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Induction of Human Embryonic stem cell derived retinal stem cells in vitro using transient overexpression of messenger RNA for BLIMP, ONECUT1 and OTX2

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Induction of Human Embryonic stem cell derived retinal stem cells *in vitro* using transient overexpression of messenger RNA for BLIMP, ONECUT1 and OTX2

By

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A culminating thesis submitted to the faculty of Dominican University of California and Buck Institute for Research on Aging in partial fulfillment of the requirements for the degree of Master of Science in Biology

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CERTIFICATION OF APPROVAL

This thesis, written under the direction of the candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

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Abstract

Age-related macular degeneration (AMD) is one of several retinal degenerative diseases that results in a person's loss of vision due to retinal damage (1). The retina contains two types of photoreceptors called rods and cones. Rod photoreceptors are light sensitive and abundant in the retina. In contrast, cone photoreceptors are active only at higher levels of light and are scarce in the retinal layer (2). AMD impairs cone photoreceptors with no means to regenerate and this may eventually lead to the damage of other retinal cells (1). Preceding research has shown that human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells *in vitro* can be differentiated into retinal progenitors, allowing these cells to eventually mature into specific cell types. The overall goal of this research is to bias cone photoreceptor fate in these hES differentiated retinal stem cells *in vitro* using transient overexpression of messenger RNA. Regenerating cone photoreceptor cells *in vitro* could facilitate cell replacement therapy and improve research on AMD.

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Dedicated to Jojana and Glaver Cifuentes, my loving parents. Without whom none of this could have been possible. Thank you for all your unconditional love and support. I love you both very much.

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Background

Age Related Macular Degeneration:

Many forms of eye diseasess causing visual impairment exist in the United States. Amongst the retinal degenerative diseases, the National Eye Institute recognizes age related macular degeneration (AMD) as the leading cause of blindness for people 50 years or older. The two main types of AMD are dry and wet. Dry AMD is distinguished by an accumulation of yellow protein deposits known as drusen, while, wet AMD is recognized by new blood vessel growth, a part of the body's natural healing response. These abnormal vessels usually grow in an unstable fashion and are leaky, allowing blood to enter into the eye, causing further visual impairment (1).

Regardless of the type of AMD, this form of retinal degenerative disease is aggressive and ultimately causes damage to the central vision as shown in figure 1. About 14% of people who have dry AMD develop the wet form over time, which is more progressive (1). Currently, there are treatment options for the wet form, including taking high doses of vitamins or minerals, injections of anti-vascular endothelial growth factor into the eye, or some form of laser surgery. However, these treatments only help ablate the abnormal blood vessel formation, and often leave some scaring which further impedes central vision. To date, there is no cure for AMD. Furthermore, the cells that are harmed or destroyed by the AMD disease do not have the capacity to regenerate on their own. Thus once a cell is damaged, it is lost forever. The ability to regenerate lost cells using stem cell therapy is seen as a potential viable solution (2). In this study, we proposed a

method for developing cells *in vitro* that could replace cells damaged by retinal degenerative diseases, such as AMD.

Figure 1

Figure 1: Age-related Macular Degeneration affecting central vision.

The Retina:

The retina, a pseudostratified epithelial membrane that lines the back of the eye, is vulnerable to a variety of retinal degenerative diseases such as AMD. This membrane is viable to vision because it is light sensitive, which allows for image processes to occur (3). The cells responsible for vision are known as photoreceptors and respond to light photons. Light travels through the thick layers

of the retina before triggering the photoreceptors (figure 2). The absorption of photons by the photoreceptors is first translated into a biochemical message and then an electrical message which stimulates all the downstream neurons of the retina. When this biochemical message reaches the ganglion cells, its long axons, that make the up the optic nerve, help transport the information to the brain, which is then perceived into an image (4).

As stated earlier, photoreceptors are the cells involved in visual response. The two types of photoreceptors are the rods and cones. Both photoreceptors are located throughout the retina including the macula, an area in the center of the retina. Rod photoreceptors are extremely light sensitive, and only detect the presence or absence of light; rods are responsible for black and white vision and are vastly abundant, with approximately 120 million rods found around the periphery of the macula (5). On the other hand, cones reside in the fovea, the central area of the macula (figure 2B). Cones make up the majority of central vision and respond to high levels of light. Often referred to as the color photoreceptors, the cones are far less abundant then rods, with an estimate of about 6 to 7 million in the retina and enriched around the macula and fovea (6). Since AMD causes damage to the area known as the macula, and more specifically, the fovea, this disease primarily targets and damages cone photoreceptors.

Figure 2A

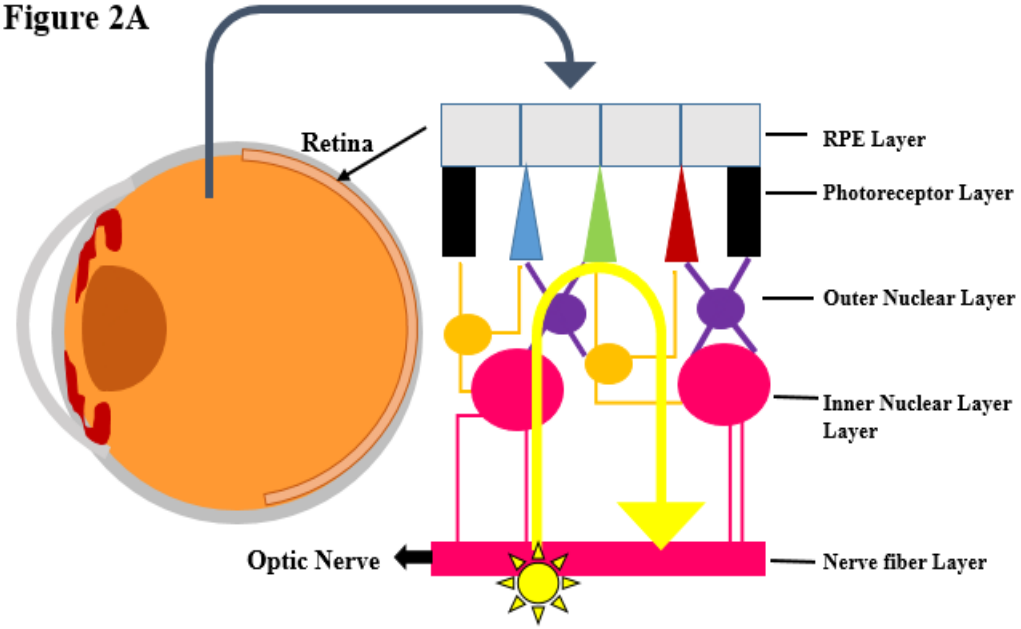


Figure 2B

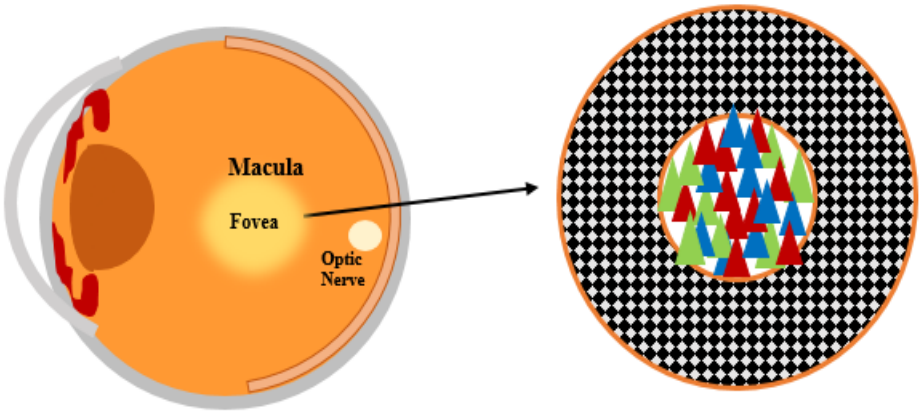


Figure 2:
(A) The picture to the left shows the light sensitive layer at the back of the eye known as the retina. The picture to the right demonstrates how light travels through this pseudostratified layer and reaches the photoreceptors, which send a signal back to the brain through the optic nerve. (B) The picture to the left shows the optic nerve and close by an area in the center of the retina known as the macula. The picture to the right demonstrates a head on view of the macula and the photoreceptors that occupy it.

Stem Cell Research:

Vision restoration through stem cell transplantation has been a vastly studied area of interest. This could be an applicable therapeutic approach for many retinal degenerative diseases such as AMD. In order for treatment to be successful, the regeneration of retinal cells needs to be accomplished first. The growth and development of retinal neural cells, including rod photoreceptors *in vitro* using stem cells have been developed by several research groups including the Lamba lab (7). However, the specific growth of cone photoreceptor cells *in vitro* has not yet been successful for several reasons, especially because the exact genes and specific pathways that regulate cone identity are unknown. Successful development of these cells are essential for many reasons. First, photoreceptor cells are in close proximity to each other and studies have shown that damage to one neuronal cell is able to affect an adjacent neuronal cell (8). These types of damages could lead to other diseases such as retinitis pigmentosa that affect rod photoreceptors. Generating and transplanting cone photoreceptors could impact rod photoreceptor health. Moreover, cone photoreceptor generation could further retinal degenerative disease studies and develop new approaches such as cell replacement therapy for retinal degenerative diseases, like AMD.

