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1970

Heath Allison K.,Morphological and Proliferative Changes That Occur in Rat Retinal Progenitor Cells Following Incubation With Retinoic Acid and RPE-Secreted Proteins. Master's of Science (Cell Biology and Genetics), August 2006, 67 pp., 12 figures, bibliography.

The principal objective of this research is to characterize virally-transformed rat retinal progenitor cells following stimulation by retinal pigment epithelial (RPE) cell secreted proteins and retinoic acid. Progenitor cells were isolated from explants of postnatal day 2 rat retinas that were cultured in either retinoic acid or proteins secreted by neonatal rat RPE cell in vitro. Isolated progenitor cells were cloned, analyzed by microscopy and proliferation bioassays, to determine if cell proliferation occurred. The isolated progenitor cells were analyzed for differentiation by Western blot analyses and immunocytochemistry. The rat progenitor cells cultured in RPE secreted proteins proliferated, but did not differentiate as shown by the presence of nestin and vimentin in these cells. Retinoic acid caused other progenitor cells to proliferate and differentiate, which is seen through the bioassays and Western blot analyses.

MORPHOLOGICAL AND PROLIFERATIVE CHANGES THAT OCCUR IN RAT RETINAL PROGENITOR CELLS FOLLOWING INCUBATION WITH RETINOIC ACID AND RPE-SECRETED PROTEINS

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# MORPHOLOGICAL AND PROLIFERATIVE RESPONSES OF RAT RETINAL PROGENITOR CELLS FOLLOWING TREATMENT WITH RETINOIC ACID AND RETINAL PIGMENT EPITHELIAL CELL-SECRETED PROTEINS

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTERS IN SCIENCE

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Fort Worth, Texas

August 2006

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# CHAPTER 1

# INTRODUCTION

## I. Retinal Development

Studying retinal development can assist us in understanding how cells of the retina develop their specific functions and morphological characteristics. The vertebrate retina is derived from the anterior neural tube and, therefore, shares the same origin as the rest of the central nervous system (CNS) (Yang, 2004). The retina develops from the walls of the optic cup, which is a derivative of the forebrain. The thick, innermost layer becomes the retina, while the outermost layer becomes the retinal pigment epithelium (RPE). As a result of the inversion that occurs during eye formation, the optic vesicle becomes inverted, leaving the photoreceptor cells adjacent to the RPE (Sheedlo, 2005). The retinal pigment epithelium exists as a monolayer that is involved in the maintenance of photoreceptors. Therefore, damage of the RPE due to a number of diseases results in loss of vision (Kubota, 2006). After 56 days, the human embryo has a developing retina and retinal pigment epithelium. The mature retina is composed of specialized sensory neurons, interneurons, and projection neurons (Sheedlo, 2005). Together, these neurons accomplish the tasks of image detection, processing, and transmission. The vertebrate

retina is composed of seven major cell types arranged in a laminar organization including retinal ganglion cells, horizontal cells, amacrine cells, cone and rod photoreceptors, bipolar cells, and Muller cells (Das, 2005). The lamination pattern of the retina is dependent on the production of a sufficient number of each retinal cell. Each retinal cell has a different function and location within the mature adult retina. For example, the retinal ganglion cells project axons that eventually form the optic nerve. Horizontal cells are located in the inner nuclear layer along with the amacrine cells. The outer segments of rod and cone photoreceptor cells respond to photons of light and, therefore, initiate the phototransduction cascade. Rod photoreceptors function in night and peripheral vision, while cone photoreceptors are concentrated in the macula and function in visual acuity and color vision. Bipolar neurons interconnect photoreceptor axons and ganglion cell dendrites, while Muller cells extend from the inner laminar membrane (ILM) to the outer laminar membrane (OLM) and are the primary glia cell of the retina (Sheedlo, 2005). The majority of retinal ganglion cells (RGCs), horizontal cells, amacrine cells, and cone photoreceptors are formed during early histogenesis, whereas the greater part of rods, bipolar cells, and the Muller glia are produced during late histogenesis (Das, 2005).

## II. Retinal Progenitor Cells

All of the neural cell types in the ocular region arise from a common precursor, the retinal progenitor cell (RPC). The formation of neural cell types from retinal progenitor cells is characterized by two important events. Each cell type is generated during a

limited period, and the number of cells that are born varies significantly across cell types (Sheedlo, 2005). There are a numerous differences between retinal stems cells and retinal progenitor cells; however, these terms are commonly used interchangeably. Retinal stem cells are undifferentiated cells and conserve the ability to differentiate into other retinal cell types. This ability allows these cells to act as a repair system for the body by replacing cells that have died. In addition, cell replenishment occurs as long as the organism continues to remain alive. Stem cells can be classified as totipotent, pluripotent, or multipotent. Totipotent stem cells are produced from the fusion of an egg and sperm cell. Furthermore, totipotent cells are produced following the first few divisions of the fertilized egg. These cells can differentiate into any type of cell. The second major type of stem cells, pluripotent cells, are descendants of totipotent cells. Pluripotent stem cells can grow into any cell type except totipotent stem cells. The third major type of stem cell is multipotent, which are considered to be the more specialized stem cell and include retinal stem cells. Adult rat retinal stem cells are considered to be multipotent which are defined as stem cells that can produce only cells that are closely related (Wikipedia, 2005). A stem cell is at the top of the lineage ladder and each stem cell divides to produce one of the following cell populations: two stem cells, one stem cell and one progenitor cell, or two progenitor cells. Under the influence of growth factors, retinal stem cells will survive, differentiate, or proliferate (Sheedlo, 2005). On the other hand, progenitor cells normally produce only one cell type and are thus considered unipotent in most cases (Wikipedia, 2005). Cell-lineage analyses have made obvious that vertebrate retinal progenitor cells are multipotent at dissimilar

developmental stages; therefore, progeny derived from individual progenitor cells can become different cell types. The multipotency of retinal progenitor cells throughout neurogenesis suggests that local environmental factors play a central role in cell fate decisions (Yang, 2004, Ahmed I, Dev. Bio., 1998). In addition, these cells have the property of self-renewal which distinguishes them from non-stem cells. Retinal progenitor cells have a more limited cell lineage and reduced mitotic activity than stem cells (Belliveau et al., 2000).

III. Retinal Pigment Epithelial Cells

The retinal pigment epithelium (RPE) differentiates prior to the underlying neural retina and is located immediately adjacent to the developing photoreceptor cell layer. RPE cells function chiefly in phagocytosis of shed outer segment photoreceptor cells, form, in part, the blood/retina barrier, direct transport between the neural retina and choriocapillaris, and are involved in the phototransduction process. Therefore, RPE can potentially influence rod and cone maturation. In addition, the RPE is in a unique position to manipulate the development of the neural retina by the release of trophic factors into the interphotoreceptor matrix (IPM). RPE cells have recently been shown to play a role in the proliferation of dividing retinal progenitor cells and the eventual organization of the retinal layers (Sheedlo, 1996). Past studies from Sheedlo et al., show that a factor secreted by cultures of both normal and transformed RPE cells causes ganglion cell-like neurite outgrowth and proliferation in transformed rat progenitor cells. The secreted

proteins also play a part in migration of retinal progenitor cells possessing photoreceptor-cell antigens from neonatal rat retinal explants (Sheedlo, 1996). It was demonstrated that a RPE-cell secreted factor promotes Muller cell proliferation and photoreceptor cell survival and differentiation. The factor and media together is known as RPE-conditioned media (RPE-CM). Additional studies have demonstrated that RPE and its conditioned media are responsible for photoreceptor cell maturation. In addition, neonatal and embryonic retinal explants treated with RPE-conditioned media from transformed neonatal rat RPE cells stimulate neurite outgrowth. Progenitor cells that exhibit a photoreceptor phenotype will proliferate in response to RPE-CM (Sheedlo, 1998). Studies have determined that a factor in RPE-CM plays a significant role in retinal cell survival and maturation. This factor appeared to accelerate the maturation and promote the survival of photoreceptor cells (Sheedlo, 1998). Numerous researchers have demonstrated that medium conditioned by cultures of RPE-CM enable round cells to emerge from explants of retinas from embryonic and neonatal rats and therefore populate culture dish surfaces in large numbers. In the presence of RPE-CM, defined medium, or growth factors, progenitor cells continue to express predominately differentiated photoreceptor cell markers and survive and proliferate in low density cultures (Sheedlo, 1996). Molecules secreted by RPE cells promote essential developmental and repair mechanisms between the RPE and neural retina, especially photoreceptor cells. Also, RPE trophic factors are necessary for ordinary photoreceptor cell differentiation and regenerative repair (Sheedlo, 1998).

## IV. Cellular Differentiation

Cellular differentiation is the method by which cells mature to a particular type of cell. Thus, an unspecialized cell becomes specialized during the differentiation process. For example, a stem cell following mitotic division can be directed to become a heart cell, retinal cell, or liver cell. During differentiation, the morphology of the cell alters even though most of the genetic make-up remains the same (Wikipedia, 2005). Specific genes are activated while others are inactivated to direct the cell to a mature state, as demonstrated by its function and/or characteristics. A significant number of studies point to the fact that cell differentiation may arise during or after the final cell division. In addition, some studies suggest that cell commitment and differentiation in the developing retina are influenced by cell-cell interactions or cell interactions with the external environment (Ezeonu, 2000). Differentiation of retinal progenitor cells to rod photoreceptors is influenced by locally diffusible signals in the extracellular environment (Kelley, 1994). It is becoming increasingly apparent that cellular differentiation within the CNS and retina can be modulated by environmental factors such as the extracellular matrix, cell adhesion molecules, and trophic factors. RPE cells have been shown to synthesize and secrete several proteins in vitro, such as FGF-2. Although there is little evidence of specific novel RPE-factor secretion in vivo, these studies provide a necessary stimulus to investigate such a possibility (Sheedlo, 1998). Certain members of the family of fibroblast growth factors are emerging as strong candidates to direct neuronal development, differentiation, and function. For example, increase in the number of photoreceptor cells expressing opsin upon treatment with FGF-2 may be due to several

phenomena including increased cell survival, differentiation, or proliferation. It has been demonstrated that FGF-2 can stimulate the differentiation of immature photoreceptor cells, permitting a much greater number of cells to express the visual pigment opsin without increasing total neuronal numbers (Hicks, 1992). FGF-2, a polypeptide with regulatory activity on cell growth and differentiation, lacks a predictable secretory signal sequence, and its mechanism of release from cells remains unclear (Taverna, 2003). This protein loses its mitogenic influence on the late retinal stem cells/progenitors and instead is observed to promote their differentiation (Das, 2005). Another environmental factor, retinoic acid, influences the early sequences of photoreceptor cell maturation. The differentiation and maturation of rod and cone photoreceptor cells is also induced by retinoic acid (Sheedlo, 2005). In previous experiments, FGF-2 withdrawal and retinoic acid stimulation of adult hippocampal stem cells triggered differentiation into neurons (Akita, 2002). Thus it appears the brain stem cells differ from retinal stem cells in their response to FGF-2. To determine whether or not a cell has differentiated, researchers examined these cells for different proteins using immunocytochemistry and other biochemical methods. For example, the intermediate filament protein, nestin, is characteristic of immature, undifferentiated cells. Nestin-positive cells have been shown to be significantly greater in populations of embryonic progenitor cells when compared to postnatal progenitor cells (Sheedlo, 1996). Therefore, if nestin is present in cells, the cell can be classified as immature and undifferentiated. On the other hand, markers suggestive of photoreceptor differentiation include rhodopsin (rods) and recoverin (rods and cones) (Qui, 2005). Retinal cells, determined to be photoreceptor cells by opsin

immunocytochemistry have been produced by differentiation in the presence of media conditioned by confluent cultures of normal neonatal rat RPE cells (Sheedlo, 1996).

