroid may also be responsible for thermoregulation, adjustment of retinal position, and secretion of retinal growth factors (Anand-Apte, 2010).

2. Cone-Rod Homeobox (Crx)

The Cone-Rod Homeobox (Crx) gene lies downstream from the transactivator gene Orthodenticle Homeobox 2 (Otx2), which is required for assisting in the production of photoreceptor cells (Chen, 1997; Kimura, 2000). Crx is a structural gene that encodes a transcription factor (CRX) expressed during early retinal cell development and in newborn photoreceptor cells. CRX interacts with other transcription factors including NRL, RORB, and RAX to regulate transcription of photoreceptor cells (Kimura, 2000). The Crx gene is located on chromosome 7 in the mouse (*Mus musculus*) and consists of 14,009 base pairs (Figure 2). There is a total of two known splice variants of Crx, both containing four exons.

Figure 2: Outline of Cone-Rod Homeobox (Crx) in *Mus musculus*. The Crx gene is located on chromosome 7, totaling 14,009 base-pairs in length. The gene contains four exons, with the fourth exon being the longest.

Normal CRX function is necessary for the maintenance of cone and rod photoreceptors. Mutations in Crx result in photoreceptor cell degenerative diseases including Cone-rod dystrophy, Leber congenital amaurosis, and in some cases retinitis pigmentosa (Huang, 2000, Decembrini, 2014). A previous study has shown that a complete absence of CRX led to defects in the development of photoreceptors (Ohsawa, 2007). A complete Crx knockout in animal models have also been linked to retinal degeneration. CRX-deficient mice also fail to produce the outer segments of the photoreceptor layer and therefore lack proper maturation of rod and cone photoreceptor

Figure 3: *Crx^{ivrm65}* Mice Exhibit Lack of Production of Crx Protein, as well as Mature Photoreceptors at Postnatal 5 Weeks. (A'-A'') Presence of Crx protein, as well as abundant presence of Otx2, a cofactor for Crx located upstream of the gene. (B'-B'') Lack of production of Crx protein as well as rod cell opsin protein, Ret-P1, in *Crx^{ivrm65}* mice at 5 weeks. Otx2 expression remains consistent between both control and *Crx^{ivrm65}*.

cells (Furukawa, 1999). Although no obvious morphological differences can be discerned between young CRX homozygous and CRX-deficient mice, one-month-old CRX-deficient mice display less than 1% of normal rod activity and a 90% reduction of cone

function (Furukawa, 1999).

In the lab, we have generated a Crx mutant *Mus musculus* animal model exhibiting retinal degeneration due to a single nucleotide mutation (Crx^{tvrm65}). Figures 3 and 4 below demonstrate the retinal degeneration in a 5-week-old mouse retina compared to control mouse retina. It has been shown that photoreceptor cells form in Crx mutant mice;

Figure 4: Crx^{tvrm65} Mice Exhibit degradation of Photoreceptor Cells at Postnatal 5 Weeks. (A'-A''') Control mice show proper lamination of Blue Cone Opsin (BCO), as well as pan-photoreceptor protein Recoverin at postnatal 5 weeks. (B'-B''') Crx^{tvrm65} mice, however, show degradation of both BCO and Recoverin as they both lack proper lamination. (A and B) The yellow bars indicate degeneration of photoreceptor layer. DAPI stains nuclei. Scale bars 100µm.

however, the lack of proper function of the gene results in either hindered maturation of photoreceptors or

degeneration of photoreceptor cells. Figure 3 shows a comparison between a control mouse retina and a Crx mutant mouse retina at 5 weeks postnatal. At 5 weeks, Crx mutant mice exhibited no production of the Crx protein (Fig 3B') or the rod cell opsin protein, Ret-P1 (Fig 3B''). Protein expression of Otx2, being upstream of Crx, remained constant in both the control and knockout mouse model (Fig3B''). There were also signs

of degeneration of photoreceptor cells in general in the Crx mutant compared to control (Figure 4). Compared to of control (Fig4A'), there was a loss of proper lamination of Blue Cone Opsin (BCO) and the pan-photoreceptor protein Recoverin in the Crx mutant mice at 5 weeks (Fig 4B'). There was also reduction in size of the photoreceptor layer in Crx mutant mice, as shown by the yellow bar (Fig 4A and 4B). The Crx mutant mouse models show that there are instances where photoreceptor cells are produced in the absence of Crx, however, Crx is still necessary for the proper functioning and maintenance of photoreceptor cells.

3. Traditional Mouse Embryonic Stem Cell Culture and Maintenance

Mouse embryonic stem (ES) cells are found in the inner mass of the mouse blastocyst. When isolated and cultured *ex vivo*, ES cells have the ability to remain pluripotent, which allows ES cells to become any cell type (Bradley, 1984). In addition to their ability to remain pluripotent, ES cells are able to retain a normal diploid karyotype even after 140 cycles of division (Bertacchi 2015; Eiraku, 2012).

Mouse ES cells are traditionally cultured *in vitro* using culture dishes coated with solubilized basement membrane and maintained using stem cell growth medium containing fetal bovine serum (FBS) and leukemia inhibitory factor (LIF). They can remain undifferentiated by inhibiting the glycogen synthase kinase 3 pathway as well as the mitogen-activated protein kinase pathway. ES cells can also be prevented from over proliferating by passaging upon reaching approximately 70% confluency. Passaging requires the cells to be dissociated from their colonies using a protease solution, such as accutase, trypsin, or papain. Following dissociation, the cells are collected and resuspended in a new culture plate coated with solubilized basement membrane and

growth medium.

4. Three-Dimensional Tissue Engineering

Since the emergence of *in vitro* eukaryotic cell culture approximately 40 years ago, scientists have relied on glass or polystyrene coated dishes for culturing cells and tissues (Freshney, 2005) to allow cells to adhere and grow in two dimensions. Although two-dimensional tissue cultures continue to be used for most studies within basic research labs, such cultures are proving to be unreliable for studies involving translational research and regenerative medicine (Decembrini, 2014). For example, two-dimensional cell cultures fail to mimic the extracellular environment found in native tissues which hinders the ability of scientists to accurately measure gene expression, cellular migration, and apoptosis found *in vivo* (Haycock, 2010). The emergence of three-dimensional tissue engineering has provided a more direct link between *in vitro* studies and their translation into clinical settings. With the use of appropriate biocompatible scaffolds such as polymers, stem cells can be used to form any three-dimensional tissue, mimicking *in vivo* developmental processes (Haycock, 2010).

In terms of *in vitro* retinal development, three dimensional retinal organoids have opened new gateways in terms of replacement therapies (Eiraku, 2011; Ali, 2011). Due to the diverse population of cells located in the retina, it is important to have the ability to obtain the most homogenous population of cells for *in vivo* transplantation (Eiraku, 2011). In previous studies, researchers found that it is possible to generate photoreceptor cells in a two-dimensional culture system and have some integration when transplanted into the mouse retina (Lamba, 2009; West, 2012). However, the embryonic developmental process of photoreceptors is more closely mimicked in a three-

dimensional retinal organoid than a two-dimensional culture system. A previous study has shown that the production of correctly staged photoreceptors in 3D retinal organoids allowed for better cell integration following transplantation (Gonzales-Cordero, 2013). Studies conducted thus far have indicated the benefits of three-dimensional organoids over two-dimensional culture systems, and show their usefulness in regenerative medicine.

