Proteins affecting rod photoreceptor differentiation in the murine retina: a proteomics approach with emphasis on mTOR

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Proteins affecting rod photoreceptor differentiation in the murine retina: A proteomics approach with emphasis on mTOR

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience

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Iowa State University
Ames, Iowa
2008

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ABSTRACT

Retinal diseases involving degeneration of photoreceptors are an increasing cause of blindness in this country, particularly among the aging population. A solid understanding of retinal development is central to combating photoreceptor degeneration on several fronts. Defining developmental processes may have therapeutic relevance in ‘rehabilitating’ photoreceptor cells that can be rescued from degenerative processes by the application of exogenous trophic or survival factors. Further, advances in stem cell research may someday make replacement of photoreceptors a feasible therapy for the treatment of retinal degeneration. In this regard, it will be crucial that we have a clear understanding of the developing retinal environment, and the combination of intrinsic signals and extrinsic factors that influence retinal cells to adopt a photoreceptor cell fate.

The purpose of this study is to identify molecules important to rod genesis and differentiation. As a first step, we used 2 dimensional gel protein expression to identify proteins that are dynamically expressed or modified during rod genesis.

Further we tested the role of mammalian target of rapamycin (mTOR) during retinal development particularly during the ages of rod genesis and differentiation. mTOR is involved in cell growth. We found that mTOR was expressed throughout retinal development and although was found in rod photoreceptors did not appear to be specific to them. We also found that when retinal explants were exposed to rapamycin, an mTOR inhibitor, for 10 days, rod differentiation decreased as assayed by rhodopsin expression. This indicates a modulatory role of mTOR on rod photoreceptor differentiation.
CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Retinal diseases involving degeneration of photoreceptors are an increasing cause of blindness in this country, particularly among the aging population. A solid understanding of retinal development is central to combating retinal disease. Defining developmental processes may have therapeutic relevance in ‘rehabilitating’ retinal cells that can be rescued from degenerative processes by the application of exogenous trophic or survival factors.

The goal of this dissertation is to begin to understand the factors involved in rod photoreceptor differentiation. We approached this goal using two different methods. The first was a holistic proteomics method to identify proteins present at developmental ages important to rod photoreceptor genesis and differentiation. We utilized ages E17, prior to the peak of rod genesis, P0, the peak of rod genesis and P5, when rods have become irreversibly fated to become rods.

The second approach was a more specific one, characterizing a particular protein, mTOR, and its role in rod photoreceptor differentiation. We characterized mTOR in developing retinal tissues using immunohistochemistry and found that it is expressed throughout retinal development. It is found in photoreceptors but is not specific to them. We also examined the two main binding partners of mTOR, raptor and rictor. They are also expressed throughout retinal development. To determine the functional role of mTOR we applied the mTOR inhibitor, rapamycin to E17 retinal explant cultures. The
result was a decrease in rod photoreceptor differentiation suggesting a modulatory role of mTOR in rod differentiation.

1.2 Dissertation Organization

This dissertation is organized into four chapters. The first chapter is a general introduction followed by a review of the literature. The second chapter is a manuscript characterizing the retinal proteome during rod photoreceptor genesis prepared for submission to Investigative Ophthalmology and Visual Science. Chapter three is a manuscript characterizing the effect of the molecule, mTOR, on rod photoreceptor differentiation prepared for submission to Vision Research. Finally, chapter four is a summary and discussion of the results with concluding remarks and comments for future directions.

