Efficient In Vitro Development of Photoreceptors from Human Pluripotent Stem Cells

Joseph C. Reynolds
Dominican University of California

Follow this and additional works at: https://scholar.dominican.edu/masters-theses

Part of the Developmental Biology Commons, Developmental Neuroscience Commons, Eye Diseases Commons, Laboratory and Basic Science Research Commons, Optometry Commons, and the Structural Biology Commons

Survey: Let us know how this paper benefits you.

Recommended Citation
https://scholar.dominican.edu/masters-theses/169

This Master's Thesis is brought to you for free and open access by the Theses and Capstone Projects at Dominican Scholar. It has been accepted for inclusion in Master's Theses and Capstone Projects by an authorized administrator of Dominican Scholar. For more information, please contact michael.pujals@dominican.edu.
Efficient In Vitro Development of Photoreceptors from Human Pluripotent Stem Cells

A thesis submitted to the faculty of
Dominican University of California
& The Buck Institute of Aging
in partial fulfillment of the requirements
for the degree

Master of Science
in
Biology

By
Joseph Reynolds
San Rafael, California
May, 2015
CERTIFICATION OF APPROVAL

I certify that I have read Efficient In Vitro Development of Photoreceptors from Human Pluripotent Stem Cells by Joseph Reynolds, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and The Buck Institute for Research on Aging.

Dr. Deepak Lamba
Graduate Research Advisor

Dr. Meredith Protas,
Second Reader

Dr. Maggie Louie,
Graduate Program Coordinator
Abstract

Degeneration of the rod and cone photoreceptors in the human retina is among the most common causes of blindness. Replacing these damaged photoreceptors may help to restore vision.

Repairing the damaged retina relies on the insertion of new, healthy cells. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are two possible sources of photoreceptors to restore vision. Previous data shows that human ES cells and iPS cells can be differentiated into photoreceptors and transplanted into the eye to restore some vision. However, this process is inefficient, and costly. Here, we show a new method for inducing photoreceptor production from undifferentiated cells through the use of small molecules. Additionally, we aim to mimic retinal degenerations through oxidative stress to examine diseases such as diabetic retinopathy.
# Table of Contents

Copyright Notice..............................................................................................................ii
Certification of Approval.................................................................................................iii
Abstract............................................................................................................................iv
Table of Contents.............................................................................................................v
Abbreviations Page..........................................................................................................vii

## Background

- Retinal Structure and Diseases....................................................................................1
- Pluripotent Stem Cells and Disease Progression.......................................................2
- Formation of Optic Cups............................................................................................6
- Diabetic Retinopathy...................................................................................................6

## Specific Aims

- .....................................................................................................................................7

## Materials and Methods

- Creation of Induced Pluripotent Cells from Human Lymphocytes............................8
- Stem Cell Culture......................................................................................................9
- Stem Cell Differentiation into Retinal Stem Cells....................................................10
- Three Dimensional Neural Cultures.........................................................................11
- Retinal Pigment Epithelium.......................................................................................11
- RNA Extraction, Reverse Transcription and PCR Analysis.....................................12
- Immunocytochemistry..............................................................................................13
Addition of Methylglyoxal to Neural Cells………………………………………………15

Results

Formation of iPS cells from Lymphocyte Cultures……………………………………..15

Differentiation of retinal stem cells and photoreceptors from iPS cells………………16

Establishment of Floating Cultures………………………………………………………23

Diabetic Retinopathy……………………………………………………………………..25

Discussion………………………………………………………………………………….28

References…………………………………………………………………………………32
Abbreviations

Age-related Macular Degeneration (AMD)

Retinitis Pigmentosa (RP)

Methylglyoxal (MG)

Eye Field Transcription Factors (EFTFs)

Advanced glucation end products (AGEs)

Human Embryonic Stem (hES)

Induced Pluripotent Stem (iPS)

SB43152 (SB)

LDN193189 (LDN)

Diabetic Retinopathy (DR)

Dickkopf-1 (Dkk1)

Insulin Growth Factor 1 (IGF1)

Phosphohistone-H3 (PH3)
Background

Retina Structure and Diseases

The vertebrate retina is composed of multiple cell types, including rod and cone photoreceptors, the retinal pigmented epithelium, ganglion and amacrine cells. These cells organize into layers to form the neural retina (Figure 1). Degenerations of the rod and cone photoreceptors in the human retina are a major cause of blindness and affect millions of people in the United States. Age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are two of the leading causes of blindness in adults. Both diseases involve the loss of the light sensitive rod and cone cells that line the retina, causing decreased sensitivity to light, and the progressive loss of vision. One of the main issues regarding blindness is that most people do not notice any vision impairment until roughly 90% of their photoreceptors have degenerated. There are currently no treatments to halt or delay the onset and progression of these diseases. However, the replacement of photoreceptors through transplantation into the retina has been shown to restore light sensitivity in mice (Lamba et al., 2009), and represents a possible form of treatment.
Figure 1: Anatomy of the eye displays the relative relationship between the photoreceptors and the Retinal Pigment Epithelium (RPE). Light passes through the Ganglion Cell Layer (GCL), Inner Nuclear Layer (INL), and Outer Nuclear Layer (ONL) before detected by the rod and cone photoreceptors. (Figure courtesy of Dr. Deepak Lamba)

Pluripotent Stem Cells and Disease Progression

Human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells offer a therapeutic approach to treating diseases of the retina. Human embryonic stem cells are undifferentiated cells that are collected from the inner cell mass of human embryos. These cells can be differentiated into any of the three germ layers, and are capable of virtually limitless self-renewal. However, given the nature of the method in which they are collected, there are ethical concerns surrounding the use of hES cells. In order to circumvent this issue, human induced iPS
cells were generated. These cells are derived from adult somatic cells, commonly lymphocytes or fibroblasts, and avoid any need for embryonic cells. Once the somatic cells are collected, they are transformed into undifferentiated cells, which are then capable of being differentiated similar to hES cells.