Cell replacement therapy can be achieved through use of stem cells and offers a viable approach to treating retinal degenerative diseases. Stem cells have three significant properties: 1) the cells have limitless replication capacities, 2) the cells are unspecialized and 3) the cells can differentiate into specialized cell types (9). There are no known retinal stem cells, but with innovations in human

embryonic stem (hES) cells, hES cells can be differentiated into retinal stem cells. These hES cells are derived from embryos that have been artificially inseminated *in vitro*. These cells have been shown to have the ability to self-replicate and are pluripotent. Pluripotency allows for an undifferentiated cell to be differentiated into any cell type. Additionally, the generation of induced pluripotent stem (iPS) cells by Shinya Yamanaka is a beneficial new way to regenerate specific cell types from different cell lineages. Induced pluripotent stem cells are derived from adult somatic cells and can then be regenerated into undifferentiated pluripotent stem cells using four genes, Oct4, Sox2, cMyc, and Klf4 (10). Advances such as iPS cells have become useful when overcoming obstacles in both research and ethical concerns.

Equally human embryonic and induced pluripotent stem cells have the capabilities of differentiating into specialized cells (9, 11). Previous studies in our lab have demonstrated that both hES and iPS cells can differentiate into retinal cells and mature into transplantable rod photoreceptors successfully (12). Mammalian retinal differentiation can be achieved in many ways by altering several signaling pathways. Forebrain and retinal development involves the co-inhibiting bone morphogenetic protein (BMP) and Wnt signaling pathways (13). In support of this Barrantes and others demonstrated that mice expressing mutated *dickkopf1 (dkk1)* and *noggin*, two known BMP and Wnt antagonists, developed major head and eye defects.

Furthermore, the Notch-Delta pathway has been shown to be essential for inducing retinal progenitor cells (14), whereas insulin like growth factor-1 (IGF-1)

has been shown to be necessary for eye development (15). Osakada and others established a protocol demonstrating the use of specific small molecules to induce retinal differentiation as an efficient and cost effective way to produce photoreceptors, which has been replicated in our lab (16). The small molecules used were SB4131542, IWR-1, and LDN193189 along with IGF1 (a recombinant protein). These small molecules and protein are collectively known as ISLI. Neural retinal differentiation induction can be traced through many markers. For example, *LIM homeobox (LHX2)* and *retinal homeobox protein (RX)* are eye field transcription factors that play essential roles in mammalian eye development. Moreover, photoreceptor differentiation can be seen through the upregulation of several transcription factors including *cone-rod homeobox (crx), neurod1 (ND1),* and *Recoverin (REC)* (17). More specifically *thyroid hormone receptor beta 2 (Thrb2)* and *retinoid receptor X gamma (RXRG)* are cone photoreceptor specific markers (18).

Cone Photoreceptor Development:

During neural retinal differentiation via hES or iPS cells *in vitro*, many specific genes are upregulated at various time points, which likely explains the genesis of some retinal cells (17, 18). Cone photoreceptors are one of the first cells developed during retinal neurogenesis (19). However, the exact genes that bias cone photoreceptor development still remain unclear. Understanding which photoreceptor genes are expressed early on could offer insights into the origin of

cone cells and could elucidate the development of cone photoreceptor cells from hES or iPS cells *in vitro.*

Unpublished data from our collaborator, Dr. Joseph Brzezinski (Colorado University, Colorado, CA), identified three early possible cone photoreceptor transcription factors through RNA sequencing. This initial data was obtained using differentiated mouse retinal stem cells where notch signaling was inhibited at day 14.5, and thus driving retinal neural induction. Birthing studies demonstrated that this specific time point was seen to give rise to a peak in cone photoreceptor development (20). OTX2, a known photoreceptor gene, ONECUT1, and BLIMP were all upregulated synchronously at 14.5 days during 10,12, and 18 hours following notch inhibition. *Trb2*, a mature cone photoreceptor marker, was upregulated later at 48 hours. This preliminary analysis suggested that OTX2, ONECUT1, and BLIMP could potentially be responsible for biasing cone photoreceptor cell fate. Further studies were necessary in order to develop a solid understanding of whether and how these genes may contribute to retinal cell progenitor fate and cone photoreceptor development.

The goal of this study is to investigate whether cone photoreceptor cells can be developed *in vitro* by overexpressing OTX2, ONECUT1, and BLIMP using differentiated retinal neural stem cells. This overexpression was done through messenger ribonucleic acid (mRNA) transient transfection of the three genes. Using mRNA has many advantages, in that 1) mRNA will not harm the host genome, 2) mRNA does not need to be localized in the nucleus and 3) it allows for faster interpretation of experimental results (21). Although mRNA has its

benefits, RNA is known to rapidly degrade, hence DNA transfections have been traditionally used more often. However, a recent study bypassed this problem where researchers confirmed their product (a stable RNA vector) could be transfected in human cells for a longer duration of about 36 hours (22). Using mRNA for this research allowed us to study cone cell origin. Developing cone photoreceptor cells *in vitro* could potentially transform the way retinal degenerative diseases are studied and may potentially be used as a means for cell replacement therapy for diseases such as age related macular degeneration.

Materials and Methods

Plasmid Transformation

DNA plasmids (OTX2, ONECUT1, and BLIMP) were provided by Dr. Joseph Brzezinski (University of Colorado, Boulder, CO). Plasmids were used to transform competent One Shot *E. coli* bacteria (Thermo Fisher, Cincinnati, OH), using manufacture's protocol. Bacteria were plated on Luria Broth (LB) media (Thermo Fisher, Cincinnati, OH) supplemented with either ampicillin or kanamycin, depending on resistance. Plates were incubated overnight at 37˚C. From each transformation, three bacterial colonies were picked the following day and grown for 1 hour at 37˚C in 15-ml of LB media containing the matching antibiotic. This bacterial culture was then used to inoculate 250 ml of LB media containing the corresponding antibiotic (1:1000 µg/µl). The flask was incubated overnight at 37˚C in a Thermo Scientific MaxQ 8000 bacterial shaker.

DNA Extraction

Plasmid DNA was purified using the Maxi kit (IBI Scientific, Peosta, IA) according to the manufacturer's protocol. The DNA pellet was re-suspended in 300 µL of elution buffer. The DNA was quantified via Thermo Scientific NanoDrop 2000 Spectrophotometer.

DNA Enzyme Digest and Extraction

DNA enzyme digestion reactions were setup as follows: 24 µg of purified plasmid DNA, 6 µl of Nhe1, 10 µl of BSA, 1 µl of 2.1 Buffer, and diethylpyrocarbonate (DEPC) water to bring the total volume to 100 µl. Nhe1, bovine serum albumin (BSA), 2.1 Buffer were purchased through New England BioLabs (Ipswich, MA). The samples were placed in a 100-µl PCR tube and incubated for 4 hours at 37˚C, followed by 15 minutes at 65˚C in a T100 Thermo cycler (Biorad, Hercules, CA) to inactivate the NheI restriction enzyme. Enzyme digestions were confirmed with a 1.5% LE quick dissolve agarose electrophoresis gel (GeneMate, Kaysville, UT), running at 100V for 30 minutes. 100 µl of the digested DNA was mixed with 100 µl of 1:1 phenol-chloroform in a 1.7-ml eppendorf tube for DNA extraction. The mixture was centrifuged for 5 minutes at 11,000 RPM. The upper layer was transferred to a new eppendorf tube and washed with 1 mL of 100% ethanol. This was centrifuged at 11,000 RPM for 10 minutes to obtain a DNA pellet, which was re-suspended in 30 µl of (DEPC) water and quantified via nano spectrometry.

Messenger RNA Synthesis

Nhe1 cut DNA plasmids (control CHERRY, BLIMP, ONECUT1, and OTX2) were run through the HiScribe T7 ARCA mRNA kit according to the manufacturer's protocols for mRNA synthesis. Synthesized mRNA was re-suspended in 50 µl of RNase-free water and quantified through nano spectrometry. A 1.5% agarose electrophoresis gel (GeneMate) was done in order to confirm the quality of RNA bands.

Retinal differentiation of human embryonic stem cells

Human embryonic stem cell lines (hESC) H1 and WA01) were purchased from WiCell Research Institute (Madison, WI). Cells were maintained in 6 well plates in essential 8 media (ES8), which consisted of 250 ml of Essential 8 basal media (Thermo Fisher, Cincinnati, OH), 1% penicillin-streptomycin amphotericin B (Lonza, City and State), and 1% Essential 8 Supplement (Gibco). The hES cell differentiation media contained 10% knockout serum replacement (KSR), 0.1% recombinant Insulin Growth Factor 1 (R&D Systems, Minneapolis, MN), and small molecules in the following concentrations: 0.1% SB413542 (Stemgent, City and State), 0.1% IWR-1 (Sigma Aldrich, St. Louis, MO), and 0.01% LDN193189 (Stemgent). Cells were kept on differentiating media for 5 days and then dissociated using 1 ml of Tryple (Corning, Manassas, VA). Half media changes with neural stem cell (NSC) media plus differentiating media would be done up until day 7, followed by full media changes with only NSC media. Differentiated neural retinal cells were sustained indefinitely in NSC media consisting of

DMEM/F-12 1:1 (Hyclone, Logan, Utah), 1% penicillin-streptomycin amphotericin B (Lonza), 0.5% Fetal Bovine Serum (FBS; Atlanta Biologicals), 1% sodium bicarbonate (Corning), 1% HEPES Buffer (Corning), 1% MEM nonessential amino acids (Corning), and 1% N1 Supplement (Sigma Aldrich).