V. Cell Proliferation

Retinal cell proliferation can be shown as an increase in the number of cells after a designated incubation time period. Early and late retinal stem cells/progenitors are distinct in their proliferative responses to different mitogens and possess variate potential to generate neurons and glia. Proliferative potential of early and late retinal stem cells/progenitors is different because of the number and types of cells generated during early and late histogenesis (James, 2004). Proliferation can occur under the influence of many factors including FGF-2, retinoic acid, and proteins secreted by RPE cells. RPE cells appear to play a role in the proliferation of retinal progenitor cells and the eventual organization of the retinal layers (Sheedlo, 1998). One of the salient features of neural stem cells/progenitors is their ability to proliferate in the presence of EGF and FGF-2. The early retinal stem cells/progenitors preferentially express FGF receptors and, therefore, are more responsive to FGF-2 than EGF. In contrast, the late retinal stem cells/progenitors prefer EGF over FGF-2 for proliferation and such responsiveness to EGF may be due to dominant patterns of expression of EGF receptors relative to those for FGF-2 (Das, 2005). Both EGF and FGF-2 have been shown to promote proliferation of retinal progenitor cells and rod photoreceptor survival; however, none of these factors stimulated differentiation of photoreceptor cells from embryonic retinal progenitors

(Kelley, 1994). Retinoic acid has been shown to influence cell proliferation during development of the nervous system and this molecule has been detected in the embryonic retina. Addition of exogenous retinoic acid has been shown to cause a dose-dependent, specific increase in the number of cells that developed as photoreceptors in culture throughout the period of retinal neurogenesis (Kelley, 1994). The use of all-trans retinoic acid in the culture medium has a striking effect on the development of photoreceptors in embryonic rat retinas. Both 100 and 500 nanomolar all-trans retinoic acid (RA) levels produce a significant increase in the number of photoreceptor cells (Kelley, 1994). The RPE plays a role in the proliferation of dividing retinal progenitor cells and the eventual organization of the retinal layers, apparently through the release of diffusible factors. Nevertheless, the generation of large numbers of progenitor cells from retinal explants promoted by RPE-secreted proteins has important implications for possible therapeutic approaches in retinal diseases (Sheedlo, 1996).

## VI. Cell Proteins

### A. Vimentin

Vimentin is an intermediate filament protein that is crucial for the structure of eukaryotic cells. Intermediate filament proteins are non-polar structures that can be found in cells such as fibroblasts and endothelial cells. All intermediate filament monomers, including vimentin, have a basic α-helical domain that is located on each end in the amino (head)

and carboxy (tail) terminal region. The role of vimentin has been thoroughly studied by numerous scientists throughout the years. It was found that vimentin is bound to the nucleus, endoplasmic reticulum, and mitochondria. In this position, vimentin plays a chief role in supporting and anchoring the arrangement of organelles in the cytoplasm and maintaining cell shape. In addition, vimentin allows for the flexibility and resilience of the cell. Therefore, when a cell is stained with an antibody against vimentin, staining is observed in most of the cell. In Western blot analyses, vimentin is located at 58 kDa and is easily represented by most cells (Wikipedia, 2005).

B. Pax-6

Pax-6 is a tissue specific transcription factor with vital functions in the development of the eye, nose and central nervous system. PAX genes program nuclear transcription factors which are known to be the major controllers of developmental processes in both vertebrates and invertebrates. Transcription factors are proteins that bind to DNA at a specific promoter or enhancer, where it regulates transcription. In addition, Pax-6 is involved in early development of the optical vesicle and is approximately 47 kDa in molecular weight. Therefore, Pax-6 is an excellent antibody to assess retinal cell differentiation. (Wikipedia, 2005).

C. Opsin

Rhodopsin is one of five types of G-protein coupled receptors found in vertebrate photoreceptor cells and has a molecular weight of 36 kDa. A G-protein coupled receptor, also known as a transmembrane receptor, transduces an extracellular signal into an intracellular signal. Opsin uses a photoisomerization reaction to translate electromagnetic radiation into a cellular signal the photoreceptors can use. This particular type of opsin is monochromatic and allows night vision because it is found in rod cells. In addition, rhodopsin in the retina is responsible for both the formation of photoreceptors and the perception of light (Wikipedia, 2005). Opsin is found as a membrane protein in retinal cells and therefore in immunocytochemistry shows up faintly with fluorescence.

VII. RPE Proteins and Molecular Growth Factors

Growth factors are proteins that act as signaling molecules linking cells, such as cytokines and hormones. The factors bind to specific receptors on the surface of target cells to encourage differentiation and maturation of progenitor cells. Growth factors usually have a positive effect on cellular growth and differentiation (Wikipedia, 2005).

A. Fibroblast Growth Factor

One major growth factor present in the retina is the family of fibroblast growth factors (FGF). Fibroblast growth factors have been shown to promote cell growth, differentiation, migration, morphogenesis, and survival throughout the development of the central nervous system. Fibroblast growth factor was first found in a cow brain extract (Gospadarowicz et. al, 1996). This group noted that in a bioassay with neurons, the fibroblasts proliferated with this growth factor. In addition, they further fractionated the extract using acidic and basic pH, which caused the isolation of two slightly different forms. The two different forms of the growth factor were named acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2). These proteins share a large number of the same amino acid sequences but they are individually unique (Wikipedia, 2005). Basic fibroblast growth factor can stimulate the differentiation of immature photoreceptor cells, permitting a much greater number of cells to express the visual pigment opsin, without enhancing the total neuronal cell numbers (Ifeoma, 2000). Basic fibroblast growth factor is expressed in the retina where it can act as a rescue factor. It is located in neovascular tissue at high amounts in age-related macular degeneration (ARMD). Studies on the role of FGF-2 in the development of choridal neovascularization have proven that this growth factor can cause neovascularization (Rosenthal, 2005). Neovascularization is also known as angiogenesis which is the formation of new blood vessels from already existing vessels. Presently, there are 23 members of the FGF family that are structurally similar signaling molecules. Some examples are FGF-1, FGF-2, and FGF-3 which bind to a family of receptor molecules

consisting of 4 members including FGFR-1, FGFR-2, FGFR-3, and FGFR-4 (Wikipedia, 2005). FGF-2 is one member of the fibroblast family that is a heparin-binding protein, which has shown to possess mitogenic effects in a broad range of cells during embryonic formation (Goldfarb, 1996). This growth factor appears to have a neuroprotective effect on neurons by up-regulating the extracellular signal-regulated kinase (ERK) signaling pathway (Munoz, 2005). Accumulating evidence from numerous studies demonstrates that FGF activity is crucial for maintaining retinal stem cells. Retinal stem cells have been identified in the ciliary margin of the adult mouse retina in recent studies (Yang, 2004). Early retinal stem cells/progenitors generate more neurospheres than late retinal stem cells/progenitors in the presence of FGF-2. On the other hand, late retinal stem cells/progenitors generate a greater number neurospheres than early retinal stem cells/progenitors in the presence of EGF (James, 2004). FGF-2 release from cells occurs by vesicle shedding and, therefore, it has been proposed that FGF-2 is released from cells through alternative pathways including cell death or wounding. Membrane vesicles containing FGF-2 are shown to bud from the plasma membrane of viable cells and in addition can be purified from conditioned media generated from cell cultures. Immunolabeled vesicle-associated FGF-2 on endothelial cell plasma membranes indicates that FGF-2, present inside the vesicles, is distributed to target cell receptors following a spontaneous vesicle breakdown. A limited release of FGF-2, caused with contact between vesicle and plasma membranes, may symbolize a method for uninterrupted delivery of small amounts of FGF-2 in close proximity to the cell membrane (Taverna, 2003).

B. Retinoic Acid

Retinoic acid (RA) plays an essential role in retinal development by modulating cellular proliferation, differentiation, and survival in vertebrates. Retinoic acid supplementing to culture medium has an interesting effect on early eye morphogenesis and the development of photoreceptors. The morphology of retinoic acid-treated cells is similar to developing photoreceptors *in vivo*, such as a thin axonal-like process. Also observed is a wider process comparable to the inner segment and forming outer segment. In one study it was concluded that the number of rhodopsin immunoreactive cells was much greater in cultures treated with retinoic acid at the concentration of $10^{-6}$ M. The numbers of rod photoreceptors present was demonstrated to be elevated in cultures treated with retinoic acid when compared with control values. In addition, this study reported that increasingly greater numbers of cells developed as photoreceptor cells with higher concentrations of retinoic acid. During these experiments, there was no significant generation of rods after 2 days of culture; although, by 4 days *in vitro*, a significant increase in the number of rods was observed when compared to the control cells. It has been noted that retinoic acid may only act on those progenitor cells that have become signaled to differentiate. These cells would differentiate as late retinal phenotypes such as amacrine cells or rod photoreceptors. In addition, another study showed that in the presence of retinoic acid, certain immature cells in cultures that normally differentiated into amacrine cells were influenced instead to differentiate into photoreceptor cells (Kelley, 1994).

In immunochemistry experiments it has been shown that the percentage of vimentin-positive cells in RA pretreated groups was lower than in the non-treated groups. Also, retina-specific marker-positive cells such as rhodopsin were not present in RA pretreated cells (James, 2004).

C. Secreted Proteins of RPE

RPE cells synthesize, store, and/or secrete a large amount of factors and cytokines. Thus, RPE trophic factors are mostly responsible for normal photoreceptor cell differentiation and some forms of regenerative repair. In different experiments, it has been confirmed that neonatal rat RPE-CM promotes the survival of retinal progenitor cells (Sheedlo 1996). A majority of these cells expressed the rod photoreceptor cell marker opsin. Neonatal and embryonic retinal explants cultured with conditioned media from rat RPE cells stimulated progenitor cells to display a photoreceptor phenotype. These progenitor cells also proliferated in response to the RPE-CM. In another study, it was observed that rod photoreceptors accounted for 70% of all cells in the mature rat retina. Of these photoreceptors, 80% of the cells from retinal explants treated with RPE-CM expressed opsin. In contrast, under the control conditions, 20% of explant cells expressed opsin indicating that a component of the RPE-CM is responsible for increasing rod cell survival and promoting differentiation into rod photoreceptors (Sheedlo, 1998). RPE cells have been shown to secrete several proteins in vitro, such as FGF-2 (Sheedlo, 1996).

Although there are many studies about cell protein secretion function in the retina, there are a few studies that are similar in different parts of the human body. These studies show that certain cells in the body act similar to RPE cells secreted proteins that promote cell morphological differentiation and proliferation. One study showed that the use of a defined serum-free medium in a culture produced neurosphere clusters of progenitor cells in human pancreatic cell line PANC-1. After exposure to the serum-free medium, the pancreatic cells differentiated into hormone-producing islet-like cell aggregates. For this experiment, FGF-2 was the protein incorporated into the defined media because it is known to be secreted by the extracellular matrix. This protein is largely expressed in the section of the mesenchyme surrounding pancreatic precursor cells. FGF-2 in these cells promotes early stage development of the endocrine pancreas. In the pancreatic precursor cells, FGFs are thought to act via a paracrine mechanism to stimulate migration of cells into the mesenchyme, which initiates the development of the pancreas. PANC-1 cells express FGF receptors and FGF-2 is known to bind to several related receptors that send signals to these particular cells. In this experiment, signals arising from endocrine pancreatic precursor cells themselves were sufficient to promote cell clustering and differentiation during formation of the islets of Langerhans (Hardikar, 2003).