1.3 Literature Review

1.3.1 Structure and cell types of the retina

The retina has long been recognized as being a good structure to use in the study of the central nervous system. This is largely due to its ease of accessibility and distinct layered organization containing specific cell types (Ramón y Cajal 1892). The mature retina is composed of discrete layers: 3 nuclear layers and 2 synaptic layers (Dowling 1987). These layers are the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL) and the ganglion cell layer (GCL).
There are 7 distinct cell types in the mature retina and they are all originally derived from a common progenitor pool (Turner and Cepko 1987; Turner et al. 1990). Rod photoreceptors are the low light sensing cell in the retina and have their cell bodies in the outer nuclear layer. Rod photoreceptors comprise about 70% of the cells in the adult mammalian retina and make up 97% of the photoreceptors (Jeon et al. 1998). Cone photoreceptors are necessary for detecting color as they are sensitive to different wavelengths of light and are also found in the outer nuclear layer. Photoreceptors transduce light to neural impulses. Bipolar cells transmit the neural impulse from the photoreceptors to the ganglion cells either directly or indirectly via intermediate amacrine cells. There are both rod and cone specific bipolar cells (Dowling 1987; Dowling and Boycott 1966). Bipolar cells are found in the inner nuclear layer. The outer plexiform layer contains the synaptic connections between the photoreceptors and their respective bipolar cells as well as the horizontal cells. Cone bipolar cells have a direct path whereas rod bipolar cells transmit the neural impulse indirectly via AII amacrine cells. This is an intermediate cell that communicates between the rod bipolar cell and the ganglion cell. There are multiple types of amacrine cells, which are distinguished based on Golgi staining and morphology. They do not directly synapse with one another. Amacrine cells and horizontal cells are found in the inner nuclear layer and mediate lateral interactions (Dowling 1987). The inner plexiform layer has the synaptic connections between the bipolar cells, amacrine cells and ganglion cells (Dowling 1987; Dowling and Boycott 1965). Bipolar cells and amacrine cells can give direct input to ganglion cells (Dowling 1987). Ganglion cells in the ganglion cell layer convey the neural impulse through their axons via the optic nerve. Müller glia play a supportive role and span the retinal layers
from the outer limiting membrane to the inner limiting membrane, of which is mostly made up of the Müller glia end feet and astrocytes.

At early embryonic ages there is little to no lamination, or layering, in the retina (Pei and Rhodin 1970). Structural layering occurs as the embryo develops. Retinal development occurs in a central to peripheral manner. In the mouse, cell division, one aspect of development, ceases centrally around postnatal day 5-6 but not until P11 in the periphery of the retina (Young 1985a). Central to peripheral maturation however, is not limited to the mouse and exists in all other vertebrate species (Holt et al. 1988). Complete retinal maturation occurs in the mouse at around six weeks of age where all cell types and layers are present (Young 1985a). Eye opening occurs around 2 weeks of age.

1.3.2 Cell Fate Determination

Cells in the retina are born in a determined and conserved order (Young 1985a). In most cases the cell types born at a given time overlap but there is a definite order followed (Altshuler 1991; Cepko et al. 1996). Retinal progenitor cells pass through a characteristic set of “competency states” during which extrinsic cues can influence what type of retinal neurons the progenitor cells will become (reviewed in Livesey and Cepko 2001a). Each competency state is characterized by the limited subset of retinal neurons that can be produced at that particular time during development. For example, progenitor cells from very early stages of retinal development give rise to ganglion cells or cone photoreceptors, but are not intrinsically competent to give rise to rod photoreceptors (Cepko et al. 1996). Thus, retinal development is a complicated process with different
intrinsic and extrinsic factors thought to influence cell fate decisions of retinal progenitor cells at different times during the process (Belliveau and Cepko 1999; Cepko et al. 1996; Holt et al. 1988; Livesey and Cepko 2001a; Reh 1992; Turner et al. 1990).

1.3.3 Factors Influencing Retinal Cell Fate

Many factors have been found to affect cell fate determination, they can be thought of as either intrinsic or extrinsic. Intrinsic factors are inherent or endogenous to the cell such as transcription factors. Extrinsic factors are exogenous to the cell/system. Potential factors can be stimulatory or inhibitory in regards to a particular cell type. However, it is still unclear how these factors interact with each other and at what ages each is most influential (Reviewed in Levine et al. 2000). One study found that co-culturing rat embryonic retinal progenitors and postnatal retinal cells affected the cell fates of the differentiating progenitors. They concluded that there are two signals in the postnatal retinal environment that affected the determination of retinal progenitor cell fate. One of these signals increased cone cell differentiation as evidenced by an increase in cells positive for cone opsins and the other inhibited amacrine cell differentiation (Belliveau and Cepko 1999).