5. Genome Engineering

The notion of altering genes to study and treat diseases has been around since the early 1970s (Jaenisch, 1974). The first transgenic mouse using viral DNA was created in 1974, and this was followed by the introduction of knock-out mice a few years later (Smithies, 1985; Robertson, 1986). Retroviral gene therapy for treating patients with life threatening genetic disorders developed in the 1990s when doctors administered a retrovirus-driven gene therapy to a patient with Severe Combined Immunodeficiency (SCID). The patient showed a temporary response to the treatment, initiating an influx of donors for more gene therapy-based clinical trials (Tobita, 2015). Unfortunately, the development of a leukemia-like phenotype in a patient undergoing gene therapy caused a suspension of clinical trials around the world (Check, 2002). Although retroviral therapy was not as successful as originally anticipated, it did initiate a cascade of advancements with regards to structured genome engineering techniques. This began with the introduction of zinc finger nucleases (ZFN), followed by a more complex engineering system known as Transcription Activator-like Effectors (TALEs), and most recently the adaptation of Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR).

Zinc finger nucleases (ZFNs) were one of the first widely used genome engineering

tools (Miller, 1985). These custom designed nucleases are made by combining a DNA-binding domain with a DNA-cleavage domain (Smithies, 1985). ZFNs are able to recognize a total of 9 to 18 nucleotides, and the cleavage domain induces a double-stranded break in the target sequence. The natural repair machinery of the cell then allows an insertion via introduction of a desired DNA sequence, or deletion of the target sequence. The main limitation of ZFNs was in their construction. There are two publicly available methods to create specialized zinc-finger proteins, including the modular assembly and the combined Oligomerized Pool Engineering (OPEN) and Context-dependent Assembly (CoDA), or OPEN/CoDA (Segal, 2015). Modular assembly joins specially designed fingers that can recognize a specific 3-base pair sequence. The downfall to modular assembly is that the highly specific designs of these fingers were resulting in a reduced activity of the three-finger arrays (Segal, 2015). After discovering this pitfall with modular assembly derived fingers, scientists moved towards a more revolutionary zinc-finger protein designing tool, known as OPEN/CoDA. With OPEN/CoDA, a wide variety of fingers are designed to accommodate not only the final binding site, but also the fingers neighboring the final binding site (Segal, 2015).

The TALE DNA binding sequence was discovered in 2009, which prompted the engineering of TALE proteins for genome modifications (Boch, 2009). Naturally occurring TALE proteins are found in the bacterial genera *Xanthomonas* and *Ralstonia*, where the proteins assist in the infection of plants (Boch, 2010). The highly-conserved nature of TALE proteins allowed it to be the ideal choice in terms of personalized genome engineering nucleases. The DNA-recognition elements of TALE proteins consist of a series of tandem repeats equaling approximately 34 amino acids (Segal, 2013). In

addition, each TALE repeat can recognize a single DNA nucleotide.

While scientists were utilizing this newer, better alternative tool for genome engineering, another more robust genome engineering tool emerged. The gene editing technique known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a naturally occurring mechanism in bacteria and archaea that is used as a defense mechanism against phages (Barrangou, 2007). The short palindromic repeats of

Figure 5: Overview of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Mechanism. The Cas9 endonuclease encompasses the gRNA, which directs it to the target site on the genomic DNA, which is followed by the protospacer adjacent motif (PAM). Following location of the target site, the endonuclease induces a double stranded break in the DNA, in which the DNA's natural repair machinery reanneals the two broken ends.

DNA are approximately 20-40 base pairs in length which form hairpins during transcription. These repeats act as spacers for foreign viral DNA recognition sequences (Barrangou, 2007). Each palindromic sequence is separated from another palindromic sequence by a protospacer. Each protospacer DNA sequence is unique. In the early

2000s, scientists found that these protospacer DNA segments aligned perfectly with viral DNA (Bolotin, 2005). In addition, preceding these short palindromic repeats are helicase and nuclease genes known collectively as Cas genes.

In the presence of any foreign DNA sequence (as might be found in a phage), the CRISPR-Cas system responds by releasing a Cas protein complex containing a segment of CRISPR-RNA (crRNA), which includes the protospacer elements (Barrangou, 2014). After being guided to the target site, the Cas protein can cleave and destroy the foreign piece of DNA. If a pathogen injects a foreign DNA sequence not previously recognized by CRISPR, a class of Cas proteins known as Class I Cas proteins are released which bind to this foreign DNA strand. Before it is cleaved by the nucleases, a segment of the foreign gene is added to the palindromic repeats as a spacer.

In terms of gene editing, an RNA sequence known as the single guide RNA, or sgRNA, guides the Cas9 endonuclease to its locus of interest where the endonuclease creates a double stranded break (Figure 5). The sgRNA is made up of CRISPR-RNA (crRNA) and the transactivating RNA (trRNA). The crRNA consists of a 20-base pair protospacer element that is complementary to the target region of the gene to be edited (Barrangou, 2014). The trRNA is the segment of the sgRNA that guides the Cas9 endonuclease to its cleavage site. The three main functions of the Cas9 endonuclease are: (1) to bind to the sgRNA; (2) to bind to the target DNA; and (3) to cleave the target DNA, resulting in a double-stranded break.

Two repair mechanisms that cleaved DNA sequences can undergo include the non-homologous end-joining (NHEJ) mechanism and the homologous directed repair (HDR) mechanism. The NHEJ mechanism is employed if there are no DNA repair templates

provided with the CRISPR complex (Capecchi, 2005). As a result, the NHEJ mechanism is more error prone, resulting in random mutations. The HDR mechanism is much preferred due to the presence of a repair template that specifies modifications that need to be made on the DNA template. The HDR repair mechanism is more widely preferred for creating point mutations or inserting a gene of interest due to the repair template being provided for the repair of the double-stranded break.

MATERIALS AND METHODS

1. Culture of Mouse Embryonic Stem Cells

Mouse embryonic stem cells (mESC) were cultured on Matrigel- coated plates using mouse embryonic stem cell (MESC) media containing Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12), fetal bovine serum (FBS), non-essential amino acids, 0.1M β -mercaptoethanol, and penicillin/streptomycin. CHIR99021 and PD0325901 were also added to enhance mESC self-renewal. To allow the stem cells to remain in their undifferentiated state, we used leukaemia inhibitory factor (LIF).

To avoid stem cell crowding and death, the cells were split upon reaching approximately 90% confluency. The cells were washed once with phosphate buffered saline (PBS; Corning, Corning, NY) to rinse off residual MESC media. The cells were then dissociated using 1X trypsin (Corning). Following dissociation, the trypsin was neutralized using FBS containing media, and the cells were transferred to a 15mL conical tube (Gene Mate, Kaysville, UT). The cells were centrifuged at 270xg for 3 minutes. Following centrifugation, the media was removed, and the cells were resuspended using fresh MESC media. mESCs were added to one or more Matrigel-containing wells of a 6-well plate at a density of between 5×10^4 to 6×10^5 cells/well.