Human Embryonic Kidney (HEK) Cells

Passage 1 HEK cells were kept in media composed of 70% DMEM/High Glucose (Hyclone), 10% penicillin streptomycin amphotericin B (Lonza), and 20% fetal bovine serum (Atlanta Biologicals, Miami, FL). Cells were grown to 70% confluence for transfection experiments.

Transfection Protocol

Transfections were carried out on hES neural retinal stem cells between one and three months following differentiation (during earlier retinal development). Cells were dissociated with 1 ml of accutase (Sigma Alderich) per well (usually 2 wells from a 6-well plate needed) and transferred onto a 24-well plate coated with matrigel. When approximately 70% cell confluence was reached (about 2-3 days), transfections were performed. Using Stemgent's RNA Transfection Kit (Cambridge, MA), cells were transfected with the mRNA (CHERRY, BLIMP, ONECUT1, AND OTX2) synthesized via HiScribe T7 ARCA mRNA kit. For each reaction, 0.5 µg of mRNA, 1 µl of Stemfect reagent, and 25 µl of Stemfect buffer were mixed and incubated at room temperature for 15 minutes. This mixture was then applied to one well of the 24-well plate, along with 2 ml of NSC

media. The reactions were left overnight at 37°C and successful transfections were confirmed with the CHERRY control mRNA. If taken in by the cell, the CHERRY mRNA fluoresces red under the fluorescent microscope. Transfection experiments were left 7 days post transfection with daily changes of NSC media in order to see if mRNA production is validated after one week. Analysis of all transfections *in vitro* was done through quantitative RT-PCR.

RNA extraction, Reverse Transcription and Quantitative PCR

RNA extraction from wells was done using Direct-zol RNA MiniPrep kit according to manufacturer's protocol (Zymo Research, Tustin, CA). RNA samples were resuspended in 30 µl of DEPC water and the yield was determined using the Thermo Scientific NanoDrop 2000 Spectrophotometer. Synthesis of cDNA was done with iScript cDNA synthesis kit (BioRad) per manufacturer's protocol. 250 ng of total RNA was used, and the rest of the kit's instructions were followed. The reactions were incubated in a T100 Thermal Cycler machine per kit's instructions. Quantitative RT- PCR. Analysis was performed on the CFX Connect Real Time PCR Detection System (BioRad). Primers used in this study are listed in Table 1.

Table 1 – List of Primers

Primary Name	Forward Sequence	Reverse Sequence
β-Actin	GGA TCA GCA AGC AGG AGT AT	GGT GTA ACG CAA CTA AGT CAT AG
Lhx2	TAC TAC AGG CGC TTC TCT G	GAT AAA CCA AGT CCC GAG C

Rx	CTC CTC TCA GTT CAC CAA G	CAT CTC TTT GCC TCA GTT CT
Crx	CCT TCT GAC AGC TCG GTG TT	CCA CTT TCT GAA GCC TGG AG
BLIMP	GTG GTA TTG TCG GGA CTT TG	GGT TGC TTT AGA CTG CTC TG
NeuroD 1	GAA AGC CGT CTG ACT GAT TC	AGA AGT TGC CAT TCA TCG TGA G
Recover in	CCA GAG CAT GTA CGC CAA CT	CAC GTC GTA GAG GGA GAA GG
RXRG	GCT GAA CTT GCT GTT GAA CCA AAG	TTG GTA ACA GGG TCA TTT GTC GAG
TRB2	ACA CCA GCA ATT ACC AGA GTG GTG	TGG TCT TCA CAT GGC AGC TCA C
Nrl	ATG TGG ATT GGA CGA CTT C	TTG GCG AGA TTG TCT TGG

Immunohistochemistry

Cells were fixed with 2% Paraformaldehyde (Alfa Aesar, Sparks, NV) for 45 minutes at 4˚C in the dark. Paraformaldehyde was aspirated off, and cells were washed two times at five minutes each with 1X phosphate-buffered saline (PBS). Following the washes, samples were blocked with a block solution consisting of 1X PBS and 10% normal donkey serum (EMD; Millipore Darmstadt, Germany) for one hour. Primary antibodies were diluted in blocking solution according to Table 2. Primary antibodies were distributed to fixed cells and were incubated

overnight at 4°C in the dark. The cells were washed two times as described above. Secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA) were added at a 1:500 dilution in block solution, and then placed on cells for one hour at 4°C in the dark (not shown in table). The secondary solution was removed with two washing steps as described above. DAPI (Enzo Life Sciences Farmingdale, NY) was added to the cells for 30 seconds, and cells were washed an additional two times. Samples were then mounted on glass slides (VWR, Radnor, PA) and viewed using Fluoromount-G (Electron Microscopy Science, Hartfield PA).

Table 2 – List of Primary Antibodies

Antibody	Company	Dilution
OTX2, donkey anti goat	Santa Cruz Biotechnology	1:100
Recoverin, donkey anti goat	Santa Cruz Biotechnology	1:100
RXRG, donkey anti rabbit	R&D Systems	1:100

Animal Transplantation and Samples

Human retinal stem cells were generated using the differentiation protocol, infected with GFP plasmid and transfected using the transfection protocol. The cells were exported and implanted into IL2rg null mice (graciously performed and provided by Dr. Jie Zhu, Buck Institute for Research on Aging, Novato, CA). Upon receiving mouse retina samples, staining for photoreceptors was carried out with the immunohistochemistry previously described."

Results

Development of the Neural retinal stem cell differentiation protocol:

The Lamba lab has established several retinal neural differentiation protocols using both hES and iPS cells. The specific protocol chosen here used four molecules to drive neural retinal differentiation (Figure 3A). These molecules differentiated stem cells in a less costly and more accelerated manner, where differentiation is only happening for a few days as opposed to weeks. In this particular investigation, the human embryonic stem cell line referred to as H1 cells was the main cell type used for neural retinal differentiation. However, an iPS cell line called Lonza, generously donated by the Zeng lab at the Buck Institute (Novato, CA) was also used for the same experiments (data not shown). The results below correspond to H1 cells; however, the data for the Lonza cells was not significantly different. Neural retinal differentiation occurred best when the differentiation cocktail media was added to these undifferentiated cells at 70% well confluency and an average cell size of 4µm (figure 3B). This allowed the undifferentiated cells to properly differentiate and spread out.

The differentiation cocktail media included four main molecules, insulin growth factor1 (IGF1), SB4131542, IWR-1, and LDN193189. These are collectively referred to as ISLI and have been shown to drive neural retinal differentiation (16). When cells reached 100% confluency at day 5, they were dissociated. In order to get cells acclimated to the regular neural stem cell media (NSC), half of the media was changed until day 7. Complete NSC media changes continued after day 7. Typically by days 15-30, morphological changes were

noticeably visible in the differentiated cells. The first confirmation towards successful neural retinal differentiation occured when neural clusters or neural rosettes began to develop. These neural areas were manually picked and expanded for future experiments (Figure 3B – 3E). In this specific experiment, cells were grown and maintained until around 90 days after differentiation media was added to them at Day 0.

At the three-month time point, quantitative RT-PCR data was used to confirm effective neural retinal differentiation, where both eye field and retinal transcription factors were shown to be upregulated (Figure 1F). Taken together, this data confirmed that H1 cells could be successfully differentiated into neural retinal stem cells. These differentiated cells were then expanded until they reached the appropriate age (a potential period for cone photoreceptor development) for the next phase of the experiment, which was to conduct transient mRNA transfection of genes of interest to study their role in photoreceptor development.