1.3.4 Rod Photoreceptor Development

The majority of cells produced postnatally are rod photoreceptors and the most definitive marker for differentiated rods is rhodopsin, the molecule that absorbs light and initiates phototransduction (Cepko et al. 1996; Young 1985a). The earliest marker for rods is neural retina leucine zipper (Nrl) (Farjo et al. 1993; Swain et al. 2001; Swaroop et
Nrl is a leucine zipper transcription factor (Swain et al. 2001; Swaroop et al. 1992). In the retina it is exclusively expressed in rods and is needed for rod photoreceptor development (Farjo et al. 1993; Mears et al. 2001; Swain et al. 2001; Swaroop et al. 1992). In the Nrl knockout mouse, there is a complete loss of rod differentiation, no detectable rod function and all the photoreceptors have cone like characteristics (Mears et al. 2001). Nrl induces rod promoter activity alone and in combination with cone rod homeobox protein (Crx) (Mitton et al. 2000). In the mouse, rods are at their peak of genesis at birth, P0. They begin to express rhodopsin at P3 to P5 although the time from cell genesis to rhodopsin expression varies. As noted previously, the birth order of retinal cell types (“birth” being the time at which retinal progenitor cells undergo their terminal mitotic division) is well established. However, what is still not known is when and how they make an irreversible cell fate decision. For rods, in the developing rat retina, it seems to be in the first postnatal week coinciding with the time that they start expressing rhodopsin based on studies that were able to alter cell fate determination (Ezzeddine et al. 1997). In one particular study, addition of CNTF to rat retinal explants caused a decrease in the number of differentiated rods but once the rod photoreceptors expressed rhodopsin their fate determination could not be altered by the addition of CNTF (Ezzeddine et al. 1997). It is important to better understand how intrinsic mechanisms and extrinsic factors drive a cell to its fate. This motivates our efforts to identify proteins that peak during rod photoreceptor genesis and/or differentiation based on the hypothesis that proteins important for rod differentiation will be dynamically expressed after genesis of presumed rod photoreceptors.
1.3.5 Extrinsic Factors

Many extrinsic factors have been investigated for their effect on retinal development. These include fibroblast growth factors, Sonic hedgehog, retinoic acid, taurine, 3-isobutyl-1-methylxanthine, brain derived neurotrophic factor, leukemia inhibitory factor and ciliary neurotrophic factor. Basic fibroblast growth factor (bFGF or FGF2) is a member of the fibroblast growth factors. When bFGF is included in cell culture media it is thought to keep cells in an undifferentiated state. FGF1 and FGF2 were some of the first identified factors shown to affect rod photoreceptor differentiation. These factors stimulated rhodopsin expression in rat retinal cells cultured in vitro and increased the number of cells positive for rhodopsin (Hicks and Courtois 1988; Hicks and Courtois 1992). Additionally, FGF was found to cause a proliferation in both rat monolayer cell cultures and retinal explants with progenitor cells from younger ages being more responsive to FGF (Lillien and Cepko 1992).

Similarly, Sonic hedgehog (Shh) was found to have a positive effect on rod differentiation. When added to rat retinal cell cultures Shh resulted in an increase in the number of differentiated rods (Levine et al. 1997). A recently published study showed a similar effect when Shh was added to purified mouse embryonic stem cells positive for retinal homeobox gene and cultured in retinal culture medium (Osakada et al. 2008). This condition promoted rod photoreceptor differentiation but an even greater effect was seen with the combination of acidic fibroblast growth factor, basic fibroblast growth factor, Shh and retinoic acid (Osakada et al. 2008). Retinoic acid (RA) is another extrinsic factor that positively affects rod photoreceptor development. Like Shh, when RA was added to dissociated rat retinal cells, the number of differentiated rods increased.
(Kelley et al. 1994). Recently, another group demonstrated that RA affects rod
development by regulating Nrl (Khanna et al. 2006). They found that Nrl protein
increased in cultured rat and porcine photoreceptors as well as in Y79 cells, a human
retinoblastoma cell (Khanna et al. 2006). In a separate study, RA added to prenatal and
perinatal rat retinal cell cultures, did not have an effect on rod numbers but it did speed
up the rate of rhodopsin expression in explants and cultures of rat embryonic retinas
(Wallace and Jensen 1999). Two other extrinsic factors tested in this same study were
found to accelerate rhodopsin expression were taurine and 3-isobutyl-1-methylxanthine
(IBMX) (Wallace and Jensen 1999). All three of these factors were postulated to have an
effect on rhodopsin negative cells speeding their differentiation into rhodopsin positive
cells but not increase the total number of cells to express rhodopsin (Wallace and Jensen
1999). RA had the greatest effect followed by IBMX with the least potent effect by
taurine (Wallace and Jensen 1999). Taurine has also been shown to be expressed in
rodent retina during embryonic development (Altshuler et al. 1993). Taurine, when
added to rat retinal cultures had a stimulatory effect on rod photoreceptors (Altshuler et
al. 1993). A gene was found that was upregulated by the addition of taurine called
Taurine Upregulated Gene 1 (Young et al. 2005). When this gene was knocked down,
there was a malformation of photoreceptor outer segments (Young et al. 2005).