2. Generation of Three-Dimensional Retinal Tissue

For this study, we utilized a mouse embryonic stem cell line consisting of a GFP reporter knock-in at the Rax locus. The Rax gene is expressed in retinal stem cells. The mESC with a GFP reporter at the Rx locus were cultured until approximately 70% confluent. Cells were dissociated with 1X trypsin and neutralized with FBS containing media. Cells were transferred into a 15mL conical tube and centrifuged for three minutes

at 270xg. The media was removed, and the pellet was resuspended in 1mL of Glasgow Minimum Essential Medium (GMEM). The resuspended cells (10 μ L) were counted using the Countess II (Life Technologies, Carlsbad, CA).

A total of 1.28x10⁶ cells were seeded onto a 2% agarose gel microwell mold (MicroTissues, Inc., Providence, RI) to seed approximately 5,000 cells per microwell. The seeded cells were placed in an incubator at 37°C under 5% CO₂ for 24 hours. The next day, the formed spheroids were centrifuged at 47xg for 1 minute to transfer the newly formed spheroids onto a low attachment cell culture dish (Corning) using a 1% Cultrex BME Solution (Trevigen, Gaithersburg, MD) solution in Retinal Differentiation Media containing GMEM, 1.5% Knockout Serum Replacement (KSR), and Wnt pathway inhibitor IWR1. On the seventh day, the media was changed to Retinal Maturation Media containing DMEM, B27 Supplement, N2 Supplement, and bovine serum albumin (BSA), and incubated at 37°C with 5% CO₂ and 40% O₂. The organoids were placed on Retinal Maturation Media as well as 0.3 μ M EC23 on days 10 to 14. The cells were checked daily for morphological changes, and media was changed (Retinal Maturation Media) on days 14, 17, 20, 23, and 26 to ensure the cells received sufficient nutrients.

3. Generation of Lentiviral CRISPR Plasmid

We used the LentiCRISPR v2 plasmid which was purchased from Addgene (Addgene, cat 52961). The lentiviral CRISPR plasmid envelope used was pMD2.G vesicular stomatitis virus-G (VSV-G) expressing vector (AddGene, cat 12259), and the packaging plasmid used was psPAX2 (AddGene, cat 12260).

3.1 Digestion and Dephosphorylation of the Lentiviral CRISPR Plasmid:

Five μ g of the lentiviral CRISPR plasmid (LentiCRISPR v2) was digested and

dephosphorylated using Esp3I Fast Digest Enzyme (Fermentas, Waltham, MA), FastAP (Fermentas), 10X Fast Digest Green Buffer, 100mM DTT, and double-distilled water. The plasmid was then incubated at 37°C for 30 minutes. Following incubation, the digested and dephosphorylated plasmid was loaded onto a 0.7% low melting agarose gel (IBI Scientific, Peosta, IA). The gel was electrophoresed at 80 volts for one hour with the electrophoresis chamber (Bio Rad, Hercules, CA) set on ice. Following electrophoresis, the expected 10kb band containing the digested plasmid was excised, leaving the 2kb spacer behind.

3.2 Purification of the Digested Plasmid:

The digested plasmid was purified using the QIAquick II Gel Extraction Kit (QIAGEN, Germantown, MD). To stabilize the agarose and bind DNA, QX1 buffer, diethylpyrocarbonate water, and QIAEX II were added and incubated at 50°C for 10 minutes. Agarose contaminants were washed using QX1 Buffer. Residual salt contaminants were washed away using wash (PE) Buffer. DNA was eluted using DEPC-water and quantified using a spectrophotometer (ThermoFisher, Waltham, MA).

3.3 Phosphorylation, Annealing, Dilution, and Ligation of Oligos:

The gRNA oligos we designed were based on sequences in exon 4 of the Crx gene. Each pair of 100μM oligos was phosphorylated and annealed using 10X T4 Ligation Buffer (New England Biolabs, Ipswich, MA), T4 PNK (NEB), and double distilled water. The oligo samples were placed in a thermocycler and run at 37°C for 30min, 95°C for 5 min, and finally 25°C at 0.1°C/sec. Each annealed and phosphorylated oligo was diluted 1:500 in DEPC-water. The gRNA oligos were ligated using 50ng of

the purified and digested plasmid, the annealed and phosphorylated oligo, T4 DNA Ligase Buffer, T4 DNA Ligase, and DEPC water. The ligations were incubated for 120 minutes at 23°C.

3.4 Transformation of *E. coli* with Plasmid:

One Shot® Stbl3™ Chemically Competent *E. coli* (ThermoFisher) cells were transformed with the recombinant plasmids. One vial of One Shot was thawed on ice for each plasmid. Approximately 100ng of plasmid DNA was added to a vial and incubated on ice for 30 minutes. The vials were transferred to a 42° water bath for 45 seconds and quickly transferred back on ice for an additional 2 minutes. SOC medium (ThermoFisher) was added to each vial, and the total contents were then transferred to separate test tubes. Each tube was placed in a shaking incubator at 37°C at 225rpm for 1 hour. Samples from each tube were used to inoculate Lysogeny Broth (LB) agar plates containing 100µg/mL ampicillin and placed in a 37°C incubator overnight. The next day, single colonies were picked and placed in test tubes containing LB with 100µg/mL ampicillin. The tubes were then placed in a shaking incubator at 37°C, 225rpm. Once LB appeared turbid, the samples were mixed with equal amounts of glycerol and stored at -80°C.

4. Generation of Lentiviral Particles

Human embryonic kidney (HEK) cells were cultured in a 10cm dish using high glucose DMEM (Corning) and FBS until they reached approximately 90% confluency. Transfection reagents were prepared using calcium chloride and DEPC-water. The plasmid mix was made using 10µg target plasmid (CRX), 6.5µg psPAX2 plasmid, and 3.5µg of vesicular stomatitis virus-G (VSV-G) plasmid. HBS (pH 7.1) was added

dropwise and vortexed. The mix was finally added to cultured HEK cells following a 20-minute incubation at room temperature. The next day, the HEK media was removed, cells were rinsed once with Hank's Balanced Salt Solution (HBSS), and viral collection media was added to the cells. The viral collection media was collected in a 50mL conical tube (Gene Mate) after 24 hours for a total of two collections in 48 hours. The viral containing medium was filtered using a Steriflip-HV Filter Unit, pore size 0.45µm (Millipore, Billerica, MA).

5. Viral Infection and Generation of CRISPR Knock-down ESC Lines

Virus-containing mouse embryonic stem cell media was made using the virus-containing collection media, FBS, non-essential amino acids, 0.1M β-mercaptoethanol, penicillin/streptomycin, CHIR99021, and PD0325901. LIF was also added to prevent differentiation. The media was added to mESCs when they were at approximately 90% confluency. The mESCs were introduced to fresh virus-containing MES media for two days, with media changes every day. On the third and fourth days, a fresh solution of mouse embryonic stem cell media with 1µg/mL solution of puromycin was added to the cells to eliminate all cells not infected with the virus.

6. Immunostaining

Cells were harvested in 1.7mL microcentrifuge tubes (GeneMate) upon formation of optic cups. The cells were centrifuged at 270xg for 3 minutes, and all media was removed. The cells were resuspended and fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA), and incubated on a shaker set at 200rpm at room temperature for 45 minutes. Following incubation, the PFA was removed, and cells were washed with 1X PBS buffer three times. The cells were resuspended in 15% sucrose

(Sigma-Aldridge, St. Louis, MO) and incubated for 5 hours at 4°C. The cells were transferred to 30% sucrose overnight at 4°C. The next day, the cells were embedded onto a cryosectioning mold with Clear Frozen Section Compound (VWR, Radnor, PA) and kept at -80°C. All tissue sectioning work was performed with the cryostat (Leica, Buffalo Grove, IL). The molds containing retinal tissue were transferred to sectioning chucks and sectioned in 10-12µm slices. The sections were then transferred to micro-slides (VWR).