D. Signaling Pathways

Apart from studying the influences of both retinoic acid and the RPE-secreted proteins on progenitor cells, another important focus is to determine the mechanisms that are involved in progenitor cell proliferation and differentiation. There are numerous studies

that address the signaling pathways of retinoic acid and FGF-2, along with their affects on progenitor cells.

Retinoic acid has been shown to cause cell proliferation and differentiation in neural progenitor cells using a pathway that involves Gab2. Gab2 protein amplifies signals stimulated by numerous growth factors including retinoic acid. In neuronal differentiation and proliferation, retinoic acid causes an elevated expression of Gab2. This protein then upregulates FGF-2, which is a peptide growth factor that plays a critical role in cell proliferation and differentiation. Therefore, when retinoic acid is incubated with neural progenitor cells, a high Gab2 expression is shown which then upregulates the FGF-2. The upregulation of FGF-2 affects progenitor cells similar to RPE-secreted proteins (Mao, 2005). Retinoic acid signaling has also been found to be required for olfactory pathway development and other forebrain regions throughout life. This factor influences cell survival, proliferation, and differentiation in this region. Since neural progenitor cells are comparable to retinal progenitor cells it is hypothesized that the same signaling is found for both types of cells (Haskell, 2005).

Transdifferentiation is a cellular pathway that is influenced by members of the FGF family to regenerate parts of the retina. This procedure is currently being studied for its use in retinal transplantation therapy because of the ability to permit natural cell proliferation and differentiation of the retinal progenitor cells. For transdifferentiation to occur, the hedgehog (Hh) pathway comes into play as the chief modulator. In addition,

the hedgehog pathway plays a role in survival of the retinal pigment epithelium. During transdifferentiation, the developing RPE loses its distinctive phenotype, proliferates, and differentiates into the various layers of the retina. This latter process is stimulated by FGF-2, the stimulatory growth factor originating in RPE-secreted proteins and conditioned media. Hedgehog signaling was shown to modulate FGF-2 stimulation and the overall process of RPE proliferation and differentiation. Therefore, when FGF-2 is added to retinal progenitor cells they are extended like the RPE allowed to proliferate indefinitely (Spence, 2004).

## VIII. Retinal Diseases

Today there are numerous diseases that affect the eye, but two that most adversely affect the retina are age-related macular degeneration (ARMD) and retinitis pigmentosa (RP). Although these ailments affect the same part of the eye, the cells they affect and symptoms they cause are dissimilar.

### A. Age-Related Macular Degeneration

Age-related macular degeneration (ARMD) is an incurable eye disease, and, at least to date is the major cause of blindness for those aged 55 and older in the US. At present, this disease affects more than 10 million Americans. Age-related macular degeneration destroys the retina initially within the macula lutea. The macula lutea is the region of the retina that has primarily photoreceptor cells, with other layers either missing or

rudimentary. Photoreceptor cells of the retina respond to light and convert it to electrical impulses, which are ultimately processed by the ganglion cells, the output neurons that convey visual information to the brain (ARMD online, 2005). Vision loss can also be attributed to retinal dysfunction caused by degeneration of the retinal pigment epithelium (Kubota, 2006). Another important ocular structure, the fovea centralis, is in the center of the macula and is located posterior retina. This retinal region provides the clear, sharp, central vision that one uses to focus on objects directly in front of the eyes. Because ARMD does not affect peripheral vision, at least in the early stages, it does not lead to total blindness (ARMD online, 2005). The main indicator of macular degeneration is dim or fuzzy central vision. In both wet and dry ARMD, objects may appear distorted or smaller than their physical morphology. Also, straight lines may appear wavy or curved. Patients with age-related macular degeneration may develop a blank or blind spot in their central field of vision. ARMD permanently damages the cells that provide central vision, resulting in blindness in the fovea. Therefore, vision loss from ARMD cannot be reversed, at least presently.

The two forms of ARMD are dry and wet. Dry age-related macular degeneration, that includes more than 90% of ARMD cases, does not typically cause severe vision loss. In dry ARMD, deposits in the Bruch's membrane, called drusen, hinder the normal interaction of the retina and the RPE cells. Parts of the macula, the RPE, and blood vessels beneath the macula become nonfunctional and atrophy. This breakdown damages the fovea and changes central vision, which gradually becomes dimmer or more blurry

with age. Wet age-related macular degeneration makes up 10% of ARMD cases. It can damage the macula quickly, causing permanent photoreceptor cell loss. Wet ARMD, also called exudative ARMD, develops in areas where dry ARMD is present. Breaks develop in the deeper layers of the retina, and atypical, delicate blood vessels form in these breaks. These blood vessels leak blood and fluid under the macula, leading to a rapid loss of central vision (ARMD online, 2005).

Vision loss from both wet and dry ARMD is caused by damage to the photoreceptor cells in the macula. The process that leads to this damage is different in wet ARMD than in dry ARMD although the cause of each condition is unknown. Wet ARMD can sometimes be treated with laser surgery, but there is currently no treatment available for dry ARMD. Treatment for wet ARMD can slow down or delay further damage to central vision; however, vision cannot be restored (ARMD online, 2005).

ARMD is the most common cause of blindness because of the many risk factors associated with this disorder. Risk factors for age-related macular degeneration include smoking, nutrition and obesity. In addition, race, genetics, and gender can be associated risk factors for developing ARMD. Prevention of age-related macular degeneration includes lowering risk factors and eating healthy diets of antioxidants and zinc. Eating foods with vitamins A, C, and E could help reduce chances of developing ARMD. Exercising and maintaining one's health are preventive methods for most diseases (ARMD online, 2005).

B. Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a group of hereditary disorders that affect the retina's ability to respond to light. It is primarily a disease of rod photoreceptor cells and initially occurs in the periphery of the retina. The rod cells are responsible for night vision, dim light vision, and peripheral vision. Cone cells, which are responsible for color vision, visual acuity and seeing in bright light, may sometimes become affected as RP progresses. Retinitis pigmentosa is caused by mutations in any one of at least ten different genes. Over 70 mutations in the rhodopsin gene have been identified as contributors to retinitis pigmentosa. The end result is a malfunction in the retinal pigment epithelial (RPE) cells and a breakdown of the outer segment disc membrane of rod cells. When cells are destroyed at an abnormal rate, the build-up of waste products hinders normal retinal function. The outcome includes occlusion of small blood vessels, an abnormal increase in the number of RPE cells, and the loss of photoreceptor cells. There are three different genetic defects that can cause retinitis pigmentosa including autosomal dominant inheritance, autosomal recessive inheritance, and X-linked (RP online, 2005). Symptoms of RP usually manifest between the ages of 10 and 30 and signs of the disease start with the breakdown of rod cells. Initially, RP patients suffer a decrease in night vision and an inability to see in dimly lit places such as movie theatres. Tunnel vision is the progressive loss of peripheral sight and the gradual reduction in the ability to see peripherally can cause tripping accidents. These incidents occur when rod cells and outer cone cells are affected. Most patients are legally blind by 40 and there is currently no affective treatment or therapy for RP. Hopefully, in the next decade, the study of the

genes affecting retinitis pimentosa may provide a cure or treatment for RP (RP online, 2005).

## IX. Hypothesis and Significance

Although there are numerous studies on retinal progenitor cells, additional research needs to be performed to develop therapies in ocular medicine. The current studies have demonstrated that retinoic acid and FGF-2, present in RPE-secreted proteins, stimulate retinal progenitor cell proliferation. These experiments have determined that progenitor cells incubated in these factors caused increased proliferation when compared to the cells that were cultured in defined media lacking serum. Also, the progenitor cells incubated in either RPE-secreted proteins or retinoic acid showed signs of cell differentiation. As stated above, FGF-2 is secreted by RPE cells and promotes cell division, without morphological differentiation, of rat progenitor cells. Retinoic acid has been shown to cause cell proliferation at $10^{-6}$ M concentration. The purpose of my research is to prove that progenitor cells cultured in retinoic acid or RPE-secreted proteins will show, via immunocytochemistry, western blot analyses and other experiments, that these cells are viable candidates for transplantation. This transplantation therapy may provide sight in patients suffering from age-related macular degeneration (ARMD) and/or retinitis pigmentosa (RP).

# CHAPTER 2

# MATERIALS AND METHODS

## I. RPE Cell Culture

Transformed rat retinal progenitor cells propagated from P2 rat retinal explants were cultured in Dulbecco's Modified Eagle's Media (DMEM) and 10% fetal bovine serum at 37 C and used in these studies. The rat progenitor cells were transformed with the psi 2 E1A virus and the $12^{th}$ passage was used in these experiments. Rat retinal pigment epithelial cells were also cultured in DMEM and 10% serum at 37 C. The RPE cells were necessary to obtain the RPE-secreted proteins for these experiments.

## II. RPE-secreted Protein Collection

Retinal pigment epithelial (RPE) cell secreted proteins (conditioned medium) were collected and frozen at -20 C for later use. To obtain secreted proteins, RPE cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) containing ITS+ (I-2521

Sigma) in 6-well plates and each well of RPE cells was cultured in 2 ml of DMEM/ITS+ for two days. During this incubation, the RPE cells secreted growth factors and other proteins into the media. The media was collected after the two days and frozen until it was needed for culture experiments.

## III. Progenitor Cell Culture

### A. RPE-Secreted Proteins

These experiments were set up using rat retinal progenitor cells that were cultured in DMEM with 10% fetal bovine serum (FBS) for two days. After one day the cells were sufficiently attached, they were grown in RPE-secreted proteins. First, the medium in the wells of progenitor cells was removed and replaced with one of the following: 10% FBS media, ITS+ media, RPE-secreted proteins, or conditioned media + serum. The progenitor cells were incubated for two days and monitored with a phase contrast microscope. These experiments were repeated in quadruplicate for accuracy.

### B. Retinoic Acid

To obtain $10^{-6}$ M of retinoic acid, 50 mg of all-trans retinoic acid powder was diluted in 1 ml of DMSO (dimethyl sulfoxide). The solution was sterile filtered prior to use. In each experiment treatment with $10^{-6}$ M of retinoic acid was carried out and morphological changes in retinal progenitor cells were studied. The working RA solution consisted of 1

ul of retinoic acid to 17 ml of DMEM/ITS+ solution. Progenitor cells were incubated with three different solutions, so a comparison could be made between each group. The three different environments for cells included a control of ITS+, serum, or retinoic acid solution. After three days, the progenitor cells were microscopically observed for changes in morphology or other characteristics. These experiments were repeated in triplicate to assure accuracy for this study.

## IV. Proliferation Bioassays

Proliferation bioassays were performed to provide data on mitotic activity of retinal progenitor cells. These assays were carried out following incubation with RPE-secreted proteins and retinoic acid. Two types of bioassays were performed to determine the effects of RPE-secreted proteins, FGF-2, and retinoic acid. Before beginning each proliferation assay, a standard curve was generated to obtain the equation that determined the results of the three day bioassay. For the standard curve, progenitor cells are counted and diluted to 1000 cells per 10 ul. The standard curve was set-up with the following numbers of cells in four separate wells: 0, 500, 1000, 2000, and 3000 cells. The number of cells was placed in quadruplicate in the 96-well microtiter plate for accuracy. After the cells were added to the plates, the number of cells in each well was read by a microtiter plate reader paired with a bioreagent.