Another extrinsic factor that has been shown to affect rod development is brain-
derived neurotrophic factor (BDNF). It has an indirect effect on rod photoreceptors.
BDNF acts through the TrkB receptor. This receptor is found in Müller glia, retinal
pigmented epithelial cells, ganglion cells and amacrine cells but not rod photoreceptors
(Rohrer et al. 1999). In TrkB knock out mice, rod photoreceptors developed but were
delayed compared to the wild types (Rohrer et al. 1999). The postnatal day 16 mutants were comparable to the postnatal day 12 wild type mice in rhodopsin content, electroretinogram (ERG) analysis and outer segment dimensions (Rohrer et al. 1999). Photoreceptors cannot directly respond to BDNF, but these observations suggest that their development is dependent on interactions with cells that do. The cells that are activated by BDNF are Müller glia, ganglion cells and amacrine cells, though retinal pigmented epithelial cells could not be ruled out (Rohrer et al. 1999).

Other extrinsic factors that have been described include those that inhibit the development of rod photoreceptors. Two such factors are Müller-cell-derived leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). These factors were first discovered when it was noticed that in dissociated cell cultures of rat retina, rods would not differentiate, based on morphology and expression of rhodopsin (Sparrow et al. 1990; Watanabe and Raff 1990). Based on this observation, a study was performed in rat and mouse dissociated retinal cell cultures to determine what was causing this effect (Neophytou et al. 1997). It was found that rods would not develop in the presence of fetal calf serum (FCS) but they would develop in culture if the serum was absent and the cell density was high enough (Neophytou et al. 1997). Neophytou et al. reported that FCS had a positive effect on Müller glia cell numbers in culture and that the inhibitory effect of FCS on rod photoreceptors was likely due to the excretion of LIF from the increased number of Müller glia cells (Neophytou et al. 1997).

The second extrinsic factor to have a known negative effect on rod photoreceptor development is ciliary neurotrophic factor (CNTF). Addition of CNTF to postnatal rat retinal explants was found to decrease the number of differentiated rods (Ezzeddine et al.
1997). In this study the cells that were immunoreactive for bipolar cell markers were increased. It was presumed that the cells fated to be rod photoreceptor cells were re-specified with the addition of CNTF to become bipolar cells if it was early enough (Ezzeddine et al. 1997). However, once cells began to express rhodopsin, they could no longer be affected by CNTF. Another group using the same paradigm did not see the increase in other cell types but did observe an inhibition of rod photoreceptors in rat cell culture (Kirsch et al. 1998). In another study using early postnatal rat retinal slices, the effects of CNTF in rat on rod photoreceptors were not permanent (Schulz-Key et al. 2002). They found that rods decreased but only transiently. The rods returned to normal numbers and the ONL was intact after a delay of 3-4 days.

1.3.6 Intrinsic Factors

There are also many known intrinsic factors that affect rod photoreceptor development. Some of these include neural retina leucine zipper (Nrl), Crx, Otx2, Cyclin D1, retinoblastoma protein (Rb), Nr2E3 and NeuroD.

The factor first expressed specifically in rods is neural retina leucine zipper (Nrl). Mears et al. found that it acts with Crx to regulate rhodopsin transcription (Mears et al. 2001). In Nrl knockout mice, rod photoreceptors do not form and cone like cells that have connections with rod bipolar cells take their place (Strettoi et al. 2004).

Crx is an Otx-like homeodomain transcription factor and is expressed prenatally during retinal development (Hennig et al. 2007; Rutherford et al. 2004). Chx10 is a gene expressed in neural progenitor cells and when mutated causes microphthalmia in humans. Chx10 mutant mice do not form normal rod or cone outer segments. Crx was not
expressed in these mice during embryonic retinal development but was detected postnatally (Rutherford et al. 2004). Crx is part of a larger network of genes involving photoreceptor fate determination and development (Hennig et al. 2007).

Otx2 is another gene that is part of this photoreceptor network and it functions to regulate Crx (Hennig et al. 2007). Otx2 is expressed in the developing nervous system including the retina and retinal pigmented epithelium (RPE) (Hennig et al. 2007). Cyclin D1 is a key regulator of the cell cycle and over expressed in many cancers. Mice that are deficient in Cyclin D1 not only have microphthalmia and thin retinas but their photoreceptors have an increased death rate (Ma et al. 1998).