The generation of iPS cells allows for different cell lines to be redirected into undifferentiated cells, and then differentiated into various cell types (Takahashi and Yamanaka, 2006). Several groups have shown that these iPS cells can be used to study specific diseases (Dimos et al., 2008). By taking a lymphocyte from a patient with a disease of interest, and a person without the disease, undifferentiated iPS cells can be grown from each, and differentiated into the cell type impacted by the disease. This can be useful to study disease progression, and to compare the gene expression levels photoreceptors derived from the two individuals over time, to gain an understanding of the underlying cause of the disease. This technique can be useful to study the development and maturation of photoreceptors derived from patients with either AMD or RP.

One possible treatment for retinal degenerative disorders is cell replacement therapy. Differentiation of either hES cells and iPS cells into retinal progenitors allows these cells to be expanded and matured to become photoreceptors, (Lamba et al., 2009). Previous studies have shown that mice and human photoreceptors can integrate into the retina of adult mice and even restore function (Lamba et al., 2009; MacLaren et al., 2006). However, the rate of successful integration of these cells is relatively low. Additionally, the transplanted photoreceptors are susceptible to the same degenerations as the native cells. This issue places great importance of higher rates of integration, as well as creating mature and functional photoreceptors en masse for
sequential transplantations. Stem cells provide a self-renewing source of cells that can be differentiated on a large enough scale to provide this method of cell replacement.

Creating photoreceptors from pluripotent stem cells requires a specific protocol in order to properly induce retinal differentiation. Proper forebrain development of the nervous system relies on two critical signaling pathways: the BMP and Wnt pathways (Niehrs, 2001). More specifically, induction of retinal cells through recombinant proteins can be achieved by creating low levels of BMP and Wnt signaling (del Barco Barrantes et al., 2003). Dickkopf-1 (Dkk1) and Noggin have been shown to inhibit Wnt and BMP signaling, respectively. Although the precise mechanism of these genes is currently unknown, inhibition of Dkk1 and Noggin in mice embryos leads to a lack of eyes and other abnormalities in head development. In addition, Insulin-like growth factor 1 (IGF1) has been shown to promote eye induction (Pera et al., 2001). Wnt signaling is associated with development of the spinal cord, midbrain and hindbrain (Castelo-Branco et al., 2003; McMahon and Bradley, 1990). In contrast, inhibition of Wnt activity during this period of embryonic development is associated with forebrain induction (Mukhopadhyay et al., 2001; Sanges et al., 2013). Several studies have shown that the inhibition of the BMP pathway in the embryo pushes developing cells towards a neural fate (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Smith et al., 1993).

Inhibition of these pathways requires recombinant proteins. However, recombinant proteins are relatively expensive and slow acting in vitro. The use of small molecule inhibitors of these pathways at precise stages helps to push for specific differentiation (Nelson et al., 2007) and would allow more precise control of the cell types formed during differentiation. The progression of retinal and photoreceptor differentiation can be tracked looking at expression levels of several different genes including eye field transcription factors (EFTFs) such as \textit{Pax6}, 
Six2, Lhx2 and Rx as well as photoreceptor specific genes such as Crx, Blimp1, and Recoverin. These genes are upregulated during the early stages of differentiation and can be used to investigate whether the stem cells are taking on the desired cell fate (Lamba et al., 2006).

One possible treatment for diseases such as AMD and retinitis pigmentosa relies on transplantation of new photoreceptors into the retina. Efficient production of healthy and mature photoreceptors is crucial for the advancement of regenerative therapies for the treatment of AMD and retinitis pigmentosa. Creating these photoreceptors from hES cells and iPS cells through current protocols is time-consuming and labor-intensive. In addition, the recombinant proteins used are expensive and degrade quickly. Replacing these recombinant proteins with small molecule analogs may increase the overall percentage of pluripotent cells that differentiate into photoreceptors in a faster, more cost effective manner (Osakada et al., 2009). The current methods for transplanting photoreceptors into mouse eyes result in a low success rate of integrated cells. The two approaches to resolve this issue are to improve the environment in which the cells are being injected, or to improve the state of the photoreceptors themselves. Photoreceptor maturation is an important factor in their overall integration efficiency. At the time of transplantation, the host cells are fully mature cells. Current protocols result in the creation of various cell types found in the retina, as well as progenitor cells which are not present in the fully mature retina. Creating mature rods and cones that resemble the stage of the host retina may help increase photoreceptor integration. This research focuses on the differentiation process and photoreceptor maturation to increase the integration of stem cell derived photoreceptors into the retina during transplant. This can help to increase the light response in blind mice, as measured brain waves responding to light and can have several important therapeutic implications for the treatment of retinal diseases.
Formation of Optic Cups