The first bioassay studied the effects of RPE-secreted proteins, FGF-2, and retinoic acid on the proliferation of progenitor cells. Rat retinal progenitor cells were first cultured in 10% serum for a week following which, they were trypsinized, counted, and approximately 1000 cells were added to wells of a 96-well plate. The effects of each factor were counted using at least four wells on each plate to obtain accurate results. The plates were set up with two controls, one with progenitor cells in DMEM and one blank, without cells. The secreted proteins, FGF-2, and retinoic acid were then added to each of four wells. After addition of the factors, the cells were allowed to incubate for three days prior to analysis. After the three days, 20 ul of bioreagent (BioRad) was added to each well of 96-well plates and allowed to incubate for 60 minutes. The plates were then read using the microtiter plate reader at 490 nm. Subsequently, the equation from each standard was used to determine the level of proliferation.

The second bioassay performed was a retinoic acid saturation curve. This technique was helpful in determining the highest level of retinoic acid that caused proliferation without causing cell death. The saturation curve bioassay was set up similar to the growth factor bioassay except all of the wells, other than the two controls, contained retinoic acid. The different levels of retinoic acid added to the wells included 10 ul, 30 ul, 45 ul, 60 ul, 80 ul, 90 ul. The wells incubated with 10 ul correspond to the amount of $10^{-6}$ M retinoic acid. Each retinoic acid treatment was carried out in four wells to obtain an accurate analysis. The retinoic acid was allowed to incubate for three days and proliferation was measured by a microtiter plate reader at a 490 nm.

V. Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Western blots were used to detect proteins in cell homogenates. This technique was used to determine whether the progenitor cells differentiated, based upon expression levels of certain markers of retinal differentiation. Prior to performing the Western blot analyses, rat retinal progenitor cells were incubated in growth factors for 3 days. The factors the cells were also cultured in included RPE-secreted proteins, 10% serum, or retinoic acid. After three days, the cells were lysed using a lysis buffer containing a protease inhibitor. The lysed cells were then centrifuged and the media removed before freezing the protein pellets for later use.

Before the proteins were separated in polyacrylamide gels, a protein assay was performed to determine the level of protein in each sample. A standard for the assay was performed using bovine serum albumin (BSA) and a bioreagent (BioRad). The cell homogenate samples were analyzed and the standard curve was used to determine the protein content. For Western blot analyses, SDS-PAGE gels were cast by first pouring the separating gel (8%), which was allowed to polymerize overnight. A thin layer of distilled water (about 1 milliliter) was layered over the top of the gel solution with a 1 ml syringe to create a smooth surface. The next day, after removing the water, the stacking gel (4.5%) was poured on top of the separating gel and a comb was placed in position to form the wells. This gel was allowed to polymerize for 20 minutes while the samples with known protein content were being prepared. The samples used for each gel included low-range

molecular weight standards, progenitor cell proteins, proteins from cells incubated with conditioned media, proteins from cells incubated with retinoic acid, and the secreted proteins. Approximately 50 ug of each sample was mixed with 50 microliters of gel sample buffer and boiled for 5 minutes before loading the gel. A layer of working buffer was placed over the wells after the comb was removed, to prevent further polymerization. Each gel was then loaded with the molecular weight standard, followed by the other four samples. The samples were run in two sets on the gel; one set of samples run on the gel was stained with Coomassie blue, while proteins in the other set of the gel were transferred. After allowing the samples to reach the gel interface at 200 volts, the SDS-PAGE gel was run for approximately 5 hours at 100 volts. After the dye front of the gel had reached the bottom, half of the gel was set-up for transfer onto a nitrocellulose sheet and the other half was fixed and stained with Coomassie blue.

The gel that was transferred was placed in running buffer for 10 minutes to equilibrate for transfer. The other transfer materials were also soaked that included two scotch-brite pads, six pieces of filter paper, and one sheet of nitrocellulose. After these materials were fully saturated with running buffer, they were assembled as follows: 1 scotch-brite pad, 3 pieces of filter paper, SDS-PAGE gel, 1 sheet of nitrocellulose, 3 pieces of filter paper, and 1 scotch-brite pad. This sandwich of materials was then placed into the transfer apparatus and running buffer was added to the top. The apparatus was set at 100 volts for one hour in the cold room (-80 C) to allow full transfer of proteins from the gel to the

nitrocellulose sheet. After a 60-minute transfer, the sheet was removed and placed in a container for immunoblotting.

For immunoblotting, the transferred nitrocellulose sheet was added to the blocking solution on a shaker for 60 minutes at room temperature. The blocking solution of milk was used to block against non-specific binding. During this incubation, the primary antibody was diluted 1:500 in the antibody medium with the appropriate normal serum. After the blocking solution (with 5% milk) was removed from the dish, the primary antibody was allowed to incubate with the nitrocellulose for 18 hours. The next day, the sheet was rinsed with Tris-buffered saline pH 7.4 (TBS) for five minutes, six times. The sheet was then incubated in the secondary antibody diluted 1:10,000 in 5% milk in TBS. After two hours, the sheet was rinsed in TBS for 30 minutes and briefly rinsed with distilled water. The sheet was then treated with 0.01% diaminobenzidine for 5 minutes. The stained bands revealed the proteins that are present in progenitor cells.

## VI. Immunocytochemistry

This procedure was performed to support the Western blot analyses. Before immunocytochemistry was performed, progenitor cells were cultured on coverslips in a twelve-well plate. Cultured cells were then incubated for three days in one of three types of media: 10% serum, retinoic acid or RPE-secreted proteins. Cells on the coverslides

were fixed for fifteen minutes with 4% paraformaldeyde in 0.01 phosphate-buffered saline (PBS). The coverslides were rinsed three times for 5 minutes each with PBS to wash away the fixative. Triton-X-100 was added to each slide for 30 minutes which renders cell membranes permeable to allow the antibodies to enter the cells. The slides were washed with PBS for five minutes, three times. Glycine was then added for fifteen minutes to bind to free aldehydes, which prevented the antibodies from binding to these molecules. Immediately following the glycine treatment, a blocker was added for 30 minutes to prevent non-specific binding of antibodies. The primary antibody was then added to each coverslide and incubated 18 hours. The next day the slides were rinsed 3 times for 5 minutes each with PBS. A secondary antibody was then added to each slide and the plate was placed in the dark room for one hour. The secondary antibody is bound to a fluorescent molecule (Alexa green) and therefore must be incubated in the dark to prevent fading. Following the 60 minute incubation, PBS was used again to rinse the slides 3 times for 5 minutes each. Another fluorescent dye, DAPI was used for ten minutes to stain the nuclei. This stain allowed for the nucleus of the cells to be viewed while the other proteins were viewed at a second wavelength. After the ten minutes, PBS was used to rinse the slides. The slides were then placed on large coverslips with aquamount before viewing. The slides were viewed using a fluorescent microscope.

# CHAPTER 3

# RESULTS

## I. Response of Rat Retinal Progenitor Cells to RPE-Secreted Proteins

The four different environmental conditions that the rat retinal progenitor cells were cultured in resulted in a clear difference in morphology between the cells. Although the cells all started out with similar morphology, incubation with dissimilar proteins caused their cell characteristics to become greatly varied. The progenitor cells cultured in ITS+ did not survive the three day incubation and thus small, dead cells were observed (Figure 1A). Cells cultured in 10% fetal bovine serum appeared to be maturing normally, demonstrating differentiation, and process formation (Figure 1B). After incubation with RPE-secreted proteins, the progenitor cells took on a new morphology (Figure 1C). These cells became neurospheres and formed grape-like clusters. In addition, the cell processes were not visible after incubation. The progenitor cells cultured in RPE-secreted proteins plus serum, contained processes and also formed clusters, most likely due to the RPE-secreted proteins (Figure 1D). In addition, a number of floating aggregates were observed above the plated cells.

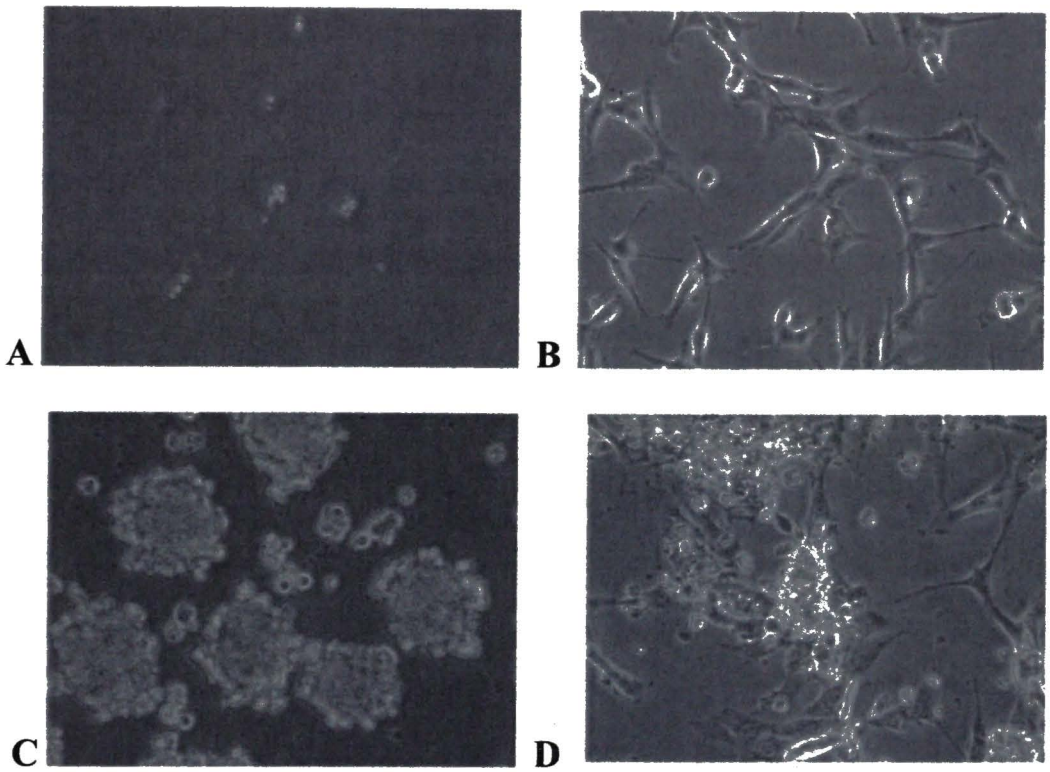


Figure 1. Effects of RPE-secreted Proteins on Cultured Rat Retinal Progenitor Cells.

A. Few of the rat retinal progenitor cells, here a passage 10, when grown in defined media lacking serum survived beyond 3 days in culture. The cells appeared round and had an irregular morphology.
B. Progenitor cells grown in 10% serum for 3 days developed thick processes. Some of these cells were round which is the morphology when initially plated.
C. Progenitor cells grown in RPE-secreted proteins proliferated and formed large clusters, resembling neurospheres in other culture systems. Few of these cells had processes that revealed their lack of morphological maturation.
D. The cells grown in RPE-secreted proteins were collected then grown in 10% serum. These cells developed processes, although many remained in clusters.

The floating neurospheres were collected and plated in serum-containing media. After 6 days, these cells attached and eventually formed processes and a differentiated morphology. The cells that were incubated in serum plus the secreted proteins, differentiated much faster than the cells that were previously cultured in the RPE-secreted proteins. The floating neurospheres remained unresponsive after three days in serum (Figure 2A, B). In contrast, after six days the neurospheres were well attached and slowly differentiating as shown (Figures 2C, D). Therefore, this shows that the secreted proteins caused the progenitor cells to remain in a dormant, non-differentiating state.