The slides were washed three times with 1X PBS containing 0.1% Triton (Sigma-Aldridge) at 5 minute intervals. Following the rinses, 1X Donkey serum (Sigma-Aldridge) was added and incubated in a humid chamber at 4°C for one hour. Following incubation, the serum was washed off once with 1X PBS containing 0.1% Triton. The primary antibody (Table 2) was added, and the slides were kept in a humid chamber at 4°C overnight. The following day, the slides were washed 5 times with 1X PBS containing 0.1% Triton. The secondary antibody was added, and the slides were incubated in a humid chamber at 4°C for one hour. Following incubation, DAPI was added. The slides were finally rinsed 5 times with 1X PBS containing 0.1% Triton and mounted with Fluoromount mounting media (Electron Microscopy Sciences). The slides were screened for morphological changes on the confocal microscope ZEISS Imager M.2 (ZEISS, Pleasanton, CA).

7. RNA Extraction and QRT-PCR Analysis for Retinal Genes

Total RNA was extracted into 1.7mL microcentrifuge tubes (Gene Mate) using TRIzol (Sigma-Aldridge). The extracted RNA was purified using the Direct-Zol Mini Prep Kit (Zymo, Irvine, CA). A 1:1 volume of 100% ethanol (Fisher Scientific, Waltham, MA) was added to the RNA in TRIzol and vortexed. The sample was then loaded on to a

Zymo-Spin IIC column in a collection tube (Zymo) and centrifuged at 16,000xg for one minute. The column was transferred to a new collection tube, Direct-Zol RNA Prewash was added, and the column was centrifuged at 16,000xg one minute. RNA wash buffer was added to the column and centrifuged at 16,000xg for one minute. The column was finally transferred into a 1.7mL micro-centrifuge tube, 50 μ L of nuclease-free water was added directly to the column matrix, and the tube was centrifuged at 16,000xg to elute the RNA. RNA was stored at -80°C.

Complementary DNA (cDNA) for quantitative RT-PCR (qRT-PCR) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer's instructions. Briefly, 500ng of the purified RNA template, 4 μ L of 5X iScript Reaction Mix, 1 μ L of iScript Reverse Transcriptase, and DEPC-water were combined and incubated in a thermal cycler as follows: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Following cDNA synthesis, qRT-PCR was performed using forward and reverse primers designed for target genes (see Appendix Table 1) in a 96-well PCR plate (Bio-Rad).

RESULTS

1. Generation of a Novel Three-Dimensional Retinal Differentiation Protocol

1.1 Initial Counting and Seeding of Mouse ES Cells into Microwell Culture

Molds

Prior studies have shown that cell count dictates retinal induction during differentiation of mouse ES cells (Eiraku, 2012; Volkner, 2016). Therefore, for our initial cell seeding step, it was important for us to have an accurate cell count. We utilized both manual and automatic cell counting techniques. For manual cell counting we used a hemocytometer (Figure 6A), and for automatic cell counting we utilized the Countess II by Life Technologies (Figure 6B). Both techniques required 10 μ L of cell solution. We were able to obtain a similar concentration of cells per milliliter of solution using both

Figure 6: Optimization of Cell Counting Prior to Seeding of Mouse Embryonic Stem Cells. (A) Manual Hemocytometer (B) Automatic Cell Counter Countess II by Life Technologies. (C) Bright field image of resuspended cells in the chamber of Countess II. (D) Screenshot of cells that were counted using Countess II.

methods, although the Countess II was quicker. Figure 6C shows a bright field image of cells in the Countess II cell counter. Figure 6D depicts the screen of the Countess II following cell counting. Countess II calculates live cells versus dead cells as an actual number and as a percentage.

Mouse ES cells were initially seeded into agarose gel microwell cell culture dishes in Retinal Induction Media (Figure 7A). Around 24 hours post seeding, we observed proper aggregation of cells. The cells exhibited proper spheroid formation in the agarose gel mold at day 1 (Figure 7B).

Figure 7: Initial Seeding of Mouse ES Cells. (A) Initial seeding of mouse ES cells into the agarose gel microwell cell culture dish at day 0. (B) Bright field image showing aggregate formation of mouse ES cells in the agarose microwell cell culture dish 24 hours. Scale bar 450µm.

In order to determine which cell seeding density resulted in better retinal induction, we differentiated mouse ES cells with an initial seeding density of 1,500 cells/well and 3,000 cells/well. At seven days post differentiation, we extracted total RNA from the organoids and performed qRT-PCR. We found that when compared to undifferentiated mouse ES cells, retinal and photoreceptor precursor genes *Lhx2* and *Otx2*, respectively, had higher expression levels in organoids with an initial seeding of

1,500 cells as opposed to organoids with an initial seeding of 3,000 cells (Figure 8).

Expression of photoreceptor precursor gene, *Otx2*, was upregulated nearly 87-fold in

Figure 8: Initial Seeding of 1,500 Cells per Microwell Results in Increased Retinal Expression at Day 7. qRT-PCR data shows increased expression of retinal precursor genes (*Lhx2*, *Rax*, and *Otx2*) as well as late photoreceptor genes (*Thrb* and *Prdm1*) compared to undifferentiated mouse ES cells. All samples normalized to mouse *B-actin*.

organoids with an initial seeding density of 1,500 cells/well compared to undifferentiated mouse ES cells, whereas *Otx2* was upregulated by only ~51-fold in organoids with an initial cell seeding density of 3,000cells/well. We also observed that *Lhx2*, a retinal precursor gene, was upregulated by 40-fold in organoids with an initial seeding density of 1,500 cells/well compared to a 13-fold expression in cells with an initial seeding density of 3,000 cells/well. Another striking observation was the presence of *Rax*, a retinal stem cell precursor gene. At day 7, *Rax* was upregulated by nearly 14-fold in organoids with an initial seeding density of 1,500 cells/well when compared to undifferentiated mouse ES cells. Late photoreceptor marker *Thrb* is upregulated by 3-fold in organoids with an initial seeding of 1,500 cells, and 6-fold in organoids with an initial seeding of 3,000 cells. Another late photoreceptor marker, *Prdm1*, is not much upregulated at 7 days, as

normally expected.

A consistent problem with every differentiation included the number of dead cells that accumulated following the day 7 media transition. We first used maturation media containing Dulbecco's Minimum Essential Media (DMEM) with no Fetal Bovine Serum (FBS), then transitioned into another maturation media which contained DMEM as well as 10% FBS at day 10 (Hiler, 2016). At first, we suspected the change into retinal maturation media with no FBS at day 7 was too harsh of a shift. This hypothesis was backed by the idea that the amount of nutrients of serum replacer found in the induction media was dramatically reduced following the media change. The change in media did not meet the nutrient requirement of developing retinal cells, which caused them to die. We then transitioned into using DMEM media containing 10% FBS from day 7 onwards instead of day 10. We found that this only caused cells to divide rapidly, resulting in the loss of the evaginated edges found in developing retinal organoids, and the presence of large masses of organoids consisting of both dead and live cells. We finally moved on to another maturation media, which included Neurobasal media with B27 supplement, Bovine Serum Albumin (BSA), and N2 supplement. All of the supplements used in conjunction with BSA allowed us to see a dramatic reduction of cell death at day 7, and a continued growth and maturation of retinal organoids.