Figure 3A

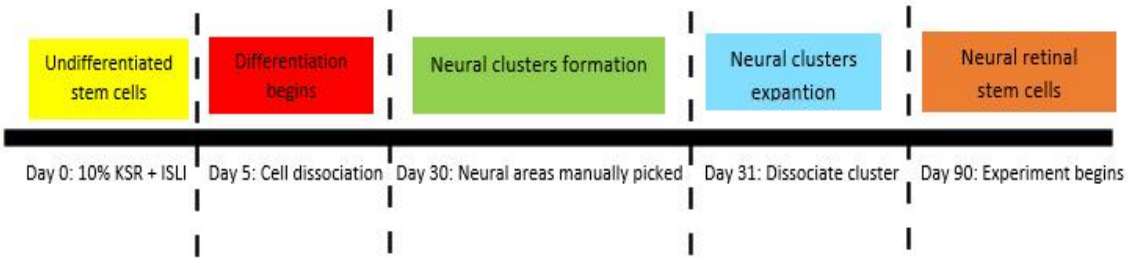


Figure 3B

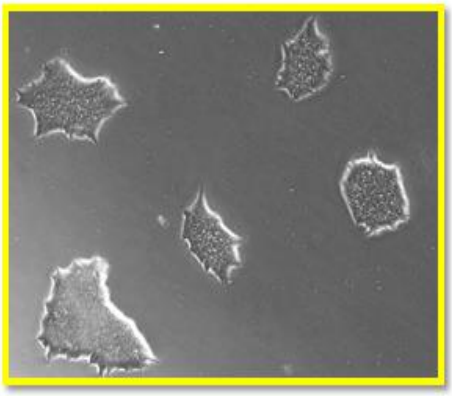


Figure 3C

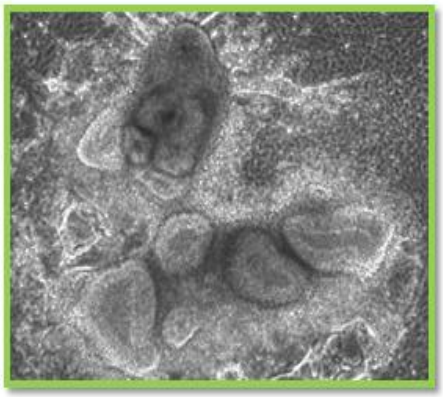
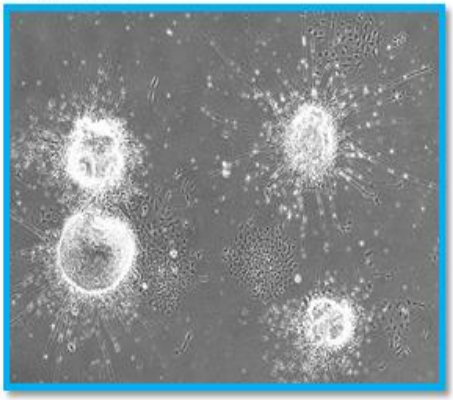


Figure 3D



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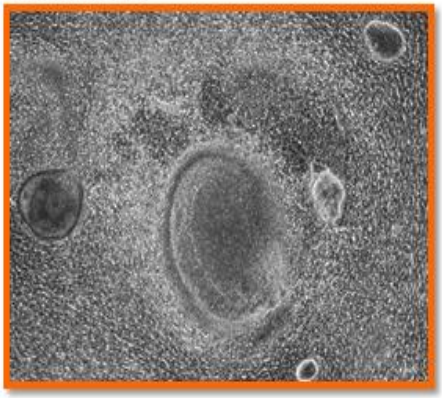


Figure 3F

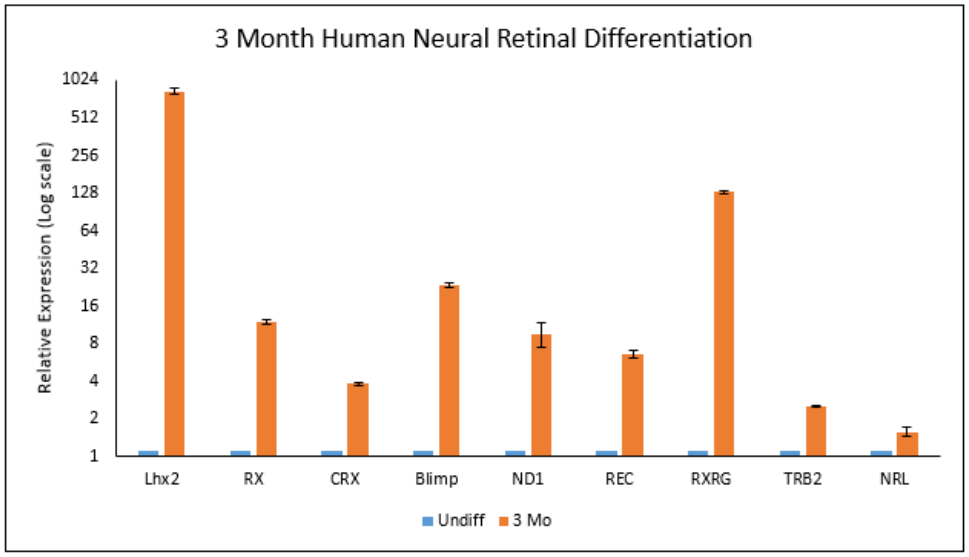


Figure 3:

(A) A schematic of hES cell retinal differentiation, highlighting the important protocol time points from Day 0 – Day 90. (B-D) Images representing neural retinal differentiation are all visible at 10X. Border color is consistent with time point on the timeline in figure 3A. (B)The hES cells were placed in a cocktail of differentiation media for 5 days. (C) At 30 days, hES cells began to develop large neural areas. (D) These neural clusters were dissociated and expanded. (E) Neural retinal cells at 3 months. (F)Three months differentiated neural retinal stem cells were crossed compared with undifferentiatied hES cells using q-RT PCR. H1 differentiated stem cells demonstrated a significant in expression of eye field transcription factors, *Lhx2* and *Rx*. Also, there was an increase in *Crx*, cone rod homeobox, and *Nrl*, a rod photoreceptor marker (24).

Synthesis of mRNA and working parameters for transient transfection:

Transfection protocols typically involve transfecting DNA, however, for the experiments in this project, mRNA was used instead. As mentioned in the introduction, there are multiple advantages for selecting mRNA. Here, we used

Toshinori's approach (22) for stabilizing RNA during the duration of the transfection. In short, our collaborators (Dr. Joseph Brzezinski, Colorado University, Aurora, CO) developed and provided pSLU expression plasmids for the following genes— BLIMP, ONECUT1, OTX2, and CHERRY *(*control) (Figure 4A). The genes were inserted into the multiple cloning site (MCS) of the pSLU vector. Downstream of this MCS were sequences for the 3′ UTR of the Venezuelan equine encephalitis virus (VEEV), which served as a stabilizing motif and recognition site for the NheI restriction enzyme that was later used for linearization. After DNA linearization, synthesis of mRNA occurred with the HiScribe kit and mRNA synthesis was confirmed by gel electrophoresis (data not shown).

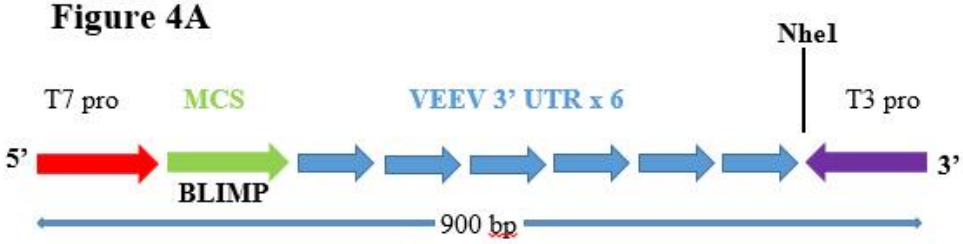
In order to save time and resources (while mRNA synthesis was in progress) a green fluorescent protein (GFP) and human embryonic kidney (HEK) cells were used to test a variety of transfection parameters for experiments to follow. We first tried using electroporation as the vehicle for transfection, however, a high degree of cell death was observed along with only a few single cells being transfected (data not shown).

After several failed electroporation attempts, different chemical transfection reagents were then tested. Stemfect (from Stemgent) was the chemical reagent with the best results. We also transition the transfection into a 24 well plate, which required less Stemfect reagent and GFP concentration, and this reduced the number of cell deaths and increased the number of positive transfectants in HEK cell (Figure 4B). Once the transfections parameters were established, GFP

transfection was then tested on the H1, the human neural retinal differentiated cell line that would be used for the remainder of the study. Results in figure 4C showed that the same conditions (Figure 4B) were able to successfully transfect the differentiated hES cell line with little to no cell death and the majority of the cells had GFP (figure 4C).

Next, the HEK cells were transfected with the control mRNA, CHERRY. CHERRY is another fluorescent marker like GFP, except it fluoresces red. Following transfection of HEK cells with CHERRY mRNA, cells showed an abundance of red fluorescence under the microscope, indicating that the transfection parameters were working (data not shown). Following the successful transfection of the control mRNA, the synthesized mRNAs for each of the gene of interest were separately transfected into HEK cells using the same conditions. Cells were collected post transfections and analyzed with qRT-PCR. Each gene (BLIMP, ONECUT1, and OTX2) demonstrated an overexpression in the respectively transfected HEK cells compared to non-transfected HEK cells (figure 4D). With these results, we moved forward with transfecting the H1 cells, first with CHERRY mRNA.