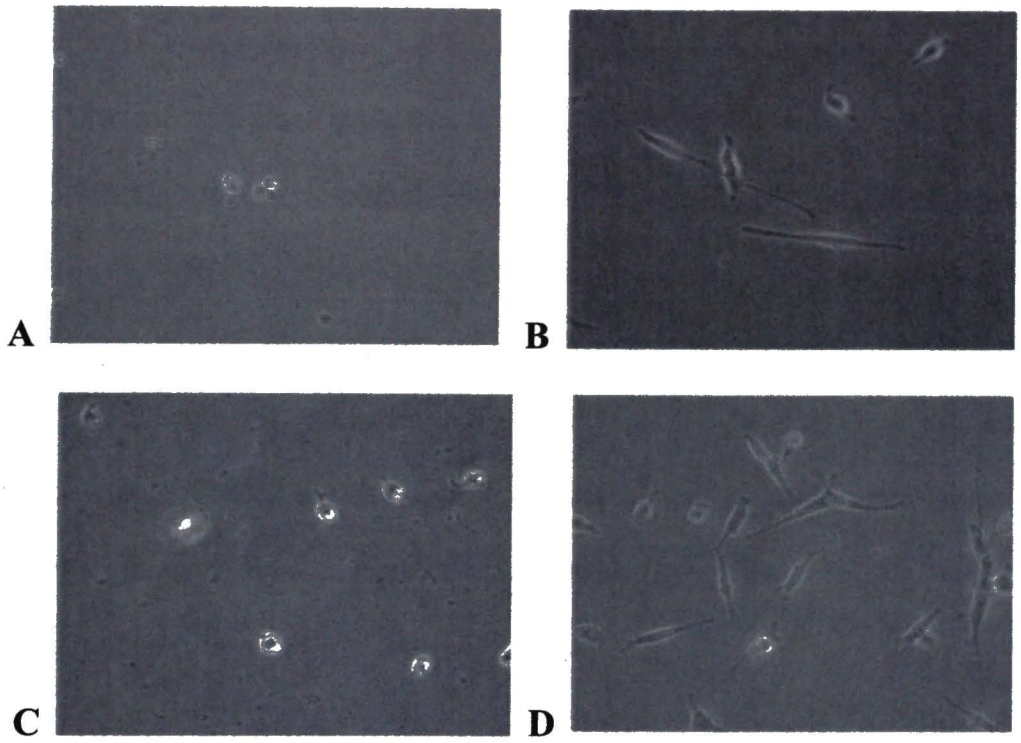


Figure 2. Effects of RPE-secreted Proteins on Cultured Rat Retinal Progenitor Cells.

A. Progenitor cells isolated from clusters developed when grown only in RPE-secreted proteins attached to the culture surface, here after 3 days. These cells were only cultured in RPE-secreted proteins.
B. After 6 days, the cells grown in conditions described in Figure 2A formed processes and appeared to undergo maturation as indicated by their bipolar morphology.
C. Floating clusters of RPE-secreted protein treated progenitor cells after 3 days incubation in serum attached to the culture surface and developed small processes.
D. After 6 days, the cells grown in the conditions described in Figure 2C appeared to proliferate and formed large, thick processes.

II. Response of Rat Retinal Progenitor Cells to Retinoic Acid

The results of the cell morphological changes after three days of this experiment are as follows. The cells cultured in ITS+ defined media were sporadically arranged on the plate surface with small processes (Figure 3A). In addition, these cells did not appear to proliferate. The progenitor cells incubated with serum containing media became confluent wells and morphologically appeared normal with differentiation and process formation (Figure 3B). In contrast, retinoic acid at $10^{-6}$ M caused the progenitor cells to become mostly confluent in the wells, but the morphology was clearly different than those cells incubated in serum. These cells formed processes, and they were clumped together in rosette-like formations across the surface (Figures 3C, D). The incubation time in this experiment was three days.

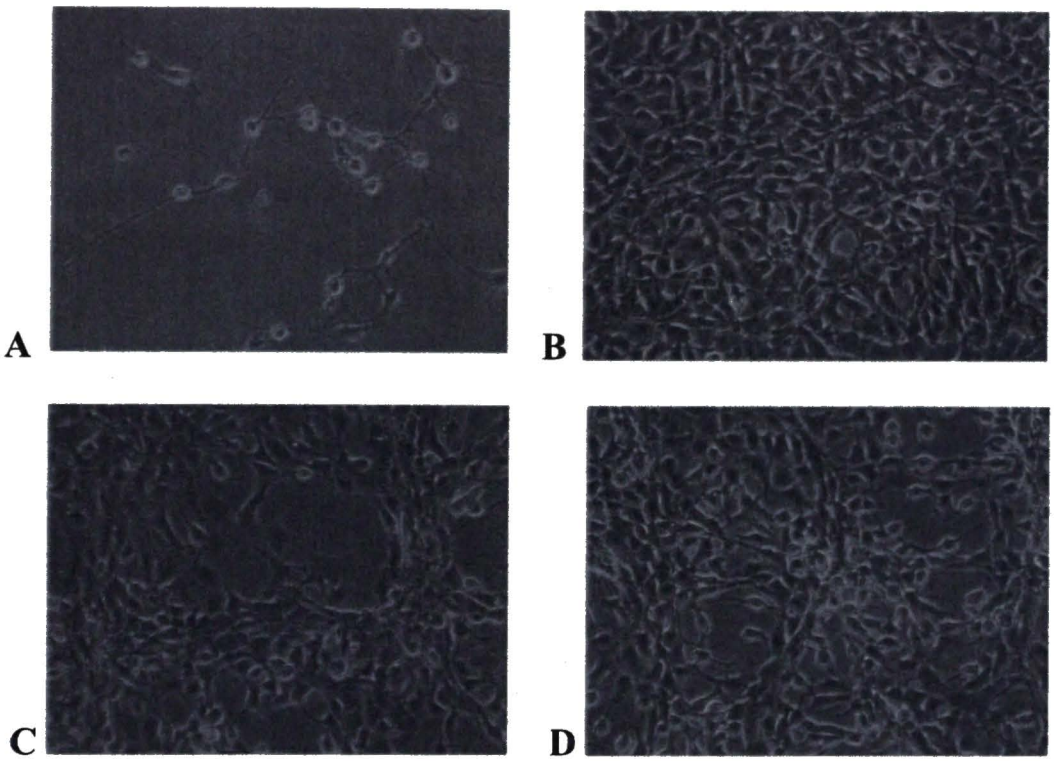


Figure 3. Effects of Retinoic Acid on Cultured Rat Retinal Progenitor Cells.

A. Rat retinal progenitor cells were cultured in $10^{-6}$ M retinoic acid for 2 days, and then grown in defined media for 3 days. These cells exhibited a neuronal morphology with thin processes that interconnected adjacent cells.
B. Rat retinal progenitor cells were cultured in $10^{-6}$ M retinoic acid for 2 days, and then grown in 10% serum for 3 days. These cells became confluent and exhibited few processes.

C, D. Rat retinal progenitor cells grown only in $10^{-6}$ retinoic acid demonstrated extensive proliferation. A few of these cells showed process formation.

III. Proliferation of Rat Retinal Progenitor Cells

The proliferation bioassays determine the proliferation rate of retinal progenitor cells grown in RPE-secreted proteins and retinoic acid. The growth factor bioassays were performed with one control (without cells), serum, defined media, RPE-secreted proteins, FGF-2, and retinoic acid. These experiments were repeated in triplicate to assure accuracy. After incubations, the controls were used as baseline numbers to compare with the experimental results. As shown in Figure 4, the cells incubated in serum proliferated, while cells incubated in defined media were less than this density after three days. The growth factors incubated with the progenitor cells stimulated significant proliferation. The RPE-secreted proteins caused a 12 fold increase in progenitor cell number, whereas serum promoted a 17-fold increase. Both FGF-2 and retinoic acid caused proliferation, 6 and 4 fold respectively, but this did not reach as high a level as those cells cultured in RPE-secreted proteins or serum.

A saturation curve for retinoic acid was performed. As shown in Figure 5, this procedure using retinoic acid caused an optimal cell proliferation (4-fold) when the cells were incubated in 10 microliters ($10^{-6}$ M) RA. Before this amount, the cells did proliferation but did not reach the highest level. After 10 microliters the bioassays showed that retinoic acid promoted significantly less proliferation of retinal progenitor cells.

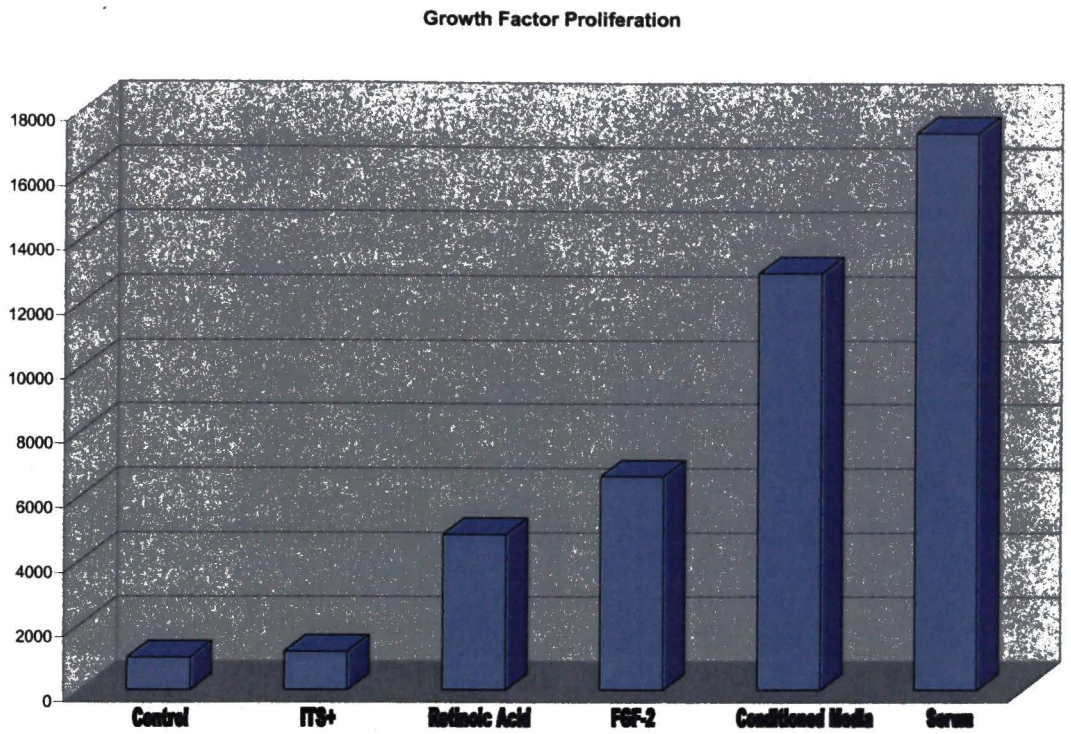


Figure 4. Proliferation Effects of Retinoic Acid, Basic Fibroblast Growth Factor, and RPE-secreted Proteins on Cultured Rat Retinal Progenitor Cells.

Proliferation bioassays revealed that rat retinal progenitor cells responded to RPE-secreted proteins by undergoing extensive proliferation, over 12-fold above plating density after only 3 days. Retinoic acid and FGF-2 also promoted a 4-fold and 6-fold, respectively, proliferation of the rat progenitor cells. Serum was the most effective vehicle that stimulated proliferation of these cells as evidenced by a 16-fold increase above plating density. These bioassay results supported the conclusions based on microscopic observations. These experiments were performed in quadruplicate.