In order to quickly influence the maturation of the neural stem cells into photoreceptors, these neural progenitor cells can be cultured in floating low-attachment conditions. In a three dimensional structure, the neural retina can self-organize into optic cup structures (Nakano et al., 2012). These hES derived cells organized into a layered structure, with the light sensitive rod and cone cells lining the outer ring of the spherical cell clusters. Neural spheres, such as these, mimic the natural organization and structure of the developing eye *in vivo*. A key feature of these floating optic cups is the short time they take to form. Within days, the neural progenitor cells begin to self-organize. Therefore, a three-dimensional cell culture can enhance differentiation and maturation of these cells, allowing a more efficient method of preparing these cells for transplantation.

Diabetic Retinopathy

Damage to the retina due to complications of diabetes is a rising concern. Diabetic Retinopathy (DR) is the leading cause of blindness in people ages 20-64, and accounts for roughly 12% of new cases of blindness per year (Engelgau et al., 2004). Furthermore, 25% of diabetic patients reported visual impairments, which is twice the proportion compared to individuals without diabetes (Saaddine et al., 1999). DR is characterized by an increase of the permeability in the capillaries and other small vessels in the eye (Singh et al., 2009). While AMD is the leading cause of blindness among the elderly, DR is predicted to be the leading cause of blindness among the younger, population (Aiello, 2003; Moss et al., 1998).
Patients of both type 1 and type 2 diabetes are susceptible to DR. Considering cases with disease duration over twenty years, 95% of patients with type 1 diabetes have DR, while 60% of type 2 diabetic patients have DR (Sayin et al., 2015). Hyperglycemia plays an important role in DR by causing vascular endothelial dysfunction (Matthews et al., 2004), although the precise mechanism of action is largely unknown. One proposed factor for the negative effects of DR is chronic oxidative stress (Baynes, 1991; Cui et al., 2006; Kowluru et al., 2001; Zong et al., 2011). Methylglyoxal (MG) has been tested as a source of oxidative stress (Shipanova et al., 1997) and leads to the accumulation of advanced glycation end products (AGEs), which are implicated with retinal complications (Kyselova et al., 2004). Accumulation of AGEs leads to apoptosis in retinal cells (Denis et al., 2002). MG has been shown to cause accumulation of AGEs and increases apoptosis of many cell types found in the eye both in vitro and in vivo (Kim et al., 2012).

**Specific Aims**

**Aim 1:** To generate mature photoreceptor cells from human-derived pluripotent stem cell lines

Current protocols use the recombinant proteins DKK1, Noggin and IGF1 in order to differentiate pluripotent cells into retinal cells. Under these protocols, recombinant proteins are added to the growth medium for two weeks in order to promote retinal stem cell markers such as CHX10, LHX2 and PAX6. It can take several months for this subset of cells to express mature photoreceptor markers such as Blue Cone opsin. In contrast, small molecules offer the advantage of being relatively cheap, more stable, and their ability to easily cross the cell membrane make them fast-acting. Replacing recombinant proteins with specific small molecules can enhance the differentiation of stem cells into photoreceptors. Creating, expanding and maturing these stem cell-derived photoreceptors can greatly improve the number of integrated cells after
transplantation into Crx-/- mice and theoretically lead to an increased response to light. Here, we assess the ability of small molecule analogs such as IWR1, SB43152 (SB), and LDN193189 (LDN), to increase the rate of differentiation of retinal stem cells and in turn affect maturation of the rod and cone photoreceptors (Table 1). Photoreceptors derived from undifferentiated cells from a healthy patient as well as a family member with RP are used in order to determine any differences in the maturation process. Theoretically, genetic differences between these family members may be linked to the disease itself. Since these are degenerative diseases, having photoreceptors derived from these individuals can allow us to compare the formation and maturation of these photoreceptors over a long period of time.

**Aim 2:** Model retinal degenerations such as those associated with Diabetic Retinopathy using photoreceptors derived from human induced pluripotent stem cells

Access to human cells through stem cell technologies allows us unprecedented ability to directly study human development and degenerations. Here, we study the effects of diabetes on the human retina. Using Methylglyoxal (MG), a chief metabolite that accumulates in tissues in diabetes, we explore the disease development, in vitro, in human stem-cell derived retinal cultures.

**Materials and Methods**

Creation of Induced Pluripotent Cells from Human Lymphocytes
Lymphocytes from both healthy and diseased patients were obtained (Coriell Biorepository, Camden, NJ) and an iPS cell line was made as previously described (Okita et al., 2011). iPS cells were made from a patient with RP, as well as an unaffected cousin to serve as the control. Briefly, fibroblast cultures were electroporated with episomal plasmid vectors. Following 48 hours of electroporation, the media was changed to hES media, consisting of DME/F-12 1:1 (HyClone Logan, UT) with 20% Knockout Serum Replacement (KSR; Gibco, Grand Island, NY), 1% Penicillin Streptomycin Amphotericin B (Lonza, Basel, Switzerland), 1% MEM Nonessential Amino Acids (Corning, Corning, NY) 100 uM Beta-mercaptoethanol (Sigma Aldrich, St. Louis, MO) and 5 ng/mL basic FGF (Stemgent, Cambridge, MA). Once the cells began to take on an undifferentiated morphology, they were manually picked and grown in Essential 8 media (Gibco).