Figure 4A


NheI
T7 pro
MCS
VEEV 3' UTR x 6
T3 pro
5'
3'
BLIMP
900 bp

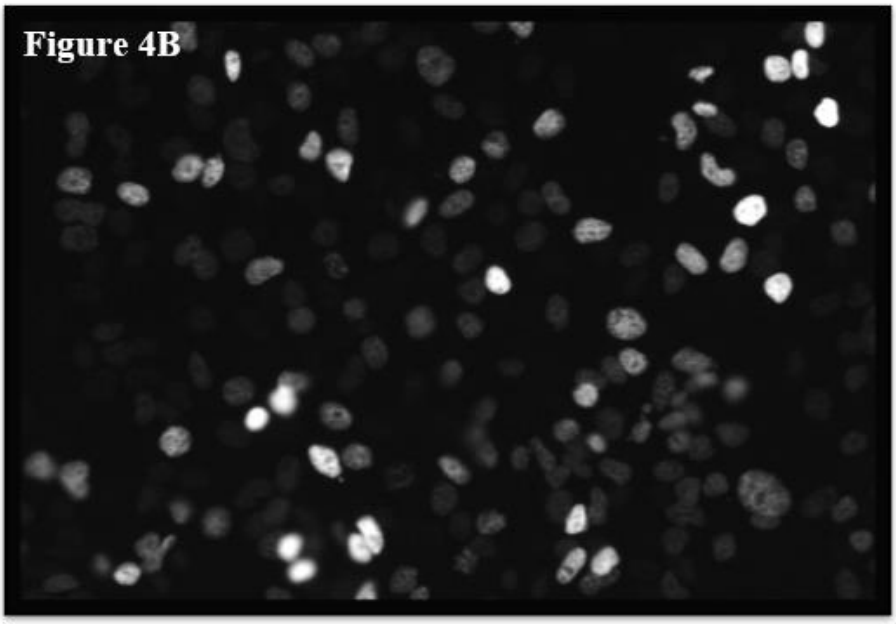


Figure 4B

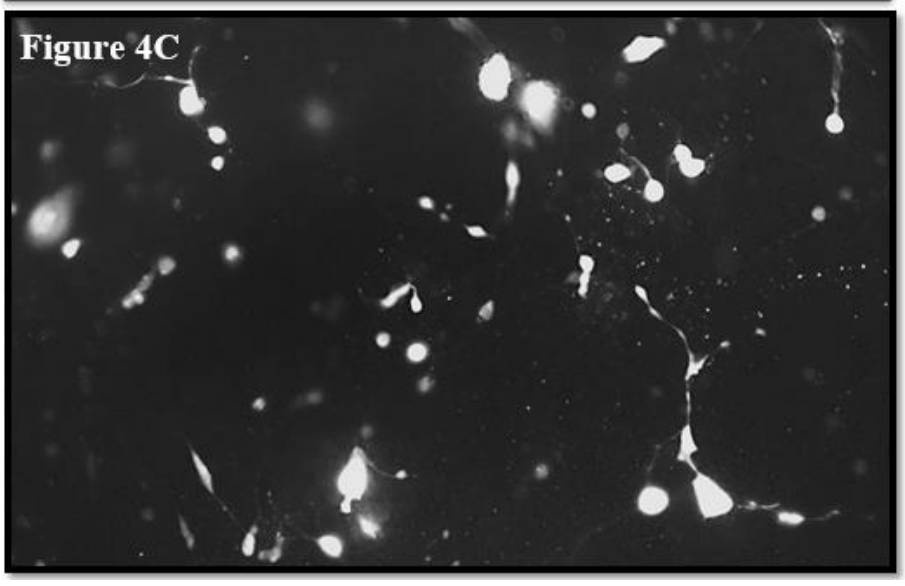


Figure 4C

Figure 4D

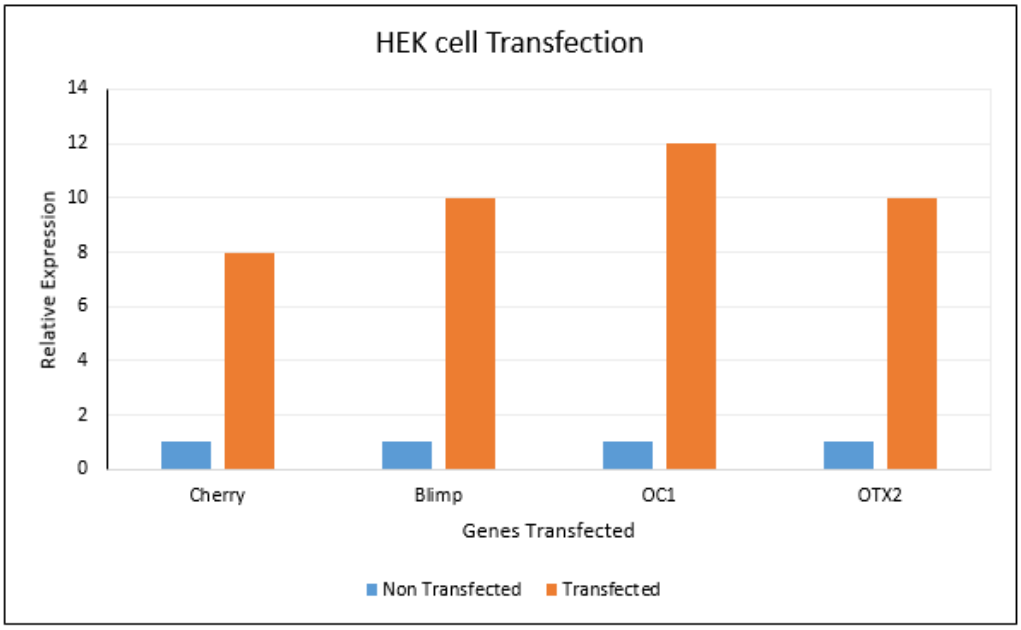


Figure 4:

Figure 4: (A) A schematic of a pLSU vector, showing the relative location of the T7 and T3 promoter with the multiple cloning site (showing *BLIMP* as an example), the six repeat sequences of VEEV 3'UTR, and the Nhe1 restriction enzyme. (B) Fluorescent positive HEK cells indicated that GFP transfection using the Stemfect reagent was effective. (C) Fluorescent positive cells indicated that GFP transfection using the Stemfect reagent was effective on the H1 line. All images were taken at 10X. (D) Transfected HEK cells showed an increase level of gene expression versus the non-transfected.

Transfection of H1 differentiated neural retinal cells with CHERRY mRNA:

Having established the parameters for neural retinal differentiation and transient transfection, the next phase of the experiment could proceed, transfecting the control CHERRY mRNA into hES (H1) cells. Transfections where done on differentiated neural retinal cells 3 months of age, a peak in human cone

photoreceptor development. These cells were plated on a 24 well matrigel plate and grown until about 70% confluence, and cells were transfected with CHERRY mRNA. Both microscopy and PCR analysis were used to confirm transfection and expression of the CHERRY (Figure 5). CHERRY expression (red fluorescence) was observed the first day after transfection (Figure 5A).

The strong expression of CHERRY protein was sustained five days after transfection, then the expression slowly decreased after 5 days, until it completely disappeared around day 14. Normal media changes were done and no cell death was observed during the 14 day period (data not shown). RNA was extracted on day 5, day 7, day 10 and day 14. On day 7, qRT-PCR data showed a seven-fold increase in CHERRY expression in transfected H1 cells compared to mock transfected cells (Figure 5B). RNA was also analyzed for the other days using qRT-PCR, and consistent with the fluorescent microscopy, the CHERRY expression gradually decreased until 14 days post-transfection (data not shown).

This data confirmed that the transient mRNA transfections were successful in the human embryonic stem cell-derived retinal neural stem cells (3 months of age) and that expression of the gene of interest occurred after no more than 7 days post transfection. Therefore, we proceeded with the main objective of our experiment, overexpressing the three early retinal genes (BLIMP, ONECUT1, OTX2) in order to bias cone photoreceptor cell fate.