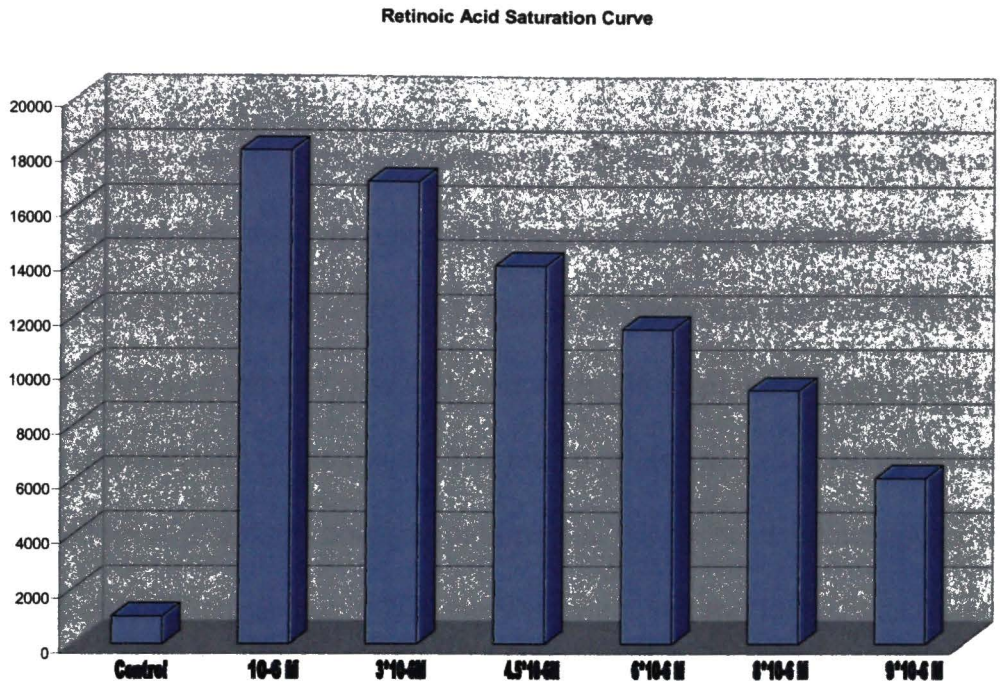


Figure 5. Effects of Retinoic Acid on Rat Retinal Progenitor Cells at Different Concentrations.

As evidenced by proliferation bioassays, the most effective concentration of retinoic acid was $10^{-6}$ M. Clearly, higher concentrations of retinoic acid, although stimulating proliferation, were less effective than the lower concentration. Future studies will be needed to determine if cell death is being promoted by the higher concentrations of retinoic acid.

## IV. Western Blot Analyses of Rat Retinal Progenitor Cells

Western blots were performed to determine whether rat retinal progenitor cells differentiated after incubation with RPE-secreted proteins and retinoic acid. For a loading control on the Western blots, β-actin antibody was used and all three cell types were positively stained (Figure 6B). Vimentin (58 kDa) was strongly positive in those cells incubated with retinoic acid. The retinal progenitor cells incubated with RPE-secreted proteins did test positive for vimentin, but the band did not appear as strong. Serum-cultured progenitor cells express vimentin at lower levels, which was confirmed in the immunocytochemistry experiments. This antibody was tested five times at protein amount of 50ug/ul per sample. Vimentin was added at a 1:500 dilution for primary incubation followed by a secondary antibody (goat anti-mouse hrp) at 1:10,000 dilution. The blot was read using DAB colormetric staining (Figure 7A). The membranes were stained for opsin at a 1:100 dilution. Opsin (36 kD) was detected in progenitor cells cultured in retinoic acid, RPE-secreted proteins, and serum which supported the immunocytochemistry data. The Western blot analyses were repeated five times each with light opsin staining, except for the control human retina which appeared darker. This result was due to the small amount of opsin protein being present in the progenitor cells and it is found within the cell membrane. (Figure 7B).

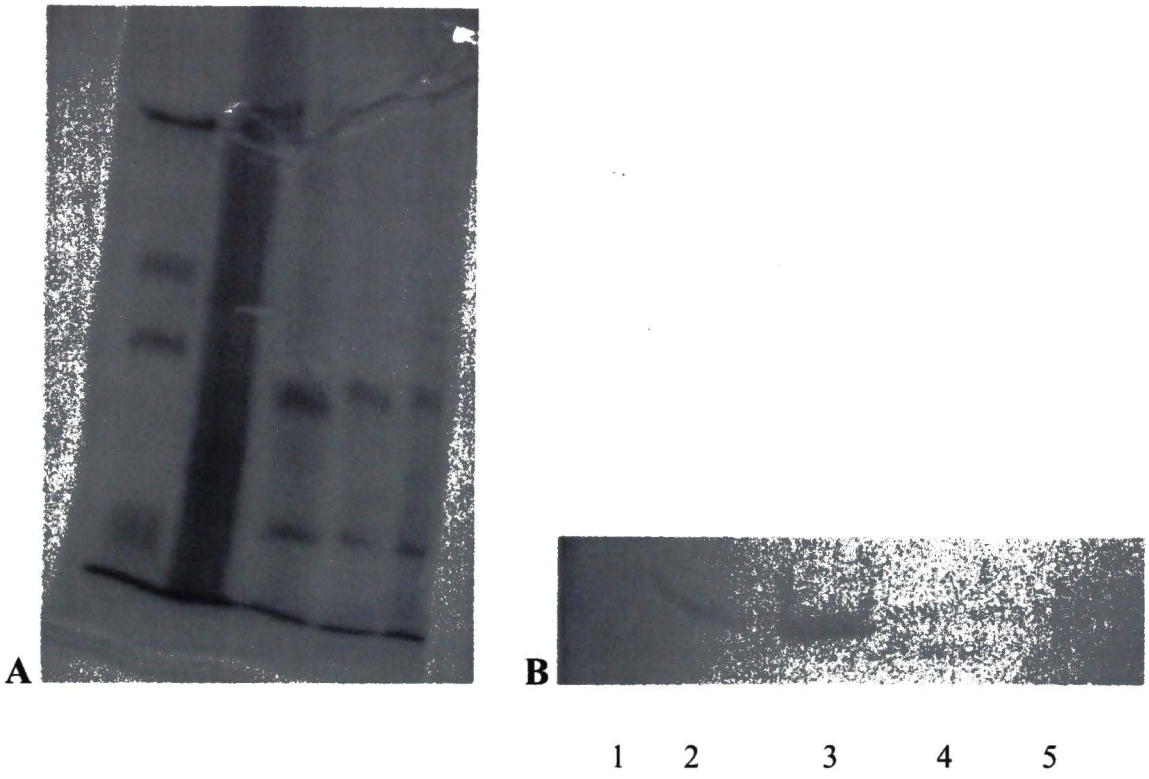


Figure 6. Polyacrylamide Gel Electrophoresis of Total Proteins of Rat Retinal Progenitor cells Grown in RPE-secreted Proteins and Retinoic Acid.

A. Total proteins of rat retinal progenitor cells were separated in 8% acrylamide gels under denaturing conditions. Lane 1: protein standards, Lane 2: rat progenitor cells incubated in serum, Lane 3: rat progenitor cells incubated in RPE-secreted proteins, Lane 4: rat progenitor cells incubated in retinoic acid, Lane 5: RPE-secreted proteins.
B. The proteins were transferred to nitrocellulose and probed with antibodies to β-actin to determine the loading levels. The lanes are the same as indicated in 6A above.


Figure 7. Western Blot Analysis of Rat Retinal Progenitor Cells Treated with RPE-secreted Proteins and Retinoic Acid.

A. Homogenates of cells treated with 10-6 M retinoic acid (lane 4) showed a denser immunolabeled band for vimentin than homogenates of either RPE-secreted protein (lane 3) or serum (lane 2) treated cells. Lane 1: protein standards.
B. Homogenates of cells treated with 10-6 M retinoic acid (lane 4) showed a lightly immunolabeled band for opsin. The adult human retina (lane 2) displayed the most intense immunolabeling for opsin.

## V. Immunocytochemical Analysis of Rat Retinal Progenitor Cells

Immunocytochemistry was performed to determine the response of rat retinal progenitor cells to RPE-secreted proteins and retinoic acid. Three different factors and media were used when culturing the progenitor cells prior to treatment using fluorescence. Cells were incubated in RPE-secreted proteins, retinoic acid, or serum. The incubation period for these cells was three days.

After performing immunocytochemistry similar immunolabeling results for each of the three factors were observed. Cells incubated in serum (control cells) were not only morphologically differentiated, but also expressed mature cell markers. The serum-cells were positive for both vimentin (55%), and opsin (70%). Figure 8C shows the progenitor cells contain vimentin and Figure 8D shows that opsin is also present. Although vimentin is also found in immature cells, opsin is only found in differentiated photoreceptor cells.

The retinal progenitor cells used in these experiments were the cells that were incubated in either RPE-secreted proteins or retinoic acid. About half of the cells influenced by RPE-secreted proteins were morphologically differentiated. This was revealed by these cells having processes, while other cells were in neurospheres. The RPE-secreted proteins appear to influence the progenitor cells to become mature. Vimentin and opsin appeared when these cells were immunocytochemically evaluated as shown (Figures 9C, D). Morphologically this observation demonstrated that although some progenitor cells

appeared to be immature, the RPE-secreted proteins influenced most of the progenitor cells to differentiate.

The progenitor cells incubated in retinoic acid also differentiated morphologically and became mature. Opsin was shown in most of the cells while vimentin was represented in 72% of the cells. This data is represented in Figures 10 C, D.

Immunostaining for a transcription factor, Pax-6, provided further proof of the immaturity of progenitor cells. Pax-6 was found to densely immunostain progenitor cells incubated in serum, RPE-secreted proteins, and retinoic acid (Figures 11 A, B, C). The percentage of cells that expressed each of the markers when cultured in serum, RPE-secreted proteins, and retinoic acid is listed in Figure 12.