1.2 Modulation of Molecular Pathways to Induce Retinal Development

We also wanted to test whether the modulation of a certain pathway involved in retinal development would result in an increased expression of retinal genes (Figure 9).

Modulation of the Wnt pathway is known to drive anterior neural plate formation

Figure 9: The use of Wnt Modulator, IWR1, in Conjunction with Matrigel Increases Expression of Retinal Genes at Day 7. qRT-PCR data shows an increase in photoreceptor precursor gene Rax in organoids treated with IWR1, a Wnt inhibitor when compared to undifferentiated mouse ES cells. All samples were normalized to B-actin.

(Nakano, 2012; Fujimura, 2016). On day 1 of differentiation, we treated organoids with 2 μ M of Wnt inhibitor IWR1 for 48 hours either with or without Matrigel. We continued culturing the organoids, collected total RNA on day 7, and measured gene expression using qRT-PCR. We found that a 48-hour treatment of organoids treated with 2 μ M IWR1 combined with 2% Matrigel resulted in a 102-fold increase in Lhx2 and a 142-fold increase in Otx2 expression compared to undifferentiated mouse ES cells. We also found that Rax, a photoreceptor precursor, was also upregulated nearly 117-fold at day 7.

Conversely, the absence of Matrigel resulted in a 94-fold increase in Otx2 expression compared to undifferentiated mouse ES cells. Though no other significant expression level differences were observed in organoids with an absence of Matrigel, we concluded that the presence of Matrigel in conjunction with IWR1 is necessary for retinal induction.

1.3 Final Retinal Induction Protocol

Figure 10: Retinal Development Timeline. Cells are initially seeded at Day 0. From Day 0 to Day 7, retinal clusters are cultured in Retinal Induction Media. A 2% matrigel solution is added on Day 1. 2µM of Wnt-modulator IWR1 is administered on Day 1, and is removed from the media on Day 3. The organoids are transitioned to Retinal Maturation Media on Day 7 until Day 28. Synthetic retinoid analogue EC23 is administered on Day 10, and is removed from the culture media on Day 14.

Our final retinal induction timeline begins on day 0 and ends on day 28 (Figure 10). Day 0 is the initial mouse ES cell seeding, wherein 5,000 cells are seeded per microwell of a gel microwell cell culture mold. Following aggregate formation of these cells in the gel microwell cell culture mold on day 1, the formed spheroids, or retinal organoids, are transferred into ultra-low attachment culture dishes. At this stage, 2% Matrigel solution is administered into the cell culture media at day 1. The retinal organoids are cultured in a Retinal Induction Media between day 0 and day 7, which includes Glasgow's Minimum Essential Media and 1.5% Knockout Serum Replacer (KSR). From day 7 until day 28, the retinal organoids are cultured in a Retinal Maturation Media, which includes Dulbecco's Minimum Essential Media, B27 supplement, Bovine Serum Albumin (BSA),

and N2 Supplement. A 0.3 μ M EC23 treatment of the retinoids between days 10 and 14 is administered to promote proper lamination of photoreceptors.

Figure 11: Bright-field Images Depicting Retinal Development. (A) Day 2 organoids exhibit semi apparent lamination along outer edge. (B) Day 4 organoids show proper lamination around outer edge of organoid. (C) By day 7, evaginations from the outer edge appear. (D) Retina-like structures visible at outpouching areas. (E) Day 16 organoids exhibit a fully stratified retina. Scale bars 100 μ m.

To keep track of morphological changes in organoids, bright-field images were taken using the 4X objective magnification at various time points (Figure 11). At day 2 post seeding, the organoids exhibit a laminated outer edge, which remains consistent throughout all retinal induction stages (Fig 11A). By day 4, the retinas show proper lamination across the outer edge of the organoid (Fig 11B). Evaginations around the laminar edges are expected to appear around day 7 (Fig 11C) and continue to expand into maturing retinas (Fig 11D). By day 16, a fully developed retina is seen, exhibiting all morphological characteristics as *in vitro* retinal organoids (Fig 11E) (Volkner, 2016).

2. Optimizing Maturation of Retinoids: Retinoid Analogues Promote Photoreceptor Lamination

Figure 12: Synthetic Retinoid Analogues Result in Similar Photoreceptor Lamination as Natural Retinoids. (A', B', C') Pan-photoreceptor marker Recoverin is expressed in retinal cells, however proper lamination is lacking in organoids not treated with retinoids. (A'', B'', C'') Mature rod opsin, Ret-P1, expression remains consistent across all conditions. Arrowheads show areas where proper photoreceptor lamination is either absent (A') or is present (B', C'). DAPI stains nuclei. Scale bars 50 μ m.

Following retinal fate induction, we focused our efforts on methodologies to promote photoreceptor development and lamination in our cultures. Previous studies have suggested that the use of retinoid analogues assist in the lamination of photoreceptors *in vitro* cultures (Zhao, 2006; Eiraku, 2012). We wanted to confirm whether the use of retinoids (natural and synthetic) assisted in a proper lamination of maturing photoreceptor cells in our system. We administered either 0.5 μ M of an *all-trans* Retinoic Acid (ATRA), a natural retinoid analogue, or 0.3 μ M EC23, a synthetic retinoid analogue, to the organoids from day 10 to day 14. The organoids were then fixed with 4% PFA on day 28,

and immunohistochemistry was performed. Using the pan-photoreceptor marker recoverin (Rec) and the rod photoreceptor opsin marker rhodopsin (Ret-P1), we determined that there were similar photoreceptor lamination patterns in organoids treated with either ATRA or EC23. As expected, organoids not treated with retinoids lacked proper lamination of photoreceptor cells, as shown in Figure 12A-12A'''. The recoverin marker indicates that in untreated organoids photoreceptor cells are scattered throughout the organoid, as opposed to exhibiting a proper laminated edge (Fig 12A'). However, organoids that were treated with ATRA exhibited a proper lamination in the outer edge, with most photoreceptor cells residing along the outer edge (Fig 12B-12B'''). A similar expression pattern was noted among organoids treated with synthetic retinoid analogue EC23 (Fig 12C-12C''').

3. Three-Dimensional Retinal Development Following CRISPR Knockdown of Crx

3.1 Generation of Guide RNA (gRNA) for CRISPR Knockdown of Crx

As previously stated, Crx is a homeobox gene that is essential for the production of photoreceptor cells in the presence of its coactivator, Otx2 (Ohsawa, 2007). We first designed gRNAs with the promotor sequence targeting exon 1 of the Crx gene. Following generation and differentiation of the mouse ES cell CRISPR knockdown lines, we saw that photoreceptor expression remained consistent between both the control line with no knockdown, as well as the knockdown lines. There are a total of two known spliced variants of the Crx gene in *Mus musculus*. With the first set of gRNAs we designed, we found that we were only targeting the first isoform, and the Crx gene was still being expressed, and in turn, the protein was still being produced with the second isoform of the gene still remaining fully functional. We then redesigned and optimized our gRNAs

with the protospacer sequence of the gRNA targeting both isoforms of Crx. Exon 4, being included in both isoforms, was the ideal target region for gRNAs.