Figure 5A

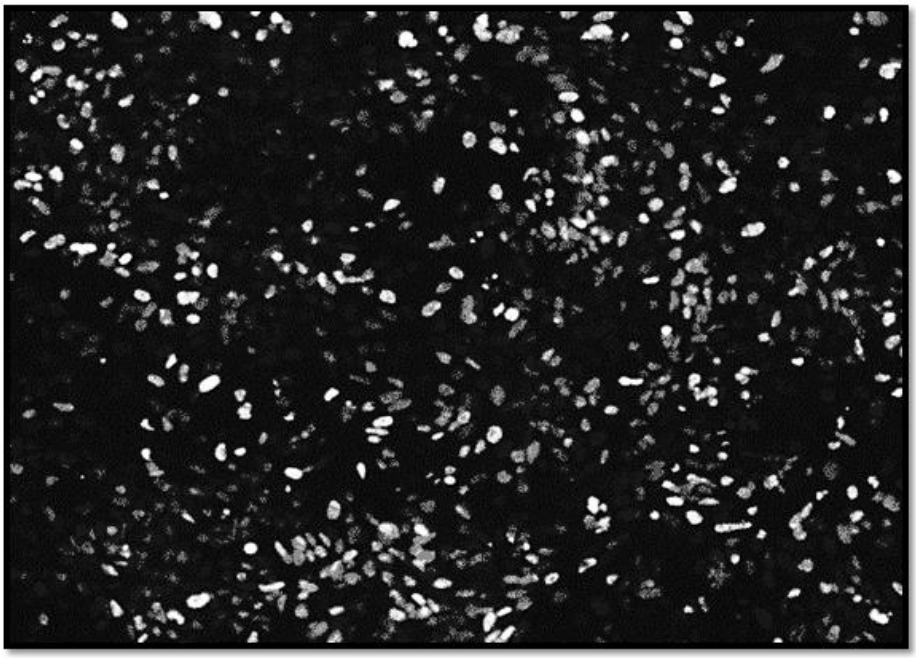


Figure 5B

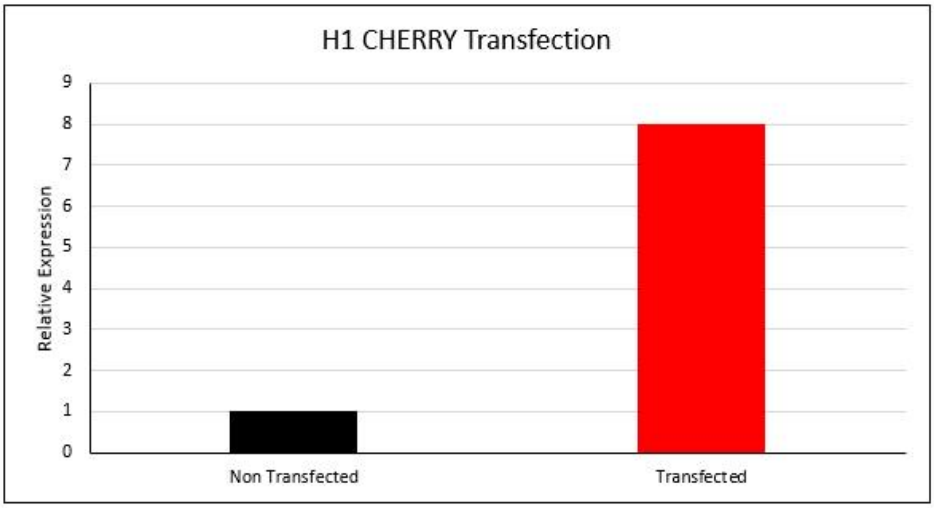


Figure 5:

(A) CHERRY control transfection proved effective via fluorescent cells. Picture was taken at 10x, measuring 4µm cells. (B) Quantitative RT-PCR done 7 days post transfection demonstrates an overexpression of CHERRY in CHERRY transfected H1 cells.

Transfection of H1 differentiated neural retinal stem cells confirms BLIMP, ONECUT1 and OTX2 transient mRNAs:

After positive CHERRY transfection was confirmed, H1 cells were transfected with mRNA for BLIMP, ONECUT1 (OC1), and OTX2. Following the same protocol established earlier, transfections were done first with single genes (i.e. BLIMP or OC1 only), then with a combination of two genes (i.e. BLIMP and OC1), and finally with all three genes (BLIMP, OC1, OTX2, also referred to as BOO). Since BLIMP, OC1, or OTX2 mRNAs do not have a fluorescent tag, validation of mRNA overexpression was seen through qRT-PCR 7 days post transfection. Results in figure 6A confirmed that transfected H1 cells did indeed overexpress BLIMP, OC1, and OTX2. CHERRY mRNA was used as our control, and cells transfected with CHERRY mRNA did not express any of the genes of interest— BLIMP, OC1, and OTX2.

Following transfection, we further analyzed how the transfection of each individual mRNA translated to gene expression. As expected, H1 cells transfected with BLIMP mRNA overexpressed BLIMP. The same was observed for OC1 and OTX2 mRNA single transfections. More specifically, the results for the single mRNA transfections showed approximately 16-32 fold increase for OTX2, and approximately a 4 fold increase BLIMP and OC1 (figure 6A). Similar to CHERRY transfections, single transfections (BLIMP, OC1 or OTX2 alone) decreased overtime until no expression was observed 14-days post transfection (data not shown). Interestingly, OC1 and OTX2 mRNA transfected cells showed an overexpression in BLIMP, while BLIMP transfected cells exhibited an

increased expression of OTX2 (6A). Taken together this data suggest that these three early retinal development genes may work together in the same pathway, either upstream or downstream from one another.

To further analyze whether these three early genes work together, we performed additional transfection of these genes in combinations or two or three. OC1 and OTX2 mRNAs were transfected together using the established protocol. This double transfection resulted in a four to five fold increase in OC1 and OTX2 expressions and a one fold increase in BLIMP expression. It was interesting to note that the double transfection did not drive OC1 or OTX2 expression significantly more than the single mRNA transfections; similarly, BLIMP was also expressed in the double transfection (OCI and OTX2 together) as it was in the single OC1 or OTX2 mRNA transfections. When all three mRNAs (BLIMP, OC1, and OTX2) were transfected, cells showed an equal expression (4-64 fold increase) of all three genes (Figure 6C). Furthermore, transfecting all three mRNA consistently resulted in similar expression levels for all three genes, while the double transfection often resulted in more varied results (data not shown). Therefore, subsequent transfections in this study consisted of all three mRNAs, which will be referred to as "BOO," for BLIMP, ONECUT1 and OTX2 (Figure 6C). Using this data, we created an H1 cell mRNA transfection protocol which is depicted in figure 6B.

Figure 6A

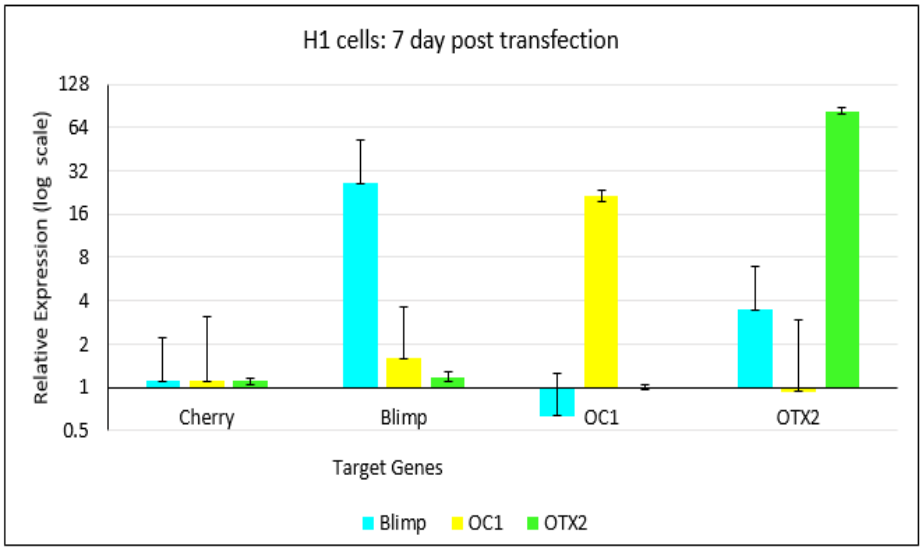


Figure 6B

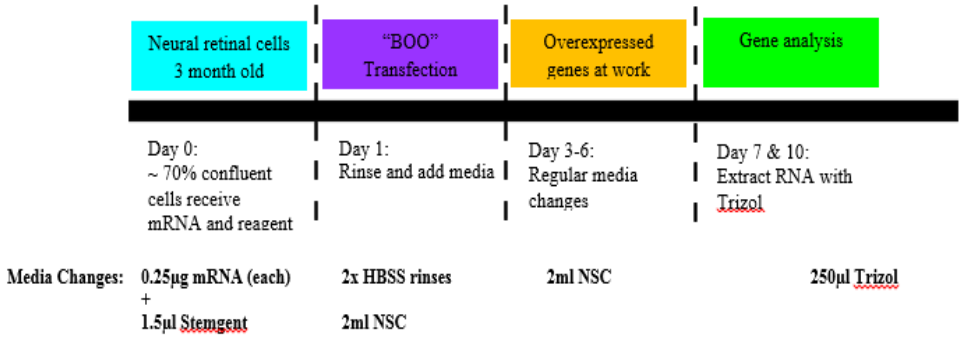


Figure 6C

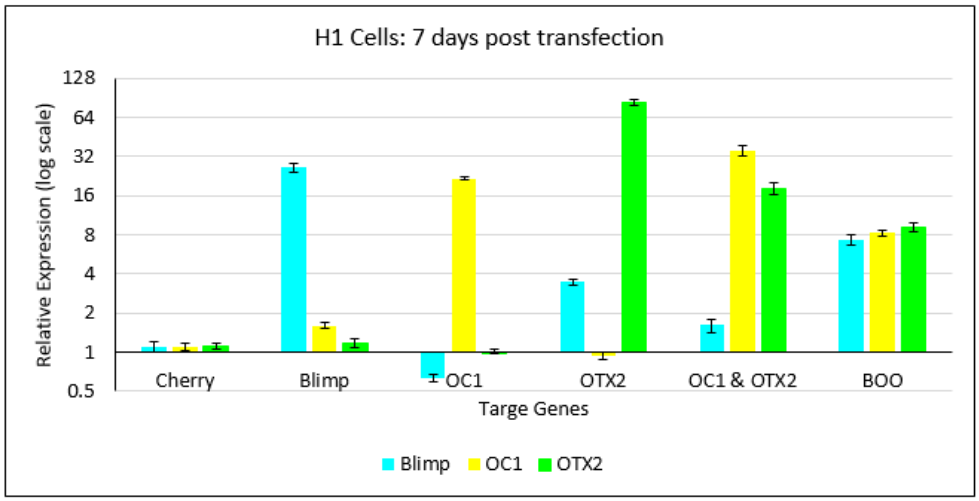


Figure 6:

(A) Quantitative RT-PCR analysis indicates that mRNA transfected cells overexpressed each target gene accordingly compared to the CHERRY transfection control that expressed none of the target genes. Quantitative RT-PCR illustrated duplicate transfection overexpressing all genes, more so OC1 and OTX2. (B) A schematic a "BOO" mRNA transfection protocol. (C)The triplicate transfection, "BOO," demonstrated a consistent overexpression for all three genes.