Another known intrinsic molecule that affects rod photoreceptor development is retinoblastoma protein (Rb). Retinoblastoma protein is responsible for both proliferation and differentiation in the central nervous system (Ferguson and Slack 2001; Slack et al. 1998). Knockout Rb mice do not survive after birth (Zhang et al. 2004b). In mice in which Rb has been inactivated by a replication-incompetent retrovirus in single retinal progenitor, knock out cells will proliferate but no rods will differentiate to maturity (Zhang et al. 2004b). Zhang et al. hypothesized that Rb’s role in cell fate determination is due to binding to the E2F transcription factor regulating cell cycle exit using this pathway (Zhang et al. 2004b).

Nr2e3 is a nuclear receptor that is specific to photoreceptors (Chen et al. 2005). Nr2e3 is necessary for normal rod cell fate specification and development while it also suppresses cone cell proliferation (Haider et al. 2006). In retinal degeneration (rd7) mice, lack of Nr2e3 causes a proliferation in cone photoreceptors as well as retinal dysplasia (Haider et al. 2001). In humans, an analogous condition called enhanced S-cone
syndrome is also characterized by a retinal degeneration and an increase in cones. It was reported that Nr2e3 was expressed only in vertebrate rod photoreceptors but, Haider et al. found that in mice, Nr2e3 is expressed in late retinal progenitor cells as well as differentiating photoreceptors (Chen et al. 2005; Haider et al. 2006). Nr2e3 was also found in mouse mature rod and cone cell bodies (Haider et al. 2006).

NeuroD is a basic helix loop helix (bHLH) transcription factor that is found throughout the central nervous system and retina (Pennesi et al. 2003). It is involved in cell fate determination and differentiation (Cepko 1999; Pennesi et al. 2003). NeuroD is expressed in several cell types of the retina including amacrine cells, developing photoreceptors and in the adult mature photoreceptors, potentially having differing roles dependent on cell type (Inoue et al. 2002; Morrow et al. 1999). It likely works with other transcription factors such as Math3, to bring about changes in non-photoreceptor cell types (Inoue et al. 2002). Alone its effect is mainly in photoreceptor fate determination in the developing retina (Cepko 1999; Inoue et al. 2002; Pennesi et al. 2003). One study reported an increase in Müller glia in NeuroD knockout explants (Morrow et al. 1999). Another group did not see similar changes in amacrine cell fate or Müller glia differentiation unless both Math3 and NeuroD were knocked out (Inoue et al. 2002).

NeuroD knockout mice displayed reductions in both rod and cone driven ERG’s in prenatal, postnatal and adult age groups (Pennesi et al. 2003). These NeuroD null mice also showed evidence of photoreceptor degeneration and coupled with its presence in mature photoreceptors suggests a role in photoreceptor maintenance as well (Pennesi et al. 2003).
1.3.7 Mammalian Target of Rapamycin (mTOR)

The Target of Rapamycin (TOR) proteins, specifically TOR1-1 and TOR2-1, were first isolated in yeast with a specific point mutation in the FRB domain of the protein that was not affected by the growth inhibitor rapamycin (Heitman et al. 1991; Schmelzle and Hall 2000). This mutation did not allow the normal binding of the FKBP-rapamycin complex. The signaling pathways are distinctively different between the TOR’s in yeast and the mammalian TOR (mTOR). There are 2 TOR proteins in yeast and they both perform the same function in regards to increasing cell growth in response to nutrient availability (Abraham 2002; Thomas and Hall 1997). TOR2 has an additional function in controlling the actin cytoskeleton that is not sensitive to rapamycin (Abraham 2002; Thomas and Hall 1997). Both proteins also signal initiation of translation via phosphatases and TAP42 (Abraham 2002).

There is a TOR in Drosophila known as dTOR (Bateman and McNeill 2004). In the Drosophila eye, the dTOR pathway was reported to be responsible for activation of neuronal differentiation. dTOR has a conserved structure and has 56% of its amino acids in common with the mammalian homolog (Oldham and Hafen 2003; Oldham et al. 2000; Zhang et al. 2000). Drosophila spp. have been used to mine the function of the TOR pathway in metazoans (Bateman and McNeill 2004). Mammalian Target of Rapamycin (mTOR) is a 235 kDa Phosphatidylinositol kinase (PIK)-related kinase with multiple functions in cell growth (Schmelzle and Hall 2000). Mammalian TOR has been identified in rat, mouse and human. mTOR is regarded as a central regulator of cell growth as cyclin dependant kinase is a central regulator of cell proliferation (Schmelzle and Hall 2000). Downregulation of mTOR allows differentiation as shown in certain cell lines,
vascular smooth muscle and the CNS (Martin et al. 2007; Que et al. 2007; Swiech et al. 2008).