The Crx knockdown lines were generated following Lentiviral infection of mouse ES cells with plasmids containing gRNA (P1 and P24) targeted at specific sites of Crx exon 4 (Figure 13). We then co-infected mouse ES cells with Lentivirus containing the P1 and P24 plasmids (Figure 14). By doing so, we hoped to either generate two

Figure 13: Target Regions of Expected Crx Knockdowns on Exon 4. gRNA “P1” was designed to target the 12,452-12,474 base-pair region of Crx exon 4. gRNA “P24” was designed to target the 12,144-12,166 base-pair region of Crx exon 4.

individual single nucleotide polymorphisms (SNPs) (Figure 14), or completely excise an with the protospacer sequence of the gRNA targeting both isoforms of Crx. Exon 4, being included in both isoforms, was the ideal target region for gRNAs.

Figure 14: Proposed CRISPR-mediated Crx Knockdown Schematic 1. With the co-infection of mouse ES cells with CRISPR lentivirus containing gRNA targeting both P1 and P24, respectively, we propose that we can excise the approximate 300 base-pair region located in between the P1 and P24 region. The reannealing of the target strands would result in altered or hindered Crx protein function.

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Figure 15: Proposed CRISPR-mediated Crx Knockdown Schematic 2. Proposal 2 involves the co-infection of mouse ES cells with CRISPR lentivirus containing gRNA targeting both P1 and P24, respectively. We propose that by doing so, we can induce two individual SNPs resulting in two point mutations. The outcome can result in either an altered or hindered Crx protein function.

3.2 CRISPR Crx-Knockdown Mouse ES Cell Lines Exhibit Hindered Photoreceptor Development

Differentiation of mESC lines with a functional Crx gene as well as the Crx knockdown lines, Clones A through C, into three-dimensional retinas was attempted. Unfortunately, Clone B failed to differentiate. At day 21, total RNA was extracted from

Figure 16: Decreased Photoreceptor Development Following CRISPR Knockdown of Crx P1/P24 at Day 21. Compared to Day 28 organoids with no knockdown, pan-photoreceptor gene *Recoverin*, as well as all mature photoreceptor genes (*S-Opsin*, *Rhodopsin*, *Cone/Arr3*) were downregulated in Day 28 knockdown organoids. All samples were normalized to *B-actin*.

differentiated Crx knockdown organoids (Clone A and Clone C), and gene expression levels were quantified using qRT-PCR. We found that photoreceptor genes (*Recoverin*, *S-Opsin*, *Rhodopsin*, and *Cone/Arrestin 3*) were down-regulated compared to organoids with no knockdowns (Figure 16). *Recoverin* was down-regulated 2-fold in both clones

compared to control. An interesting observation was S-Opsin expression in Crx Knockdown Clone C, which was downregulated nearly 20-fold compared to organoids with no knockdowns. Rhodopsin expression was down-regulated in both clones. Cone/Arrestin3 was also downregulated 5-fold in both clones. This confirmed to us that the expression of the photoreceptor genes located downstream of Crx was hindered in our knockdown lines.

To determine if expression of photoreceptor proteins were likewise down-regulated in the Crx knockdown lines, immunohistochemistry was carried out on mature three-dimensional retinas. Otx2 is a gene that is found upstream of the photoreceptor developmental pathway. We found that, as expected, there was a significant number of cells expressing Otx2 in the control with no knockdown (Fig 17A-3C). A zoomed in region of this organoid shows Otx2 expression patterns in more detail (Fig 17A'-17C'). We also found that there was no difference in Otx2 expression between both knockdown lines, clone C (Fig 17D-17F) and clone A (Fig 17G-17I), compared to organoids without knockdowns (Fig 17A-17C). Zoomed in regions of Crx knockdown Clone C (Fig 17D'-17F') and Crx knockdown Clone A (Fig 17G'-17F') show Otx2 expression in detail.

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S-Opsin, Rhodopsin, and Cone/Arrestin 3) were down-regulated compared to organoids with no knockdowns (Figure 16). Recoverin was down-regulated 2-fold in both clones compared to control. An interesting observation was S-Opsin expression in Crx Knockdown Clone C, which was downregulated nearly 20-fold compared to organoids with no knockdowns. Rhodopsin expression was down-regulated in both clones. Cone/Arrestin3 was also downregulated 5-fold in both clones. This confirmed to us that the expression of the photoreceptor genes located downstream of Crx was hindered in our knockdown lines.

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Figure 17: Expression of Genes Located Upstream of Crx in the Developmental Pathway Remain Unaltered at Day 28. (A-C) Day 28 Control organoids were stained with OTX2, an upstream cofactor. (D-I) Day 28 knockdown organoids exhibit similar OTX2 expression as control. DAPI stains nuclei. Scale bars 50 μ m.

Following confirmation that Crx knockdown organoids express early retinal markers, we wanted to observe if there was a decrease in protein production downstream of Crx expression. On day 28, after three-dimensional retinal differentiation, immunohistochemistry was used to detect expression of the pan-photoreceptor marker Recoverin and opsin marker Ret-P1. As shown in Fig 18A to Fig 18D, we found that our control line consisted of an abundance of cells expressing both Recoverin and Ret-P1 (Fig 18B and Fig 18C). In both Crx knockdown clones, however, there were fewer cells expressing Recoverin (Fig 18F and Fig 18J). There were also fewer cells expressing rod opsin marker Ret-P1 compared to control (Fig 18C and Fig 18K).

Currently, our lab is in the process of genotyping the knockdown lines to determine whether the 300 base-pair region is truly excised or if point mutations have been induced. In efforts to clarify the PCR results, PCR primers have been designed, targeting both the P1 and P24 regions independently. These primers may allow us to visualize point mutations at both sites.

In addition to optimizing PCR primers, we have also started optimizing protocols for genotyping CRISPR knockdowns. A recent study suggests the use of polyacrylamide gel electrophoresis (PAGE) as opposed to traditional agarose gel electrophoresis result in better image quality of bands when genotyping CRISPR generated cells (Zhu, 2014). The study suggests that PAGE assays are able to detect a variety of indel mutations generated via nuclease systems such as CRISPR/Cas9 with high efficiency, regardless of sensitivity. We will validate the efficiency of genotyping with PAGE versus agarose gel electrophoresis in our knockdown mouse ES cell lines.

In addition to using PAGE assays, we also plan to utilize specific techniques developed for detecting point mutations. A recent study suggests that the SURVEYOR nuclease assay is efficient in detecting indels and specific point mutations in CRISPR knockdown cell lines (Ran, 2013). We will utilize this nuclease assay to confirm whether we were able to induce point mutations.

Figure 18: Crx P1/P24 Knockdown Clones Show Restricted Photoreceptor Development. (B, F, J) Day 28 knockdown clones show lack of proper lamination of photoreceptor cells, as shown by pan-photoreceptor marker Recoverin when compared to control organoids with no knockdown. (C, G, K) In addition, there was limited expression of mature rod opsin protein, Ret-P1, in both knockdown lines compared to control DAPI stains nuclei. Scale bars 50µm.