"BOO" increased expression of early photoreceptor factors:

Once we have established the successful transfection and expression of "BOO," our next step was to determine if the expression of "BOO" could bias cone photoreceptor development. To accomplish this, we again transfected H1 differentiated neural retinal cells at 3 months of age with BLIMP, OC1 and OTX2 using established protocols. Cells were collected seven days post transfection for RNA extraction and subsequently analysis using qRT-PCR. Gene expression analysis of "BOO" transfected cells showed that there was an increase in

expression of multiple photoreceptor factors in comparison to non-transfected control cells (Figure 7A). These photoreceptor factors included *cone-rod homeobox (CRX), neuronal differentiation factor (ND1),* and *recoverin* (*REC*). Moreover, the expression of specific cone photoreceptor factors *thyroid hormone receptor beta (TRB2*) and Retinoid X Receptor, Gamma (*RXRG*) were also upregulated (Figure 7A).

While qRT-PCR confirmed that the "BOO" triplicate transfected cells were expressing photoreceptor factors at the mRNA level, immunohistochemistry was carried out to assess expression level at the protein level for each of the corresponding genes. One week after transfection, higher levels of *otx2* and *recoverin* proteins, both photoreceptor markers, were seen in "BOO" transfected cells when compared to non-transfection control cells. More importantly, *RXRG* protein, an indicator of cone photoreceptor expression, was also noticed (Figure 7B). This translation further demonstrated that expression of "BOO" not only influenced mRNA expression levels of cone photoreceptor associated genes, but also resulted in a corresponding expression at the protein level. Furthermore, these results confirmed that "BOO" mRNA expression could be sustained over a period of about a week. This data suggested that one week of "BOO" overexpression may play an important role in cone photoreceptor development in H1 differentiated neural retinal stem cells.

Figure 7A

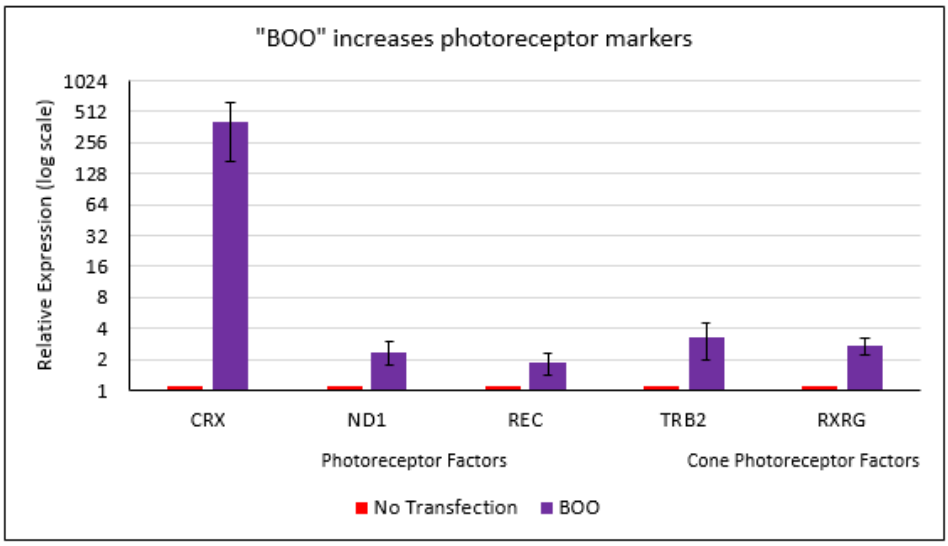
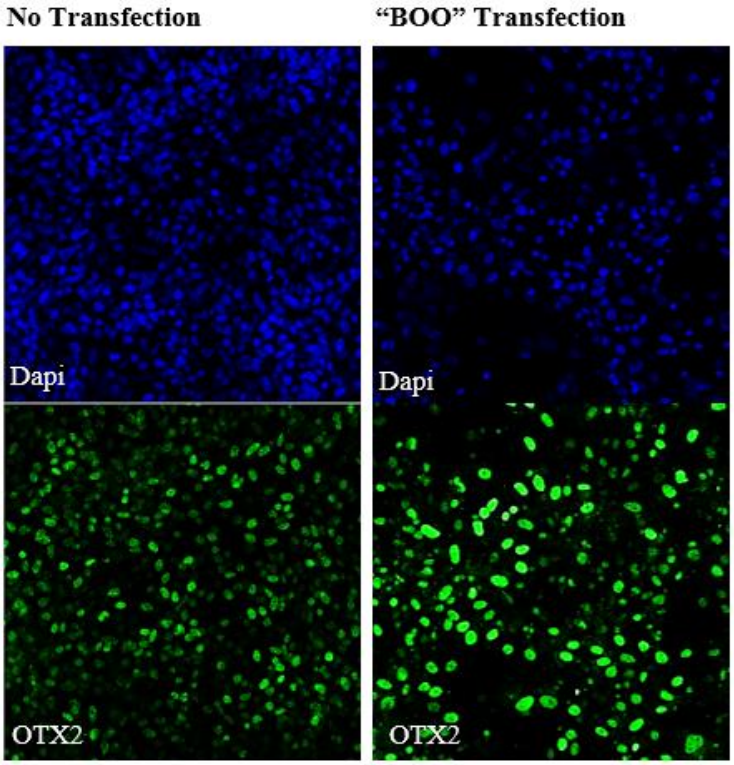


Figure 7B



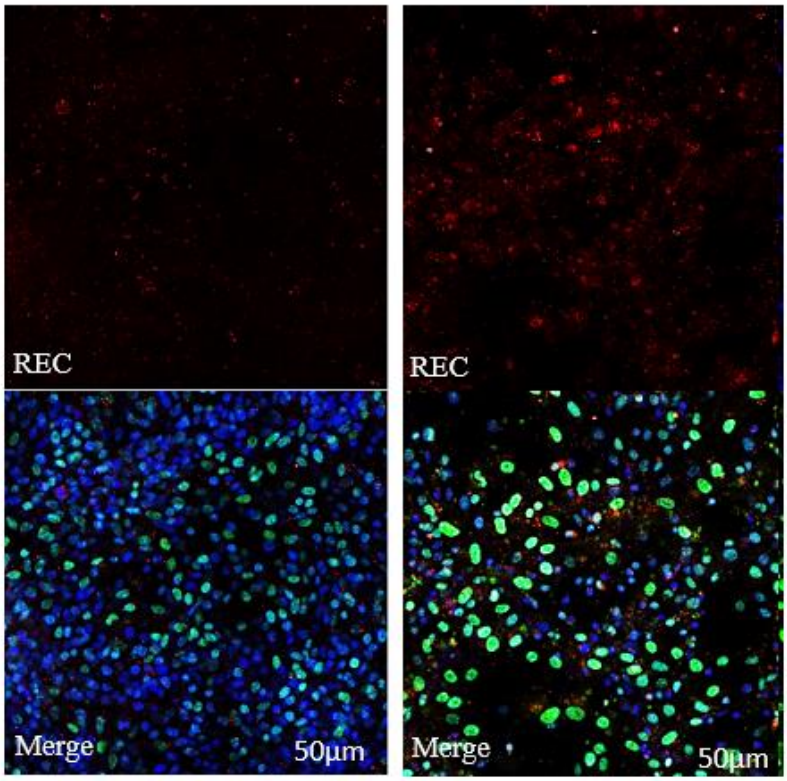


Figure 7:

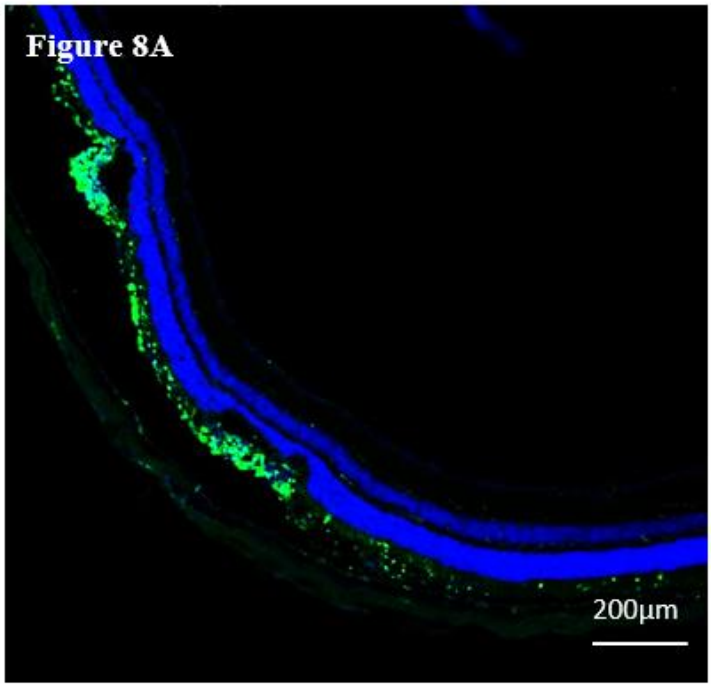
(A) "BOO" transfected cells demonstrated an increase in photoreceptor factors after 7 days. "BOO" demonstrated an increase in photoreceptor factors, specifically for cone markers. (B) Protein expression level increased for *OTX2* and *recoverin* photoreceptor factors via "BOO" transfected cells 7 days post transfection.