1.3.8 mTOR Pathway and Signaling

Cell growth is an increase in cell size in contrast to cellular proliferation which increases cell numbers. The mechanisms behind cell growth have not been as widely studied as those of cellular proliferation. One major way that mTOR affects cell growth is through initiation of translation. It does this in two main pathways via either p70S6K or 4E-BP1. First, in the presence of amino acids and growth factors, mTOR activates p70S6K which then phosphorylates S6, a 40s ribosomal protein that drives the translation of 5’ terminal oligopyrimidine tract (TOP) mRNA’s (Meyuhas 2000; Oldham and Hafen 2003). mRNA’s that contain the 5’ TOP, called TOP mRNA’s, encode components needed for translation (Meyuhas 2000). Second (also in the presence of amino acids and growth factors) mTOR inhibits 4E-BP1, a translation inhibitor that exerts its effect on eIF4E. eIF4E is also important is translating mRNA’s with 5’ untranslated regions.

There are two main binding partners for mTOR both with differing roles. mTOR will bind either raptor (regulatory associated protein of mTOR) or rictor (rapamycin-insensitive companion of mTOR). When bound, these complexes are called mTORC1 and mTORC2. Binding with raptor is necessary for phosphorylation of 4E-BP1. Binding of p70S6K seems to be needed for the phosphorylation of 4E-BP1. The raptor pathway is responsible for regulation of cell growth, an increase in the mass of each cell. If mTOR binds to rictor it activates Akt or protein kinase C (PKC) alpha and the actin cytoskeleton. Akt along with protein kinase B (PKB) is downregulated in many cancers and activation
causes more aggressive tumors so PKB/Akt is thought to be an important part of the pathophysiology of neoplasia (Sarbassov et al. 2005b). The rictor pathway is responsible for regulation of cell proliferation, an increase in the number of cells, metabolism and cytoskeleton (Sarbassov et al. 2005a). This pathway has been considered rapamycin insensitive but a recent study showed that this may not be the case (Sarbassov et al. 2006). When rapamycin is applied for a long period of time eventually all of the free mTOR is bound leaving none for rictor to bind with (Sarbassov et al. 2006).

The mTOR signaling pathway can be activated by the binding of insulin like growth factor (IGF) to the IGF-1 receptor. IGF-1 receptors are tyrosine kinases (White 2003; Yi et al. 2005). In order to exert a downstream effect they must attract and phosphorylate other proteins (White 2003). Insulin receptor substrate (IRS) proteins are activated by the IGF-1 receptor. When IGF-1 binds to its receptor, IGF-1 receptor dimerizes and autophosphorylation of the receptor occurs. This causes a phosphorylation of the insulin receptor substrate (IRS) protein downstream. In retina it is insulin receptor substrate-2 (IRS-2) and this protein has been implicated in photoreceptor survival and maturation (Yi et al. 2005). In this study, Irs2 knockout mice had a reduction of 50% of their photoreceptors by postnatal week two and a total loss by 16 months (Yi et al. 2005). IRS binds the p85 PI3K adaptor which then binds the p110 PI3k catalytic subunit (Oldham and Hafen 2003). This binding at the plasma membrane converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3 (Oldham and Hafen 2003). A negative regulator of PIP3 is PTEN, when PTEN function is lost then overgrowth occurs in Drosophila (Oldham and Hafen 2003). Protein kinase B, also known as Akt, is a
potential regulator of the PIP3/PTEN complex (Oldham and Hafen 2003). It is Akt that activates mTOR.

1.3.9 Proteomics

Proteomics is a broad experimental approach that is a useful tool in the study of retinal development. Proteomics, particularly 2 dimensional gel electrophoresis has been used successfully for over 30 years (O'Farrell 1975). As genomics is mapping all of the genes in a certain organism, proteomics is the study of all the proteins present in a cell at a particular time. Proteomics gives a snapshot of the genes that are expressed at certain times or under certain conditions. Proteomics is more context dependent because, one gene, considering post-translational modifications can encode up to 50 different proteins (Cho 2007b). Having the genome helps understand the possible protein combinations but only proteomics gives a true picture of what proteins are actually translated at any given time.

Proteomics includes many different techniques such as two dimensional polyacrylamide gel electrophoresis (2-D PAGE), liquid chromatography (LC) and isotope-coded affinity tag (ICAT) (Cho 2007b). The first set of techniques separates the proteins while mass spectrometry is needed for identification of proteins. 2-D PAGE allows separation of protein on gels based on 2 criteria, molecular weight and isoelectric focusing point (the pH at which the net charge is zero). Because it is separated according to two different criteria, the majority of spots on the gel represent one protein. Liquid chromatography (LC) is one way to separate and quantify the proteins in a sample (Cho
2007b). LC can be combined with mass spectrometry for best results in a complex mixture.