**Stem Cell Culture**

Human Induced Pluripotent stem cells as well as human embryonic stem cells were maintained in Essential 8 basal medium (Gibco) supplemented with 1% Essential 8 supplement (Gibco) and 1% Penicillin Streptomycin Amphotericin B (Lonza). Cells were grown on culture vessels coated with Matrigel (BD Biosciences, San Jose, CA). The undifferentiated cells were grown in a 37°C incubator with 5% CO₂ and 5% O₂. Daily media changes were made for the growth of these cells. The stem cells required passaging when they reached approximately 70% confluence, generally after 4-5 days of growth. Cells were passaged by an initial wash with 1x PBS (Corning), followed by the addition of a 1 mM solution of sodium citrate (Sigma Aldrich). Sodium citrate was aspirated off after 5 minutes and cells were removed by manually scraping the cells with a 200 uL disposable pipette tip (BioExpress, Kaysville, UT) in the presence of Essential 8 medium. The cell suspension from a single well was collected and directly passaged onto all 6 wells of a 6 well plate for a 1:6 passaging ratio.
Stem Cell Differentiation into Retinal Stem Cells

The iPS cells were differentiated into Neural Stem Cells (NSC) by adding differentiation media consisting of DME/F-12 1:1 (HyClone) with 10% Knockout Serum Replacement (KSR; Gibco), 1% Penicillin Streptomycin Amphotericin B (Lonza), 1% Sodium pyruvate (Corning), 1% Sodium Bicarbonate (Corning), 1% HEPES Buffer (Corning), and 1% MEM Nonessential Amino Acids (Corning) (Anchan et al., 1991). Following filtration in a 500 mL vacuum-driven filter system (Argos), 5 mL of N1 Media Supplement (Sigma Aldrich) was added (Price et al., 2002). Finally, we added the small molecules IWR1 (Sigma Aldrich), SB431542 (Stemgent), LDN193189 (Stemgent) and the recombinant protein IGF1 (R&D Systems). Cells were allowed to grow in this medium with daily changes for 5 days. On the fifth day, cells were passaged by washing the cells once with PBS (Corning), adding 1 mL of Accutase (Global Cell Solutions, Charlottesville, VA) and letting the Accutase sit for 5 minutes. Then, the Accutase was aspirated, and the cells were washed twice with PBS. Next, 2 mL of PBS was added and cells were manually scraped off the plate using a 200 uL disposable pipette tip (BioExpress). The cell suspension was centrifuged for three minutes at 1500 rpm at room temperature. The supernatant was aspirated off and the cell pellet was resuspended in NSC media, and re-plated onto 2 wells of a 6 well plate, for a passaging ratio of 1:2. NSC media is comprised of DME/F-12 1:1 (HyClone), 0.5% Fetal Bovine Serum (FBS, Atlanta Biologicals), 1% Penicillin Streptomycin Amphotericin B (Lonza), 1% Sodium pyruvate (Corning), 1% Sodium Bicarbonate (Corning), 1% HEPES Buffer (Corning), and 1% MEM Nonessential Amino Acids (Corning). Following
filtration in a 500 mL vacuum-driven filter system (Argos), 5 mL of N1 Media Supplement (Sigma Aldrich) was added. Cells were cultured in this media indefinitely.

**Table 1: Recombinant Protein Replacement Strategy**

<table>
<thead>
<tr>
<th>Recombinant Protein</th>
<th>Pathway Involved</th>
<th>Role</th>
<th>Related Small Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dkk1</td>
<td>Wnt Pathway</td>
<td>Anterior head formation and eye induction</td>
<td>IWR1</td>
</tr>
<tr>
<td>Noggin</td>
<td>BMP Pathway</td>
<td>Neural Induction, TGF-β Inhibition</td>
<td>LDN193189, SB431542</td>
</tr>
<tr>
<td>IGF1</td>
<td>many</td>
<td>Proliferation, differentiation and survival of retinal neurons</td>
<td>--</td>
</tr>
</tbody>
</table>

**Three Dimensional Neural Cultures**

In order to grow the neural progenitor cells as a floating, three dimensional culture, clusters were removed from the Matrigel coated plate by manually removing the areas of interest with a 200 uL pipette tip (BioExpress). Selected clusters were moved into an Ultra-Low Attachment Plate (Corning), and were allowed to grow floating in 2 mL of NSC media. Media was added twice a week until a volume of 5 mL was reached in the floating well, at which point the media containing the cells was collected and the cell cultures were allowed to aggregate at the bottom of a 15 mL centrifuge tube. Spent media was aspirated, and cells were replenished with 2 mL fresh media and returned to the low attachment plate.