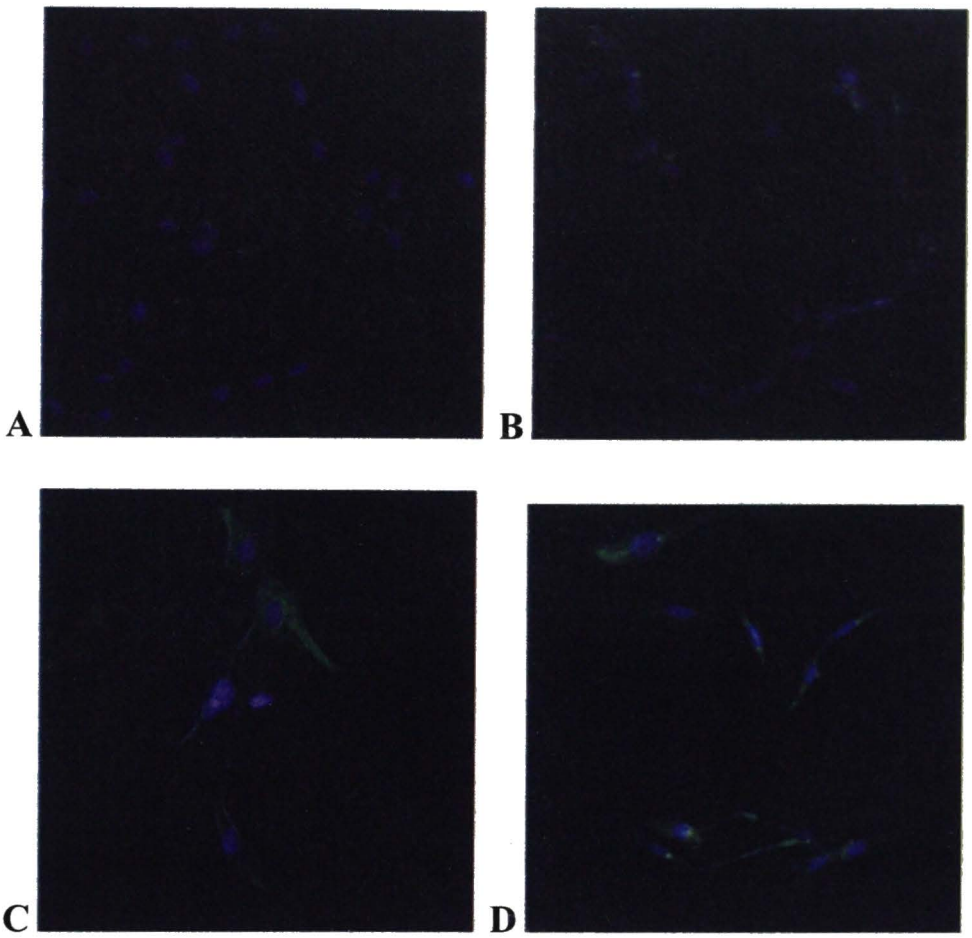


Figure 8. Immunocytochemical Analysis of Rat Retinal Progenitor Cells following Treatment with Serum.

A. For one immunocytochemical control, progenitor cells grown for 3 days in serum were treated with diluent, without the primary antibody. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
B. For a second immunocytochemical control, progenitor cells grown for 3 days in serum were treated with diluent, with an IgG. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
C. Rat progenitor cells treated with serum for 3 days showed dense vimentin immunolabeling within the cytoplasm. Note that the cells that have processes show the densest immunoreactivity.
D. Rat progenitor cells treated with serum for 3 days showed light opsin immunolabeling, a marker for rod photoreceptor cells.

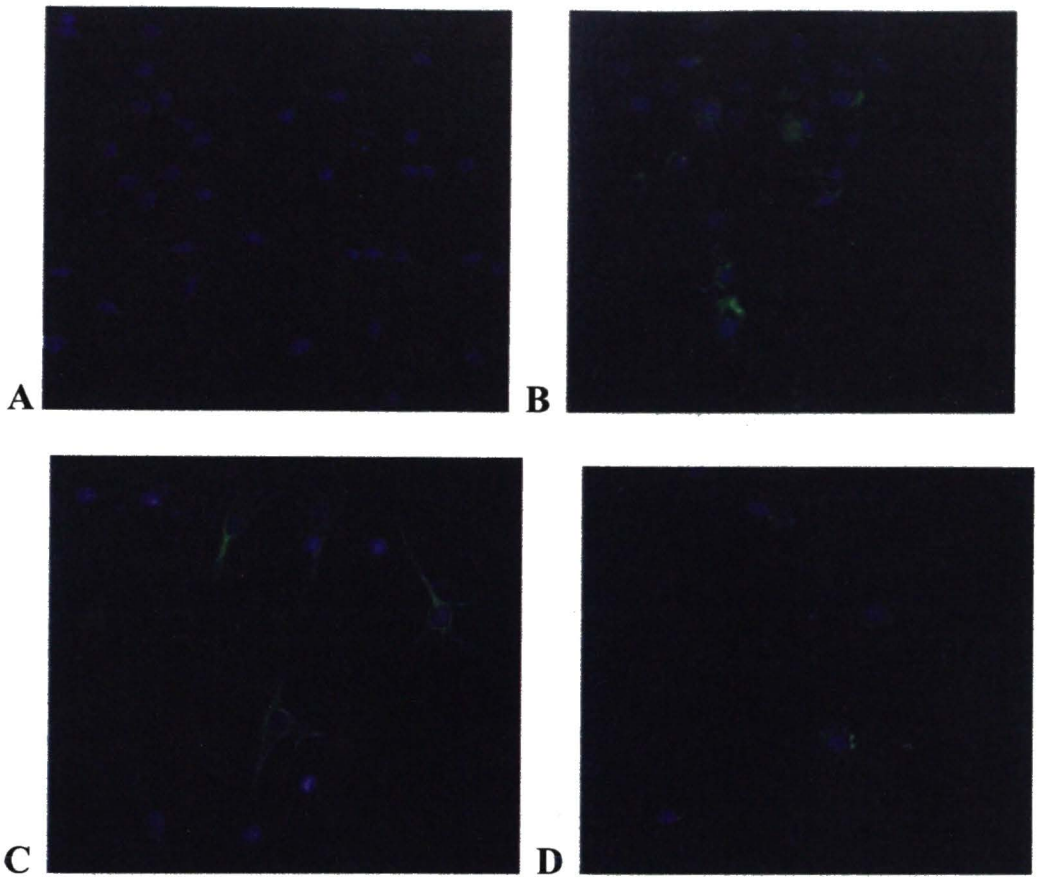


Figure 9. Immunocytochemical Analysis of Rat Retinal Progenitor Cells following Treatment with RPE-secreted Proteins.

A. For one immunocytochemical control, progenitor cells grown for 3 days in RPE-secreted proteins were treated with diluent, without the primary antibody. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
B. For a second immunocytochemical control, progenitor cells grown for 3 days in RPE-secreted proteins were treated with diluent, with an IgG. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
C. Rat progenitor cells treated with RPE-secreted proteins for 3 days showed dense vimentin immunolabeling within the cytoplasm. Note that the cells that have processes show the densest immunoreactivity.
D. Rat progenitor cells treated with RPE-secreted proteins for 3 days showed light opsin immunolabeling, a marker for rod photoreceptor cells.

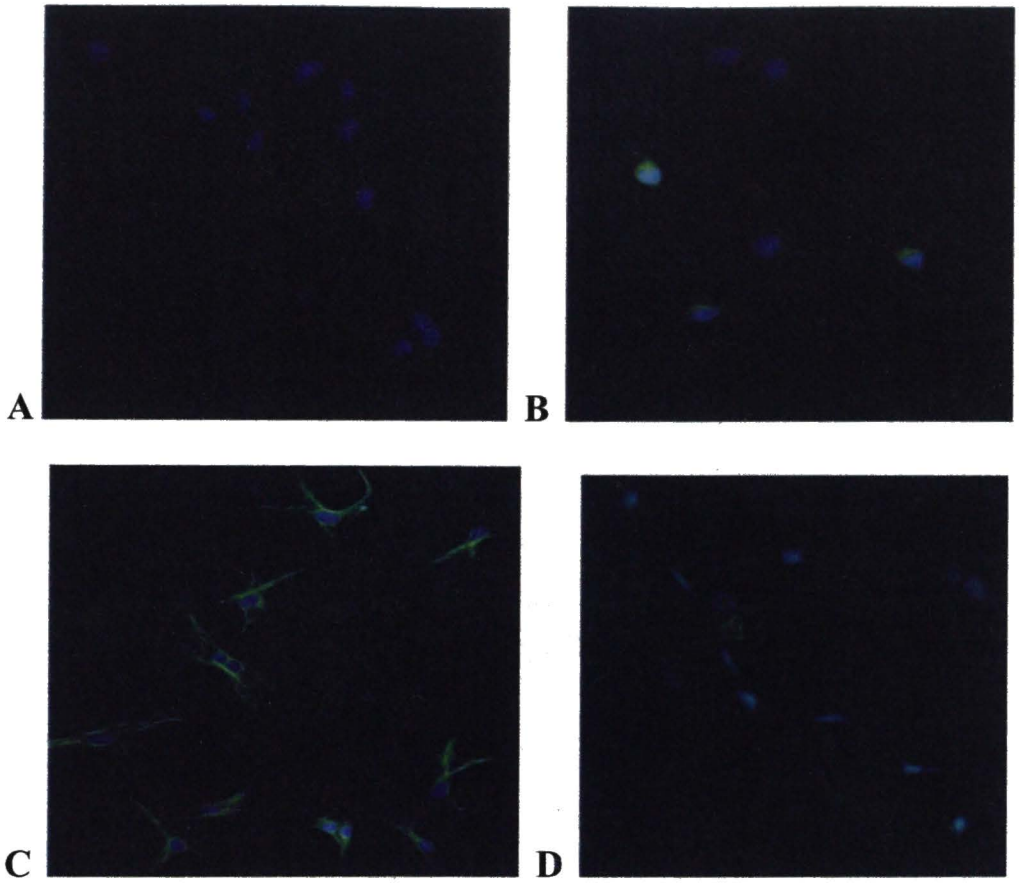


Figure 10. Immunocytochemical Analysis of Rat Retinal Progenitor Cells following Treatment with Retinoic Acid.

A. For one immunocytochemical control, progenitor cells grown for 3 days in retinoic acid were treated with diluent, without the primary antibody. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
B. For a second immunocytochemical control, progenitor cells grown for 3 days in $10^{-6}$ M retinoic acid were treated with diluent, with an IgG. All cells in these experiments were treated with DAPI to localize the nuclei. Note in this control, no specific immunolabeling was detected.
C. Rat progenitor cells treated with $10^{-6}$ M retinoic acid for 3 days showed dense vimentin immunolabeling within the cytoplasm. Note that the cells that have processes show the densest immunolabeling.
D. Rat progenitor cells treated with $10^{-6}$ retinoic acid for 3 days showed light opsin immunolabeling, a marker for rod photoreceptor cells.

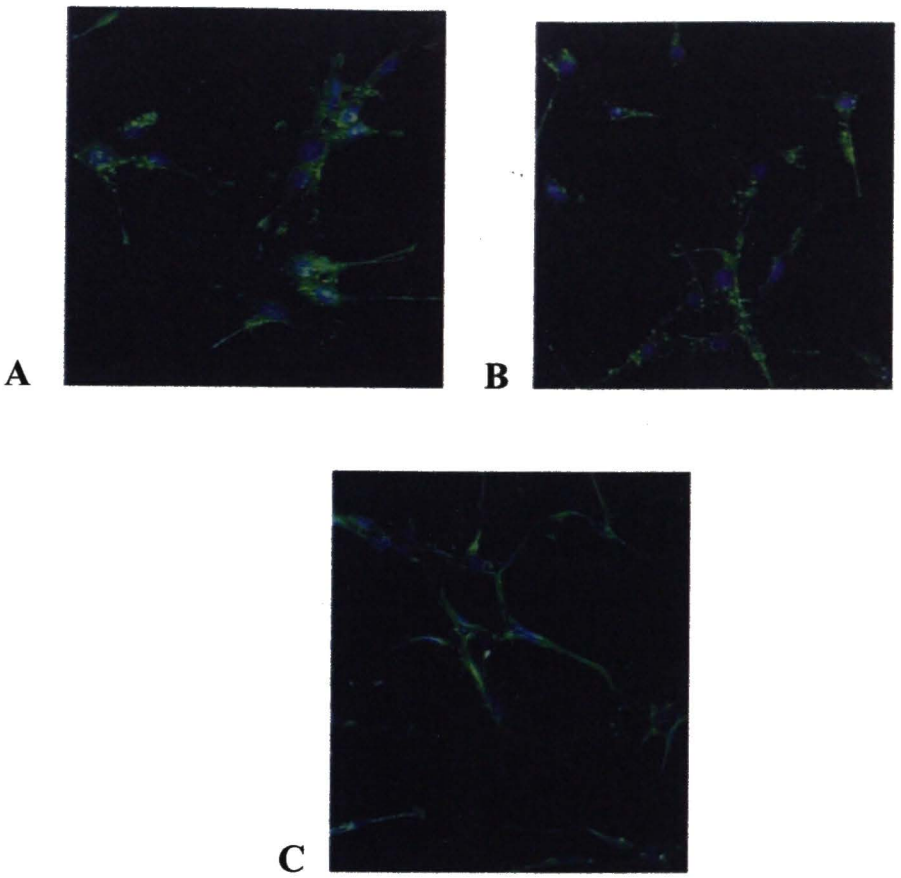


Figure 11. Pax-6 Transcription Factor Expression by Rat Retinal Progenitor Cells shown by Immunofluorescence.