Protein spots of interest are picked and trypsin digested in preparation for identification by Mass spectrometry. Mass spectrometry is the technique used to identify proteins separated by one of the previously mentioned methods. It calculates the molecular masses of protein fragments based on the mass to charge ratio (Cho 2007b).

There are different methods to prepare the proteins for mass spectrometry but two most widely used approaches are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Cho 2007b). As it’s name suggests, electrospray results in a spray of small droplets which are then made into even smaller droplets (Cho 2007b). The resulting ions are then passed to the mass spectrometer, usually with a quadropole.

MALDI dissolves the samples in a crystalline structure that absorbs UV light, the matrix. A laser excites the sample dissolved in the matrix causing the ions to be released in a gas form (Cho 2007b). The excited ions are passed through a flight tube and the molecular weights are established based on their time of flight (TOF). This concept is based on masses relation to its velocity at a constant voltage (Cho 2007b). The mass spectrometer gives a reading of the peptide fragments masses and the information is fed into a computer. The computer can be used to search available online searchable protein databases such as MASCOT to find the best match for protein identification. MASCOT searches the SwissProt and Trembl web databases. Variables for protein modifications can be set according to the users criteria.
ICAT is useful when the protein structure and function is of interest. ICAT allows labeling of specific proteins and can be used in combination with 2-D PAGE (Cho 2007b). ICAT allows the user to put two samples of interest together and run on one gel.

While only one of several techniques, reproducible separation of proteins in two-dimensions has opened the door for widespread profiling of protein expression. Recent advances in 2-dimensional gel electrophoresis technology and computer based analysis have made this an effective way to separate the hundreds of proteins present in a sample and is already being used to generate proteome maps as well as ask specific experimental questions. Some of these studies have used yeast (Ideker et al. 2001; Pandey and Mann 2000; Sarry et al. 2007; Vido et al. 2001), plants (Kersten et al. 2002; Lee et al. 2007b; Ryan et al. 2001), immune-cells (Le Naour et al. 2001a; Lee et al. 2007a), bovine eye (Nishizawa et al. 1999), cancerous cells (Celis et al. 2002; Gagne et al. 2007; Li et al. 2005a; Luo et al. 2005; Pucci-Minafra et al. 2002; Wu et al. 2002), synaptosomes (Jimenez et al. 2002), plant development (Kersten et al. 2002; Lonosky et al. 2004; Ryan et al. 2001), post-golgi compartment (Morel et al. 2000), pulmonary fibrotic tissue (Malmstrom et al. 2002) and brain tissue (Skold et al. 2002; Xu et al. 2007). There are many unanswered questions regarding cancer. Many studies are using 2-D gel separation to identify differences between neoplastic and normal tissues and cells in order to identify possible treatment strategies, therapeutic targets and biomarkers (Cho 2007a). For example, some comparative studies of normal tissue versus neoplastic tissue are breast cancer (Luo et al. 2005) and ovarian cancer (Gagne et al. 2007). A study on hepatic cancer due to hepatitis B virus used proteomics to identify potential markers for the
disease (Li et al. 2005a). There is also a study examining the proteomics of atherosclerotic plaque formation in humans (Donners et al. 2005).

Recent advances in genomics have greatly furthered the information and genomic data available today. Proteomics is a great adjunct in helping the understanding of gene function by aiding in sorting through the vast amounts of information (Cho 2007b; Miklos and Maleszka 2001; Pandey and Mann 2000; Pawson and Nash 2003). Proteomics will show which of those proteins are actually present, and how they may be modified at various stages of development and what changes are present in disease states. Proteomics combined with genomics is becoming a powerful tool to investigate biological function.

Retinal development remains an active and well studied area, yet proteomics still remains an underutilized technology in this field. One early retinal related study examined the drusen protein which is found in people with age-related macular degeneration using proteomic techniques (Crabb et al. 2002). Two recent studies have used proteomics to study the developing retina. In one study, 2-D gel electrophoresis coupled with MALDI-MS and peptide mass fingerprinting was used to create a protein database for developing chick retinas (Lam et al. 2006). In another, the proteome of the postnatal mouse retina was studied using 2-D gel electrophoresis with MALDI-MS and peptide mass fingerprinting (Haniu et al. 2006). In this study they grouped proteins according to when they are expressed. There were four groups of proteins, those that are expressed in the juvenile state, those that are expressed in the adult state, those that are expressed transiently between juvenile and adult and those that are expressed regardless of postnatal age (Haniu et al. 2006). There is currently no comprehensive data available
on the proteomic catalog of developing retina. Categorizing what proteins are expressed at each age will be helpful in understanding development and how it changes over time. A proteomics approach to rod photoreceptor development will allow us to identify the most likely proteins involved, form new testable hypotheses and better focus future studies.