**Retinal Pigment Epithelium**
Retinal Pigment Epithelium (RPE) cells were cultured similarly to the neural cells. The differentiation steps and initial passaging were carried out identically. After passaging, any cells displaying the morphology typical of RPE cells, including hexagonal shape and any pigmentation, are manually separated from the neural cells (Figure 3). Using a 200 uL pipette tip, any region showing RPE morphology was removed, and centrifuged as described above. The supernatant is aspirated, and the cells are resuspended in RPE media. A 6 well plate of differentiated cells can be combined to fill one well of a six well plate of newly picked RPE cells. A 500 mL bottle of RPE media consists of DME/F-12 1:1 (HyClone), 5% Fetal Bovine Serum (FBS; Atlanta Biologicals), 1% Penicillin Streptomycin Amphotericin B (Lonza), 1% Sodium pyruvate (Corning), 1% GLUTAMAX (Gibco), 125 mg Taurine (Sigma Aldrich), 10 ug Hydrocortisone (Sigma Aldrich) and .0065 ug Triiodo-thyronin (Sigma Aldrich). Once the cells became confluent, the FBS was reduced to 1%. After filtration in a 50 mL vacuum-driven filter system (Argos, Logan, UT), 5 mL of N1 Media Supplement (Sigma Aldrich) was added.

RNA Extraction, Reverse Transcription and PCR Analysis

RNA was extracted from cells using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). All steps were done according to the manufacturer’s recommended instructions. RNA samples were quantified and tested for purity using a NanoDrop 2000 machine (Thermo Scientific). cDNA was synthesized from 500 ng of total RNA template using the iScript cDNA synthesis kit (Bio-Rad). Reactions occurred in a T100 Thermal Cycler (Bio-Rad, Hercules, CA) according to the manufacturer’s recommended instructions. Quantitative Real Time PCR analysis was performed using CFX Connect Real-Time PCR Detection System (Bio-Rad) and
CFX Manager Software (Bio-Rad). Each sample was done in duplicate. Forward and Reverse primers (Table 2) were used at a final concentration of 400 nM with 1 uL cDNA sample, 7.4 uL of DEPC water (GeneMate) and 10 uL of iTaq Universal SYBR Green Supermix (Bio-Rad). Samples were normalized using the Beta-Actin gene.

**Table 2: RT-PCR Primers**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>GGA TCA GCA AGC AGG AGT AT</td>
<td>GGT GTA ACG CAA CTA AGT CAT AG</td>
</tr>
<tr>
<td>Blimp</td>
<td>CCA CAA GAA CTA CAT CCA T</td>
<td>CAT TGA TTC GGG TCA GAT</td>
</tr>
<tr>
<td>Brn3b</td>
<td>CGC TCT CAC TTA CCC TTA CAC ACA</td>
<td>CGG TCA TGC TTC CAA CTG CTT CTT</td>
</tr>
<tr>
<td>Chx10</td>
<td>CGA CAC AGG ACA ATC TTT ACC</td>
<td>CAT AGA CGT CTG GGT AGT GG</td>
</tr>
<tr>
<td>Crx</td>
<td>GTA CCC AGA CGT CTA TGC C</td>
<td>TTC TTG AAC CAA ACC TGA ACC</td>
</tr>
<tr>
<td>Lhx2</td>
<td>TAT CAC CTC AAC TGC TTC ACG</td>
<td>TAG ACC AGG CTG TCC TTC AT</td>
</tr>
<tr>
<td>Pax6</td>
<td>AGT GAA TCA GCT CGG TGG TGT CTT</td>
<td>TGC AGA ATT CGG GAA ATG TCG CAC</td>
</tr>
<tr>
<td>Rx</td>
<td>GTA CCC AGA CGT CTA TGC C</td>
<td>TTC TTG AAC CAA ACC TGA ACC</td>
</tr>
<tr>
<td>Ascl1</td>
<td>AGC ACT CTC TCA CTT CTG</td>
<td>CCT TCC TTT CTG ATT TCC TT</td>
</tr>
<tr>
<td>Recoverin</td>
<td>CCA CTC TTC CTC ACT CAT</td>
<td>CTC CGA GAA CTT GGT GTT</td>
</tr>
</tbody>
</table>

**Immunocytochemistry**
Cells were fixed with 2% Paraformaldehyde (Alfa Aesar, Sparks, NV) for 20 minutes at room temperature and in the dark. Paraformaldehyde was aspirated off and cells were washed three times at five minutes each with PBS (Corning) with 0.5% TWEEN (Acros Organics). Following the washes, samples were blocked with PBS with 0.5% TWEEN and 10% Normal Donkey Serum (EMD Millipore Darmstadt, Germany). Cells were blocked for one hour prior to the addition of primary antibody. The primary antibodies were diluted in the block solution. Cells were incubated in the primary antibody overnight at 4°C in the dark. The primary antibody (Table 3) was aspirated off and cells underwent three washes as described above. Secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) was added at a 1:500 dilution in the block solution. Secondary antibodies covered the cells for one hour at 4°C in the dark. The secondary solution was aspirated and cells were washed three times as described above. Finally, DAPI (Enzo Life Sciences Farmingdale, NY) was added to the cells for 30 seconds before removal and additional washes. Samples were mounted on glass slides (VWR, Radnor, PA) using Fluoromount-G (Electron Microscopy Science, Hartfield PA).