A. Rat progenitor cells treated with serum for 3 days showed dense Pax-6 immunoreactivity.
B. Rat progenitor cells treated with RPE-secreted proteins for 3 days showed dense Pax-6 immunolabeling.
C. Rat progenitor cells treated with $10^{-6}$ M retinoic acid for 3 days showed dense Pax-6 immunolabeling.

Figure 12. Opsin, Vimentin, and Pax-6 Expressing Rat Retinal Progenitor Cells Following Treatment with Retinoic Acid, RPE-secreted Proteins, and Serum.

| **Treatment** | **Opsin** | **Vimentin** | **Pax-6** |
|---|---|---|---|
| Serum | 70% | 55% | 100% |
| Retinoic acid | 80% | 72% | 99% |
| RPE-secreted Proteins | 87% | 39% | 96% |

The numbers in each condition was determined by dividing the total number of cells by the number of cells that expressed that cell marker. Retinoic acid at $10^{-6}$ M appeared to be most effective in promoting expression of opsin and vimentin by rat retinal progenitor cells. Pax-6 transcription factor was expressed by most of the cells under each of the 3 conditions.

These results show that progenitor cells cultured in normal serum possess some of the characteristics of mature, differentiated photoreceptor cells. This was shown by the cells expressing opsin. The progenitor cell cultures incubated in retinoic acid and those incubated in RPE-secreted proteins also showed differentiated cells, although there were still undifferentiated cells present. This was shown by the process bearing cells containing opsin and vimetin, whereas the neurospheres in these cultures did not possess the opsin characteristic. Therefore, neuropspheres in culture are undifferentiated and can be manipulated into any cell type following treatment with serum.

# CHAPTER 4

## DISCUSSION AND CONCLUSION

Photoreceptors are terminally differentiated neuronal cells, hence once they are lost, and they cannot be replaced. Apoptosis of photoreceptor cells is one of the key phenotypic manifestations of retinitis pigmentosa and age-related macular degeneration. Thus, it is evident that the prospects of curing blindness in ARMD and RP patients are cloudy but may eventually encompass transplantation therapy. There are several studies on retinal progenitor cells, but additional research needs to be performed for the future of ocular cell biology and therapy. These studies show that retinoic acid and FGF-2, which is secreted by RPE cells, stimulate cell proliferation. These experiments have determined that progenitor cells incubated in these factors affect a proliferation response. Proliferation is important to transplantation therapy since retinal neurons in large numbers are necessary for therapy in ARMD and RP patients. After careful manipulation, these cells should thrive to cover the posterior of the eye with new, healthy retinal cells. This procedure may require few donors to supply many blind individuals with renewed sight.

The RPE-secreted proteins, FGF-2, and retinoic acid promote proliferation of these retinal progenitor cells. Retinoic acid is a well studied differentiation factor for many cell types although, studies have shown that it could also promote cell death in other cell types. This study showed that low levels of retinoic acid elicit a proliferative response in retinal progenitor cells. The RPE-secreted proteins act similar to FGF-2 and promote progenitor cell proliferation.

In addition, the progenitor cells incubated in either RPE-secreted proteins or retinoic acid showed expression of proteins characteristic of cellular differentiation into photoreceptors. As stated previously, FGF-2 is secreted by RPE cells that promote cell division, without morphological differentiation, of rat progenitor cells. Retinoic acid was shown to cause cell proliferation at $10^{-6}$ molar concentration. The ability of these factors to promote cellular differentiation is vital for future ocular research. If progenitor cells can be directed to differentiate by factors, these cells can be the source of precursor cells for retinal transplantation.

Current Trials of Transplantation Therapy

In recent years due to increased longevity, there have been an increase number of age-related macular degeneration cases in the United States, and this trend is likely to continue in the future. In addition, since many forms of retinitis pigmentosa show effects in young, there is a pressing need to find a cure so that children do not go through life in darkness. One of the most investigated treatment modalities for these diseases is cellular

transplantation therapy. Transplantation therapy would allow new retinal cells, derived from progenitor cells, to be transplanted into the eye of a blind individual. The hope is that the transplanted cells will survive and permit sight in patients who have non-functioning photoreceptor cells. Today, numerous transplantation therapies have been attempted but, to date, all have been unsuccessful in restoration of sight. There are three main reasons why retinal transplantation therapies have not been successful. One reason for unsuccessful retinal transplantation is the cell delivery system. This is especially a problem in transplants to the sub retinal space in the eye. The sub retinal space is the preferred position for grafts that can potentially cure retinal disease. Cells can be transplanted as sheets and as a cell suspension into the retina by way of a syringe. Both delivery methods transplant progenitor cells into the diseased retina. Another obstacle in retinal transplantation is the limited survival of grafted progenitor cells. The progenitor cells cannot survive in the new host alone without stimulation by growth factors or other nutrition molecules from the host or before they are transplanted. If the cells were incubated with growth factors before transplantation, the survival rate would more than likely increase as transplanted progenitor cell grafts. The last main problem of cell transplantation into the retina is the control over differentiation of the implanted or grafted progenitor cells. Ideally, cells should differentiate prior to transplantation, rather than proliferate and differentiate as transplants in the host retina (Young 2005). Several groups have attempted cell transplantation into the retina. Some examples of these past transplantation therapies are as follows.

One experiment completed by Aoki and coworkers (2006) demonstrated that the loss and injury of RPE cells is the reason for some ocular disorders. This group used embryonic stem (ES) cells that eventually differentiated into RPE cell precursors and embedded them into the retina of ARMD patients. The reason for using ES cells was because of the presence of Pax-6 in there cells. Therefore, it was shown that the ES differentiated into RPE-like cells which then formed eye-like structures in both a chick and a rat. Although this transplantation therapy does prove useful in other vertebrates, humans have variations in signaling pathways for photoreceptors versus the RPE cells and therefore sight restoration may not occur with this type of transplantation therapy. In addition, transplanted RPE cells tend to attach to each other in the sub retinal space rather than incorporating themselves into the host as a monolayer.

Another experiment by Aisenbrey and coworkers (2006) attempted implanting iris pigment epithelial cells into the retina. Their report states that autologous iris pigment epitheial (IPE) cells may be able to replace RPE cells following sub retinal transplantation. In this study, IPE cells were transplanted into the sub retinal space of rabbits. The IPE cells formed a monolayer on top of the original RPE and phagocytized photoreceptor outer segments. In addition, the IPE cells formed connections with the outer segments of photoreceptors and were not rejected by the host rabbit after 20 days. This transplantation therapy may maintain sight because IPE cells would provide nutritional support for photoreceptors, but not restore sight.

Warfvinge and coworkers (2005) developed a transplantation therapy in pigs through retinal progenitor cells xenografts. Xenografts are transplants of cells or tissues between two different species, in this case mouse to pig. The coworkers were convinced that retinal progenitor cell xenografts survived for a sufficiently long period and integrated into areas of injury and showed morphologic differentiation. In these experiments, mouse retinal cells were transplanted subretinally as solitary cells or neurospheres into nonimmunosuppressed adult pigs. The cells survived for up to 14 days after transplantation. The use of pigs is an excellent model system for the future of transplantation therapy because the pig vitreous cavity is much larger than that of rodents, which allows for a much more detailed functional analysis. This study did run into the problem of rejection because the mouse and pig species are distantly related. However, the experiments did demonstrate that progenitor cells integrated in the host neural retina and therefore could possibly replace the diseased cells if of the same species as the host.

The most promising experimentation with transplantation therapy is the work of Liljekvist-Larsson and coworkers (2005). This group used retinal neurospheres in their retinal transplantation therapy. Previous work showed that grafted neurospheres assimilate into adult retinal tissue. Numerous growth factors were used to expand the neurospheres *in vitro*, including basic fibroblast growth factor (FGF-2), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF). After their rat progenitor cells were incubated and formed neurospheres, they were implanted into the retinal tissue of adult rats. This study showed that treated cells were not rejected.

The purpose of this study was to determine whether the rat progenitor cells cultured in retinoic acid or RPE secreted proteins can be used in the future for retinal transplantation in diseased retinas. RPE secreted proteins caused cell clustering comparable to neurospheres, which has been reported for FGF-2. In the future, the progenitor cells that are incubated with RPE secreted proteins could be transplanted into adult diseased retinas after an appropriate incubation period. The cells would have already been stimulated by these factors and, thus, would be proliferating and differentiating prior to harvesting for transplantation. Thus, the progenitor cells would be committed to forming differentiated cells and would then be able to survive in the host retina. Progenitor cells incubated in retinoic acid also formed clusters together with process-bearing cells. These cells, it is hypothesized, will attach in the host retina and survive and function normally. The future of this technique in transplantation would be to stimulate the progenitor cells with retinoic acid and then harvest them. The harvested differentiated cells would then be implanted into the host retinas to take the place of diseased photoreceptors. Retinoic acid along with RPE secreted proteins have been shown *in vitro* to cause progenitor cells to proliferate and differentiate, thus these cells bear the characteristics of mature photoreceptors. It is hypothesized that transplantation of these stimulated progenitor cells would allow replacement of cells in diseased retinas and pave the way for development of stem cell therapies of neurodegenerative diseases. For example, since retinal progenitor cells are comparable to neural progenitor cells the success of retinal transplantation could promote development of similar approaches for the treatment of other neural disorders such as Alzheimer's and spinal cord injury.

There are other studies underway that study the effects of retinoic acid and RPE-secreted proteins, mainly FGF-2, on neuronal cells. For example, Wang and coworkers (2005) found that retinoic acid receptors permit efficient regeneration in the adult rat spinal cord. The authors determined if adult spinal cord cells could regenerate if exposed to the same factors that stimulate embryonic spinal cord cells during embryogenesis. The results of the study showed that regenerating fibers entered the spinal cord after upregulation of the retinoic acid receptor. They proposed that spontaneous spinal cord regeneration after injury was due to the formation of new intraspinal circuits and local axonal sprouting.

Ikeda and coworkers (2005) transplanted neural cells that were pre-incubated with retinoic acid into hemiplegic mice. This study showed regeneration in the mice who suffered a stroke-like brain injury. In addition, it was proven that retinoic acid induced differentiation of transplantable cells. Both differentiation and proliferation occurred after treatment with retinoic acid, which is necessary for viability of transplantable cells after they injected in the diseased animal. With studies such as these being performed throughout the world, the use of progenitor cells is seen as an important component in the future of clinical medicine.

In conclusion, developing methods to differentiate retinal and neuronal cells from their progenitor cells can lead to therapies for both ocular and neurodegenerative disorders. These cell types can be used to replenish specific neuronal subpopulations that are lost in neurodegenerative diseases. In conclusion, this research using retinal progenitor cells

will assist toward developing a method of treatment for retinal degenerations, for which there is currently no cure.

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