DISCUSSION

As indicated by the results, we have been successful at developing a protocol for differentiating mouse embryonic stem cells into three-dimensional retinas *in vitro*. When developing our three-dimensional protocol, we first considered all limitations of currently published protocols (Decembrini, 2014; Eiraku, 2012; Hiler, 2016; Volkner, 2016). The first limitation was the use of low attachment 96-well plates. According to all three-dimensional retinal differentiation studies conducted by Eiraku (2012), the use of 96-well low attachment culture plates at the initial seeding step resulted in proper aggregate formation of cells, leading to the proper development of a three-dimensional neural retina. However, we found this to be a limitation for our differentiating protocol for several technical reasons, all of which affected output efficiency. Theoretically, we should expect a maximum output of 96 spheroids from a 96-well plate. However, the rinsing steps in our protocol had a tendency to remove the coating from the wells, causing the newly seeded cells to stick to the outer walls of the wells and not cluster at the bottom of the wells as aggregates. In addition, it was quite labor intensive to individually seed cells into all 96-wells of the plate. Finally, transferring the formed aggregates onto low attachment culture plates without disrupting or damaging the spheroid involved meticulous and gentle flushing of the spheroids out of each individual well. Not only time consuming, this method could not guarantee that all spheroids would remain undamaged by the end of the procedure.

In our revised protocol, we found agarose gel culture dishes to be more efficient compared to traditional 96-well plates for initial aggregate formation. It is a more cost-

effective method, and cells are simply seeded directly onto the mold, whereupon the cells naturally gravitate into each of the 256 individual wells of the culture dish. Another benefit to these culture dishes is the fact that the transfer of the formed aggregates as floaters onto low attachment culture dishes is gentler on the organoids than traditional flushing. One drawback to using these agarose gel dishes is that we cannot control the movement of each individual cell into a designated well. Once the cells are seeded, they migrate to their well of choice, resulting in inconsistent number of cells in each well. One way we have overcome the inconsistency in exact cell count is to measure the diameter of each organoid at 24 hours' post seeding. Prior studies have shown that in terms of mouse ES cells, an approximate organoid diameter of 450 μ m at 24 hours is equivalent to 1,500 cells per organoid (Volkner, 2016). In our case, organoids consistently developed with a diameter of 450 μ m at 24 hours.

Our results also confirm that the developing retina has specific requirements for both the retinal induction and retinal maturation cell culture media. When developing the three-dimensional retinal differentiation protocol, we wanted to be sure that the base media was void of any unknown protein or lipid that could influence or bias the mouse ES cells towards a retinal fate. Thus, Glasgow's Minimum Essential Media (GMEM) was selected as a base media for our initial retinal induction steps. Along with using GMEM, we also used components similar to those in previously published journals including Knockout Serum Replacer (KSR) and 2- β Mercaptoethanol (BME) (Decembrini, 2014; Eiraku, 2012; Hiler, 2016; Volkner, 2016).

According to a previous study by Volkner (2016), the number of cells used in the initial seeding affects retinal differentiation. In one study, we observed that a lower initial

seeded density of mouse ES cells resulted in the presence of more retinas compared to a larger initial seeding density (Volkner, 2016). Contrary to this study, we found that organoids with a higher number of cells at initial seeding exhibited an increased expression of early retinal precursor genes when compared to organoids with a lower number of cells at initial seeding. Mouse ES cells that formed any spheroid measuring less than or equal to approximately 450 μ m in diameter on day 1 resulted in palpitating organoids when differentiating under the same conditions as their counterparts. We can conclude that a dense network of cells is initially required to drive mouse ES cells to a retinal fate.

Prior studies have also claimed that cutting each organoid during differentiation has resulted in more retinal embryoid bodies than organoids that were not cut (Nakano, 2012; Volkner, 2016). However, we found that cutting organoids resulted in massive cell death. Although cutting the organoids at later time points was then attempted, high cell death continued to occur, which demonstrated that in our system, cutting organoids do not help with the optimal growth of retinal organoids.

Multiple studies have shown that in two-dimensional differentiation of stem cells, the modulation of certain pathways in the presence of small molecule inhibitors have the ability to bias cell fate (De Longh, 2006; Perrimon, 2012; Fujimura, 2016). In our study, mouse ES cells were differentiated using a small molecule inhibitor targeting the Wnt pathway. Prior studies have shown that modulating the Wnt pathway during ES cell differentiation promotes neural induction of the retina (Liu, 2006; Liu, 2010; Sakakura, 2016; Fujimura, 2016). Consistent with these studies, we found that a small molecule inhibitor targeting just the Wnt pathway promoted retinal induction in our three-

dimensional organoids.

Studies have shown that the use of natural retinoid analogues such as *all-Trans* Retinoic Acid (ATRA) allows for a more structured layer or lamination of photoreceptor cells (Zhao, 2006; Eiraku, 2012). However, a prolonged treatment of ATRA inhibits differentiation and maturation of photoreceptor cells (Eiraku, 2012). Although we found that the use of retinoids is beneficial for photoreceptor lamination during *in vitro* three-dimensional retinogenesis, it is not known whether the addition of retinoids plays any additional role during *in vitro* development. Retinoids are traditionally known to play a role in the visual cycle. Specifically, the isomerization of retinoids in the retina allow the initial absorption of photons entering the eye via photoreceptor cells and transfers these light signals to the inner nuclear layer (Kiser, 2013). The importance of retinoids *in vivo* vertebrate models is well studied and understood, however, current studies fail to characterize the exact developmental cues that occur following the addition of retinoids *in vitro*.

Since ATRA has some technical drawbacks—it is sensitive to light and loses potency after multiple freeze-thaw cycles, a more robust, synthetic compound known as EC23 was also used in our study. As shown in Figure 12, both ATRA and EC23 resulted in laminated photoreceptor architecture. Due to its stability, EC23 was chosen over ATRA for photoreceptor lamination in our protocol.

When first co-infecting mouse ES cells with lentivirus containing gRNAs targeting the P1 and P24 regions, respectively, we initially hypothesized that this co-infection would most likely result in the removal of the 300 base-pair region, which in turn would result in the production of a nonfunctional Crx protein. As suggested by the gene

expression analysis of day 21 Crx knockdown organoids, there was a reduction of expression of mature photoreceptor genes including Recoverin, S-Opsin, Rhodopsin, and Cone-Arrestin3 (Figure 16) at the transcriptional level, when compared to day 21 control organoids. These preliminary results suggest that the induced knockdown lines have a negative effect on genes whose expression depends on Crx. Protein expression in the Crx knockdown lines was also analyzed. Our study confirmed that a protein expressed upstream of Crx—Otx2—was not affected by the knockdown of Crx (Figure 17), whereas proteins expressed downstream of Crx—namely the pan-photoreceptor protein Recoverin and the opsin Ret-P1—were downregulated in both knockdown lines compared to control (Figure 18).