**Table 3: List of Primary Antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-Pax6</td>
<td>DHSB</td>
<td>1:250</td>
</tr>
<tr>
<td>Goat anti-Otx2</td>
<td>BD Biosystems</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-NRL</td>
<td>Gift from Dr. A. Swaroop</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-ZO1</td>
<td>DHSB</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse anti-MITF</td>
<td>NeoMarkers</td>
<td>1:50</td>
</tr>
<tr>
<td>Goat anti-Sox2</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
</tbody>
</table>
Rabbit anti-AIPL1 | Gift from Ramamurthy lab, WVU | 1:1000

Rabbit anti-Recoverin | EMD Millipore | 1:1000

Mouse anti-RET-P1 | Santa Cruz Biotechnology | 1:200

Phosphohistone-3 | Santa Cruz Biotechnology | 1:200

Addition of Methylglyoxal to Neural Cells

Upon maturation of neural retinal cells, around four months, the cells were treated with one of two conditions. The first condition is a control group, in which media is changed with the traditional NSC media daily. In the second group, the cells were treated with 1.2 mM Methylglyoxal (MG) added to the NSC media. This dose was identified following dose-response cytotoxicity analysis (data not included). Cells were allowed to grow for 24 hours after MG addition. After 24 hours, cells were collected for PCR analysis and immunocytochemistry.

Results

Formation of iPS cells from Lymphocyte Cultures

Following electroporation, a small subset of lymphocytes began to show morphology similar to that of an undifferentiated human stem cell approximately 2 weeks following start of reprogramming (Figure 2, A). Each undifferentiated looking colony was manually isolated and grown as an independent clone. The best clones were selected and directly compared to H1 undifferentiated (Figure 2B) human stem cells. There was no difference in cell morphology
between the H1 stem cell colonies and the undifferentiated colonies of the iPS cells resulting from lymphocyte reprogramming (Figure 2C).

![Image of H1 Colony, Undiff from Lymphoblast, iPS Colony]

*Figure 2: Progression of Lymphocyte culture (A) three weeks after reprogramming is shown. Cell morphology is comparable to both H1 undifferentiated embryonic stem cell line (B) and a previously established induced pluripotent cell line (C).*

**Differentiation of retinal stem cells and Photoreceptors from iPS Cells**

Following five days of differentiation with IWR1, SB, LDN and IGF1 (ISLI), cells were allowed to grow with daily media changes. RT-PCR was used to analyze the differentiation progression two weeks after the beginning of differentiation. We see an increase in genes associated with neural stem cells, as well as genes associated with cell types found in the retina. There is an increase in gene expression for genes associated with neural stem cells, such as Pax6, Lhx2 and Rx. Additionally, there is an upregulation of genes coding for differentiated retinal cells including photoreceptor genes such as *Crx, Blimp1* and *recoverin* and ganglion cells such as *Brn3.*
Figure 3: Following Differentiation, neural cells and RPE cells are manually isolated and allowed to grow in homogenous cultures.
Figure 4: Neural cells derived from iPS cells from control (A) and disease (B) patients qRT-PCR results. Results normalized to Beta-Actin. All samples compared to undifferentiated cells. All
genes expressed as undifferentiated stem cells differentiate into neural progenitor cells. Results indicate $2^n$ fold expression.

Several different cell types normally found in the human eye are present at 2 weeks post differentiation (Figure 5). We find the RPE cells, as identified by pigmentation and their cobblestone-like morphology. Also present are the dark neural clusters, which are eventually manually picked and grown in floating cultures. Finally, there is evidence of translucent lens cells in the plate. Each of these cell types underwent purification via manual separation in order to create a homogenous plate, which was used for further analysis.

Figure 5: Heterogeneous differentiation of iPS cells into various cell types found in the eye. These heterogeneous cells are manually isolated and allowed to grow further in homogenous cultures. RPE= Retinal Pigment Epithelium, N=Neural, L=Lens
Once a homogenous culture was established, immunohistochemistry was performed to determine the differentiated state of the cells. The cells were stained for Pax6 and Otx2 (Figure 7) at both one and two weeks post differentiation. Cells that are positive for Pax6 are the neural stem cells, while cells that are Pax6 negative and Otx2 positive are developing photoreceptor cells. Co-staining of both Pax6 and Otx2 indicates that the cell is either immature and yet to undergo photoreceptor differentiation, or may still take up an RPE fate. The pattern of circular staining in the 1 week Pax6 cells is typical of neural rosettes, in which the differentiating cells move to the center of the rosette to complete differentiation.

In order to compare the differentiation results using the new ISLI protocol with the established method of using recombinant proteins, we analyzed the cells under identical differentiation conditions. One subset of iPS cells were differentiated using ISLI, while another was differentiated using DKK1, IGF1, and Noggin (DIN). RNA was collected after two weeks of differentiation and genes associated with retinal differentiation were analyzed by RT-PCR (Figure 6).
Figure 6: qRT-PCR results comparing cells differentiated with either recombinant proteins or small molecules. Results indicate a greater expression of relevant neural markers when small molecules were used for differentiation as opposed to recombinant proteins.