"BOO" transfected cells were able to integrate into a mouse host retina:

Previous results demonstrated that transient mRNA transfection could influence both gene and corresponding protein levels for about a period of one week post transfection in H1 differentiated neural retinal stem cells (Figure 7). Since the qRT-PCR data consistently validating "BOO" as the preferred

combination of mRNAs used for transfection, further analysis was done by injecting these "BOO"-transfected H1 cells into an immunodeficient IL2Rγ^{null} mouse cell line. Following the established transfection protocol, H1 differentiated neural retinal stem cells were grown to 3 months, infected with a viral GFP. The GFP infection would later help track the "BOO"-transfected cells for analysis. The GFP+ cells were transfected with "BOO" mRNA combination, and 3 days post transfection cells extracted for further animal studies.

Following extraction, the cells were injected into the sub-retinal space of the host mouse retina by Dr. Jie Zhu (Buck Institute for Research of Aging, Novato, CA). One month after injection, mice retinas were collected and GFP fluorescence was analyzed for retinal neural cell integration and localization. "BOO" transfected (GFP+) cells showed a good survival rate and were seen integrating from the sub retinal space into the outer nuclear layer (ONL) of the retina, an area otherwise known as the photoreceptor layer (Figure 8A and B). Some of the "BOO" transfected (GFP+) cell extensions were also seen integrated into the inner nuclear layer (INL). Photoreceptor extensions that leave the INL usually communicate directly or indirectly with other retinal neural cells, such as amacrine cells, when visual response occurs (4). Collectively, these results indicate that 3 month old neural retinal cells transfected with "BOO" increase photoreceptor markers, can survive host injections, and can localize in the photoreceptor area *in vivo.*



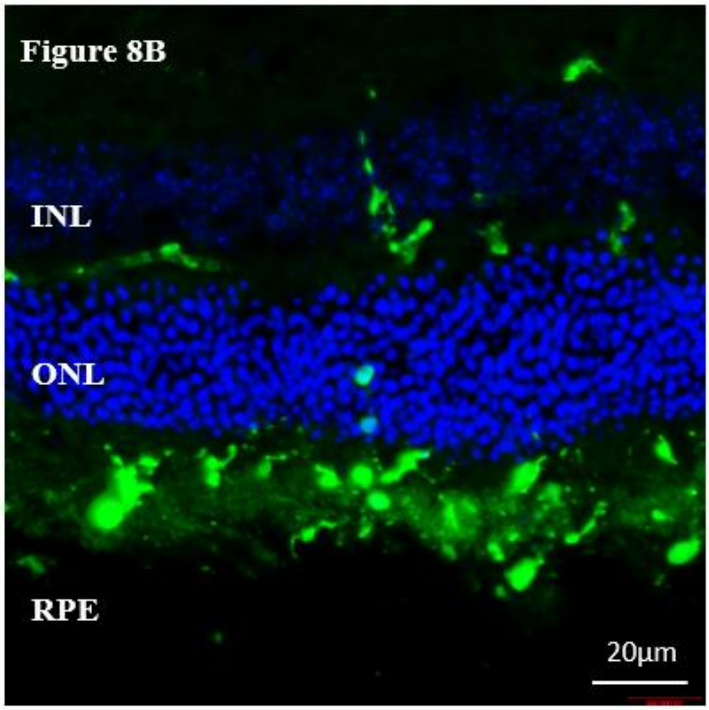


Figure 8:
(A) "BOO" transfected cells (GFP+) were seen surviving in the host retina one month following injection. (B) Cells were seen localizing in the ONL, otherwise known as a photoreceptor area.

Discussion

AMD is a retinal degenerative disease that causes an irreversible loss of cone photoreceptor cells. The mammalian retina does not have any cone cell regenerative properties on its own; therefore, transplantation of these photoreceptor cells for visual restoration is imperative. Stem cells have been used for various types of research and treatment, including retinal cell replacement therapy for AMD (23). Also, eye field studies have specifically recognized hES and iPS cells to have advanced research applications in terms of disease models and possible treatments for retinal degenerative diseases (24). However, there is minimal success reported for biasing cone photoreceptor fate during differentiation from hES or iPS cells.

The results above demonstrated that overexpression of three early photoreceptor genes could potentially bias cone photoreceptor fate *in vitro* using hES (figure 7). H1 cell line was differentiated into neural retinal stem cells and expanded over a period of three months (figure 3). This specific time point was used because of its relevance to a natural peak in cone photoreceptor development in humans (25) and was further analyzed through qRT-PCR (figure 3). Compared to the undifferentiated stem cells, the differentiated hES and iPS cell lines demonstrated an upregulation in eye field transcription factors and early retinal cell markers such as *crx*, a photoreceptor maker, and r*xrg*, a cone cell marker (figure 3F). This large fold change suggested that the cultured cells were at the appropriate early stage in development to bias only cone photoreceptor fate.

First, hES cells proved to be effective and efficient for using this mRNAs transfection protocol (Figure 5). DNA transfection has been used countless times

in past cell research; therefore, defining mRNA as an adequate transfection method here was important for future research. Furthermore, using this method in hES and iPS cells, especially from patients with retinal degenerative diseases, is valuable for future therapeutic approaches. Having a more personalized approach to medicine is the present focus for most treatments.

These studies unveiled consistently high mRNA expression levels for several genes of interest days after transfection. Three genes, BLIMP, ONECUT1 and OTX2, were overexpressed through mRNA transfection to bias cone photoreceptor cell fate (figure 6). Analysis via qRT-PCR showed at least a five fold increase for each single transfection of BLIMP, ONECUT1 or OTX2 mRNAs even after 7 days post transfection.

Second, a constant 3 fold change for BLIMP, ONECUT1 and OTX2 mRNA was seen expressed when all three mRNAs were transfected together, otherwise known as "BOO." With this constant fold change, it was easy to see the stability of these mRNAs. Although fold increases were higher in single transfections, all three mRNAs demonstrated a link between each other in terms that each gene seemed to drive one another (figure 6A) and "BOO" transfections had higher levels of expression in several photoreceptor genes when compared to a single transfection or a different combination transfection (figure 6B).

Specifically, the photoreceptor gene *CRX* always had at least a 300-400 fold increase with "BOO" transfection compared to non-transfected cells. This increase in expression for this particular gene could potentially mean that transfected cells were biasing neural retinal differentiation into photoreceptor cell

fate. Alongside *CRX*, specific cone photoreceptor factors such as *RXRG* and *TRB2* were upregulated when cells were transfected with "BOO," and this could further explain the bias of cell fate towards cone photoreceptors. To further support this conclusion, immunohistochemistry and *in vivo* analysis were done. Immunohistochemistry demonstrated that these transfected cells stained for photoreceptors factors such as *OTX2* and *recoverin* (figure 7B). In vivo analysis showed transfected cells predominantly residing in the outer nuclear layer (OPL), also known as the photoreceptor layer (figure 8B).

Many early onset retinal neural genes such as *CRX, neurod1, recoverin, RXRG and trb2* demonstrated regulation by a specific over expression of transient mRNAs. While all transient mRNAs expressed the above early genes of interest, the "BOO" transfection seemed to have more of an impact in regulation even after 7 days post transfection, leading us to believe that the combination may play a more important role in cone development. On the other hand, "BOO" post transfections that lasted longer than 7 days post transfection showed a decrease in mRNA and protein levels for the three genes (BLIMP, ONECUT1 and OTX2); therefore, there was no significant changes in photoreceptor marker expression (data not shown). This result could be due to mRNA degradation over a long period of time, in which a second round of transfection could be a possible solution. On the other hand, *in vivo* data demonstrates the cells surviving for at least one month after injection. This is promising data in which cell death is not the cause of mRNA degradation. Further data analysis needs to be done in order to see how to bypass mRNA levels lowering after 7 days post transfection.

This study explains one way to bias cone photoreceptor cell fate through a stabilized mRNA transfection method. This protocol is important because it could potentially be applied to other fields of research or different cell lines/tissues. Moreover, future research and treatment is geared towards more personalized therapeutics. Experiments that entail hES and iPS cells have grown popular for such studies. Lastly, using RNA treatments which will not harm the genome and provide a more stabilized treatment method could mean more promising therapy results.

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