From this study, we can deduce that it is possible to generate three-dimensional retinas *in vitro* in a high-throughput manner using embryonic stem cells. Retinal organoids are a step towards not only improving treatments for patients with retinal degenerative diseases, but the regenerative medicine field as a whole. Some studies have also demonstrated that it is possible to restore some visual function in mice following transplantation of photoreceptor cells derived from a 2D culture (Lamba, 2009; Pearson, 2012). These studies show it is possible for transplanted exogenous cells to integrate into the host retinal tissue. However, it is not entirely feasible to generate a homogenous quantity of cells in a two-dimensional culture system as it is in three-dimensional organoids, especially when it comes to tissues composed of multiple cell types like the retina. The *in vitro* generated retinas have the capacity of generating a homogeneous supply of cells for transplantation purposes (Decembrini, 2014).

Although we are still undergoing the process of confirming efficient CRISPR-mediated Crx knockdown in mouse ES cells, it has been shown that the CRISPR/Cas9 system is translatable into animal models, and soon, patients (Yu, 2017). The induced proliferation of cone photoreceptors in mice resulting from a CRISPR/Cas9 knockdown of rod-specific gene NRL allowed for preservation of the function of cone cells (Yu, 2017). In addition, utilizing three-dimensional organoids in conjunction with CRISPR/Cas9 shows it is possible to generate disease models *in vitro* (Ravi, 2015). As previously indicated, three-dimensional models display *in vivo* characteristics far greatly than two-dimensional cell cultures.

The use of CRISPR/Cas9 to study disease progression in three-dimensional organoids can minimize the use of laboratory animals (Pampaloni, 2007). For example, using three-dimensional models to study the progression of retinal degeneration caused by any one of the 49 mutations in Crx is far more feasible than generating animal models for each mutation (Huang, 2012). With our study, we hope to expand the use of three-dimensional organoids to study disease progression.

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Appendix

Gene Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	T _m °C
<i>B-actin</i>	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACAGAGT	59
<i>Lhx2</i>	GAATGCAAGCTCAACCTGG	GAGAACCGCCTGTAGTAGTC	54
<i>Pax6</i>	GAAGCGGCTTTGAGAAGTG	ATTCTCAGATCCCTGTGAGC	54
<i>Rax</i>	CTTCGAGAAGTCCCCTACTACC	CTGGAACCATACTGGACC	54
<i>Otx2</i>	CAATGCGGCTGTAAGTTCC	GGCGGTTGCTTTAGATAAGAC	54
<i>Recoverin</i>	GCAGCTTCGATGCCAACAG	TCATGTGCAGAGCAATCACGTA	57
<i>Rhodopsin</i>	TCAAGCCTGAGGTCAACAAGC	ACTTCCTTCTCTGCCTTCTGAGTC	58
<i>Prdm1</i>	GTTTATCTGGTGTGAAGACTGC	GGAGTCCTTGACCATGACC	54
<i>Cone/Arrestin 3</i>	CAGTGTTTAAGAAGACTAGCTCC	AGGACGACTCCATCAATGG	53
<i>Thrb</i>	ATGTCTTCAGATCGCCCAG	GTGTTCAAAGGCCAACAGG	54
<i>S-Op sin</i>	CAGCATCCGCTTCAACTCCAA	GCAGATGAGGGAAAGAGGAATGA	57

Table 1: List of Primers used for qRT-PCR

Antigen	Host	Catalog Number	Supplier	Dilution
RECOVERIN	Rabbit	AB5585	EMD Millipore	1:100
RET-P1	Mouse	O4886	Sigma Aldridge	1:500
OTX2	Goat	BAF1979	R&D Biosystems	1:500
CRX	Rabbit	ab140603	Abcam	1:250
BCO	Goat	14363	Santa Cruz	1:250

Table 2: List of Antibodies used for Immunohistochemistry

Primer Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	Tm °C	Amplicon Size (bp)
Crx Primer 1a	CTCAGATTCTTACAGCCCATCTC	CCAGGCACTCTGATCTTTGTAG	55	469
Crx Primer 1b	GTACAGATCCTTTGGGCATCTC	TTGTAGTCCAGAGGGTCCAT	55	472
Crx Primer 2a	TCCTCTGACTTGCTCAGTTTG	CTCATTGTCCTTCTGCCTCTAC	55	464
Crx Primer 2b	GAAGCAGGAGCTGATGTAGAG	GGCCTCAAACCTGCATTGTAG	55	554
Crx Primer 3a	TTCCTACCCTCAGCTCCTTTA	CTACCTCCCTCGTGTTGAAATC	55	1409
Crx Primer 3b	ATGGACCCTCTGGACTACAA	CTCATTGTCCTTCTGCCTCTAC	55	1629
Crx Surveyor 4a	CTCAGATTCTTACAGCCCATCTC	GTGATTCCGCTGGAAGGG	55	522
Crx Surveyor 4b	TTGGGCATCTCAGATTCTTAC	CCCTTCTACAAGATCTGAAACTT	55	500
Crx Surveyor 4c	TGGGCATCTCAGATTCTTACAG	GGTCCATGGGATTGTAGGTAAA	55	447
Crx Surveyor 4d	AGAGGAAGGCAGGGACAT	AGAGGAAGGCAGGGACAT	55	505
Crx Surveyor 4e	GGGTTGGATCCCTACCTTTC	CTGGAGAGATGACTCAGCAATTA	55	1737
Crx Surveyor 4f	AGTTCCTATTTTCACTGGGTTGG	GTGCCATCACTCCTGGTTTAT	55	1794
Crx Surveyor 4g	CGGCTTCTGCTTTCTGTTCTT	TTCCTGGTGTGTCTGGAGATAG	55	1683
Crx Surveyor 4h	GCTTATGCATCTCCAAGTTCCTATT	TGTGGTGGCTCACAACCTATCT	55	1694

Table 3: List of Primers used for Genotyping CRISPR Knockdowns

Symbol	Name
3D	Three-dimensional
ATRA	All-trans Retinoic Acid
BCO	Blue Cone Opsin
BME	Bismercaptoethanol
cDNA	Complementary DNA
CoDA	Context-dependent Assembly
Cone/Arr3	Cone/Arrestin 3
CRISPR	Clustered Regularly Interspersed Short Palindromic Repeats
crRNA	CRISPR RNA
Crx	Cone-rod Homeobox
DMEM	Dulbecco's Minimum Essential Media
DNA	Deoxyribonucleic Acid
ES Cell	Embryonic Stem Cell
FBS	Fetal Bovine Serum
GCL	Ganglion Cell Layer
GMEM	Glasgow's Minimum Essential Media
gRNA	Guide RNA
HBSS	Hank's Balanced Salt Solution
HDR	Homology Directed Repair
INL	Inner Nuclear Layer
KSR	Knockout Serum Replacer
LIF	Leukemia Inhibitory Factor
mESC	Mouse Embryonic Stem Cell
MG	Müller Glia
NHEJ	Non-homologous End Joining
ONL	Outer Nuclear Layer
OPEN	Oligomerized Pool Engineering
Otx2	Orthodenticle Homeobox 2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
qRT-PCR	Quantitative Reverse Transcriptase PCR
Rec	Recoverin
RNA	Ribonucleic Acid
RPE	Retinal Pigment Epithelium
sgRNA	Single Guide RNA
SNP	Single Nucleotide Polymorphism
TALENs	Transcription Activator-like Effector Nuclease
trRNA	Tracr RNA
Wnt	wingless-type MMTV (mouse mammary tumor virus) integration site
ZFN	Zinc Finger Nuclease

Table 4: Table of Abbreviations