We see a major increase in the expression of the EFTFs, as well as a marked decrease in OCT4, which is a marker of pluripotency (Figure 6). Notable, LHX2 goes from a 14 fold increase to a 50 fold increase, compared to the undifferentiated cells. PAX6 goes from a 229 fold increase to a 1077 fold increase. Additionally, RX goes increases from a 51 fold increase to a 519 fold increase when using ISLI compared to DIN. These drastic changes indicate the small molecules are more efficient at differentiating the stem cells into retinal progenitor cells.
Figure 7: Cells at both one and two weeks after start of differentiation using small molecules. Expression of Pax6, a neural stem cell marker, and Otx2, a marker of RPE and early photoreceptors increase as the cells continue to differentiate. Dapi (blue), Pax6 (green) and Otx2 (red).

RPE upon purification can be cultured at high density to allow further maturation. These cells then express various immature and mature markers of RPE including MITF, Otx2, ZO-1, CRALBP, PMEL17, BEST1 and RPE65 (Figure. 8). Upon Tissue electron microscopy analysis (TEM), the cells have typical morphology with apical microvilli, pigmented granules and abundant mitochondria. (Figure 8 D,E)
Figure 8: RPE cells display typical morphology, and ZO-1 (B,C,F) and qRT-PCR showed the expression of additional markers including TYR, BEST1, CRALBP, RPE65 and PMEL17 (G). The cells were also analyzed by TEM and the maturation features including apical microvilli, basal nuclei and apical mitochondria and melanosomes (D,E). (Figure courtesy of Dr. Deepak Lamba)

Establishment of Floating Cultures

The neural regions of the differentiated cells were manually separated from the heterogeneous culture. Neural cell colonies took up a near spherical shape within hours after being grown in the ultra-low attachment plates. Circular growths appeared on these clusters, indicating the development of self-organized optic cups (Figure 9A). At 4 weeks after suspension culture, these clusters were sectioned and analyzed via immunocytochemistry. Cells along the outer rim of the cluster were positive for photoreceptor markers Aipl1, Recoverin, NRL and Ret-P1 (Figure 9B). Additionally, Sox2, a neural stem cell marker, was only evident in the inner sections of the cluster, indicating a self-organized, layered structure.
Figure 9: Floating neural colonies (A) with growth on upper right. Layered organization of cells indicate self-organization of photoreceptors. Immunocytochemistry confirms cells on outer rim.
of floating cells are positive for photoreceptor genes. AIPL1 (D), Recoverin (E), Ret-P1 (G) are late photoreceptor markers. Nrl (F), Otx2 (B) are photoreceptor precursor markers. SOX2 is a marker of neural stem cells.

**Diabetic Retinopathy**

The inherent inability to accurately model age related diseases such as AMD and RP causes many problems when it comes to analyzing the evolution of the diseases. Any human photoreceptors developed from stem cells *in vitro* would take a similar amount of time to degenerate as they would *in vivo*. This would require decades of cell maintenance in order to observe the effects of the diseases, which is simply impractical. Therefore, it is important to develop methods to mimic the cellular environment that causes degenerations. Oxidative stress is believed to have a component in the retinal diseases, so we investigated another retinal degenerative disease, RP, in which oxidative stress has more of a well-defined role.

As stated above, one of the major metabolites to accumulate in tissues during diabetes is MG. Here, we wanted to look at the role of MG on retinal stem cells and retinal neuronal morphology as well as its potential effects on driving the intracellular oxidative stress. Several changes resulted in the addition of MG to neural cells growing *in vitro* on Matrigel plates. MG addition results in the cells exhibiting characteristics of stress including complete loss of proliferation as evidenced by a lack of Phosphohistone-H3 (PH3), a key marker of cell division (Figure 10A). Additionally, we observed alterations in the TUJ expression pattern, such as shortened or fragmented processes. In the control cells, the TUJ is present along the full length of the healthy axons. However, in the cells treated with MG, the axons appear fragmented, and their numbers are decreased. Taken together, these changes indicate that the neural cells are reducing proliferation and retracting axons, as a result of the stress caused by MG. Additionally, there is a
marked increase in TUNEL staining with the addition of MG (Figure 10B). TUNEL staining works by fluorescently labeling the ends of fragmented DNA segments, and is a marker of cell apoptosis. The control group has very low levels of TUNEL positive cells, as expected with a developing neural cell colony. Conversely, the addition of MG causes a drastic increase in the number of TUNEL positive cells, indicating an increase in apoptosis. Finally we assess if any of these changes are due to oxidative stress by looking at both NRF2 expression as well as its downstream effects, NQO1, HMOX1 and GSH components. We observed that HMOX1 was significantly upregulated within 24 hours of MG stress (Figure 10C). Each gene tested through qRT-PCR is a known effector involved in detoxification. By seeing an increase in the expression of HMOX1, there is a strong indication that this pathway specifically is being upregulated. The different effectors respond to different stressors, and our data indicates that in the presence of oxidative stress induced by MG, the iron based enzyme HMOX1 was upregulated in order to detoxify the environment.
Figure 10: (A) Control neural cells and cells treated with methylglyoxal are visualized with DAPI staining (blue). Treatment of methylglyoxal results in the loss of p-histone H3 positive cells (green). Neural axons become retracted, and there is evidence of axon fragmentation following methylglyoxal treatment, as visualized by TUJ staining (red). (B): Treatment of neurons with methylglyoxal results in a major increase in TUNEL staining (red). Cells are visualized with DAPI (blue). (C): qRT-PCR data showing relative gene expression as compared to control.

**Discussion**

Differentiation and purification of induced pluripotent stem cells into cell types present in the neural retina is an important step towards developing mature photoreceptors for transplantation. This novel method of differentiation effectively allows development of mature photoreceptors. RT-PCR analysis indicates these cells have gene expression changes similar to the photoreceptors generated using recombinant proteins, but at a faster pace. We see, in 5 days, a similar level of differentiation with the small molecules that is found after 2 weeks of using...
recombinant proteins. This is likely due to the stability of the small molecules in culture conditions, as well as their ability to easily cross the cell membrane. Additionally, further maturation is possible by creating floating cultures of these neural cells. In order for cell therapy to be a viable option for cell replacement by transplantation, a large source of replenishing cells must be available. Using small molecules, as opposed to recombinant proteins, reduces the cost of creating these photoreceptors and decreases the time it takes to grow these cells from nine weeks to four weeks.

The floating cultures and pattern of mature photoreceptors mirror the development of the eye in vivo. While the cells we develop in a dish form photoreceptors, they do not reach full maturity due to the lack of a proper cellular milieu. The self-formation of the optic cups aids in the construction of cell layers that mimic the natural development of photoreceptors in vivo. These optic cups artificially mimic the environment of the retina in an embryonic stage as it undergoes development. This process enhances the differentiation process by allowing the cells to grow as a three dimensional cluster. Establishment of this protocol has provided a source of healthy and mature photoreceptors that are prepared for transplantation. This process more efficiently generates rod and cone photoreceptors in vitro than the previously described methods. The natural ability of the cells to self-organize and form the three-dimensional structures found in the eye is an intriguing discovery. Neural clusters formed from the differentiation of iPS cells have the capability of forming the organized and regulated structure of the retina. Photoreceptors line the outside ring of these clusters, while Sox2 positive ganglion cells form the interior of these neural spheres. Although the mechanism of how the cells migrate into the correct position and orientation is unknown, the finding that they can self-orient in vitro is an encouraging sign for the possibility that they can correctly orient in vivo after transplantation. Injection of
photoreceptors into the sub-retinal space of a mouse eye should prove sufficient to allow the cells to properly integrate into the host retina. Applications for this novel process can be extended to the other cell types present including RPE cells. The relationship between RPE cells and photoreceptors plays an important role in maintaining a healthy environment to prevent degeneration of the photoreceptors. Further elucidating this relationship is now possible because of our new methods of quickly generating these cell types as described above.

A key advantage to using the iPS cell lines is the ability to compare the photoreceptors derived from a healthy individual with cells from an individual with a retinal disease. Both of the retinal diseases associated with aging and diabetic retinopathy initially require healthy photoreceptor cells, and the degeneration of these cells results from disease progression. However, it is possible to utilize the photoreceptors we have created in the lab to examine the differences in gene expression between the healthy and diseased cells. Additionally, creating photoreceptors in vitro from the lymphocytes of an individual with a retinal disease allows the cells to be monitored closely over time, in an effort to elucidate the process of their degeneration. Once the lymphocytes are reprogrammed into a pluripotent state, they essentially revert to an embryonic state. Photoreceptors that differentiate through the above protocols are young, and are not yet degenerating. We can follow these cells and monitor gene expression levels, among other indications of cell stress, to determine the age and mechanism of degeneration. Artificial stressors can be added to mimic the environment in the eye. While these diseases often take decades to progress in vivo, this process can be greatly accelerated in vitro.

The findings that treatment of neural cells with Methylglyoxal caused a decrease in cell division and increases apoptosis is expected, yet it offers a possible new approach to study diabetic retinopathy. While the mechanism of disease progression is not yet well understood,
accumulation of oxidative stress and advanced glycation end products (AGEs) that results in photoreceptor death is a plausible route. Through findings such as decrease in pH3 levels, revealing a decrease in proliferation, decreased and fragmented TUJ expression and an increase in apoptosis; it is clear that the addition of MG is having a negative effect on the photoreceptors. However, it is unknown whether this mirrors the progression of DR in vivo. Additionally, further testing is required to observe the long-term impact of a low dose of MG, mirroring chronic oxidative stress.

Taken together, the DR data can be approached in a similar fashion to retinal diseases such as AMD and RP. The replacement of photoreceptors damaged as a result of the progression of these diseases is going to be a critical component of restoring vision. Additionally, creating these photoreceptors through iPS cell lines holds several distinct advantages, including the opportunity to use the iPS lines to study disease progression. More research is required to pinpoint the specific degenerations caused by these diseases in an effort to prevent photoreceptor deterioration. However, we have made a significant step by generating a method that allows for the generation of a large number of photoreceptors. The protocol outlined here provides a cheaper, and faster method of differentiating these mature photoreceptors from ES and iPS human cell lines.

Minimizing the amount of time and money required to develop a source of mature photoreceptor cells has many benefits. In addition to a renewable source of cells for transplantation, creating three dimensional structures can be a valuable tool for further research. These models can help to discover the progression of the disease, as well as provide a more reliable method for drug screening. The ability to more closely model the eye in vivo can help find targeted drugs to combat these age-related degenerative diseases.
References