1.3.10 Clustering

Clustering is a common technique used to manage large datasets. Clustering in general can be defined as a way to group subsets of data points into groups with like data points (Jain et al. 1999; Kohonen 2001). It is also known as data segmentation. Most commonly, data points are clustered by determining the distance to the nearest cluster. In other words, the data points that are closest together in space are grouped together (Jain et al. 1999).

To understand the higher levels of learning algorithms it is necessary to start with the basics of simpler algorithm theories. There are two main groups, hierarchical and non-hierarchical both subdivided into many other categories. There are many other ways to classify the different types of algorithms but for the purposes of this dissertation I will stay with hierarchical versus non-hierarchical. A potential drawback to clustering is that it will always produce clusters, even if the data points are evenly distributed (Jain et al. 1999; Kohonen 2001).
1.3.10.1 Hierarchical Clustering

There are two basic types of hierarchical clustering: divisive, which tends to split clusters and agglomerative, which tends to merge clusters (Jain et al. 1999) (Kohonen 2001). A diagram called a dendogram is used to represent hierarchical clustering and resembles a tree diagram with branch lengths corresponding to the strength of the relationship (Jain et al. 1999; Kohonen 2001; Tamayo et al. 1999). Hierarchical clustering is accomplished through a series of successive steps grouping like data points (agglomerative). Differences happen depending on the definitions used for distance.

1.3.10.2 Non-hierarchical Clustering

There are many more types of non-hierarchical clustering. Some are simple such as simple K- (exclusive clustering algorithm) and Fuzzy K-means (overlapping clustering algorithm) and others are more elegant such as Self Organizing Maps (SOM). As mentioned previously, clustering uses distance as a means to group like data points. One way this is accomplished is using Euclidian distance, the measure of the straight line between two points (Kohonen 2001). This is not always the ideal method of measurement especially when scaling is taken into consideration. In cases of higher dimensional data like ours, another measure called the Minkowski Metric can be used where distance is measured based on a generalization of Euclidian distance (Kohonen 2001).

1.3.10.3 K-means clustering

K-means clustering is one of the simplest learning algorithms. This unsupervised learning algorithm defines K numbers of centroids, one for each cluster (Kohonen 2001).
The centroids are scattered away from one another and data points are assigned to the closest centroid. Once this is done, the centroids are recalculated and data points reassigned or assigned if it is called for. This process is repeated until the centroids no longer move. Because it is a simple algorithm it may not always group the data into the best clusters (Tamayo et al. 1999). The results are dependent on the number and placement of the initial centroids.

### 1.3.10.4 Fuzzy K-means

Utilization of Fuzzy K-means addresses some of the problems associated with K-means. As the name suggests, centroid boundaries are not clear. It is similar to K-means but the data points can have different degrees of membership to one or more centroids as opposed to K-means where a point can have only one degree of membership to only one centroid (Bezdek 1981; Jain et al. 1999). The steps are then similar but there is higher likelihood of a data point being assigned to the appropriate cluster (Bezdek 1981).

### 1.3.10.5 Adaptive Resonance Theory and Self Organizing Maps

The two types of algorithms used for my data were Adaptive Resonance Theory 2 (ART2) and Self Organizing Maps (SOM). Both of these algorithms are considered artificial neural network algorithms because they were modeled after human neural networks and the activities they are responsible for such as learning and memory (Aleshunas et al. 1994; Kohonen 2001). ART2 and SOM are both also categorized as unsupervised learning algorithms. They are called unsupervised because supervised
learning algorithms require prior knowledge or experience in the network. Since there is no expected outcome (i.e. it is unknown) these algorithms are unsupervised.

ART2 is a way to determine the appropriate number of clusters. It does this through a process of adaptation. After determining the number of clusters needed for a data set this number can then be used to run the data through SOM (Tamayo et al. 1999). A major advantage of ART2 is that it can learn new information without losing the old information unlike most previous algorithms (Aleshunas et al. 1994). This is known as the stability-plasticity dilemma a term coined by one of the developers of this algorithm. This just means that stability is the preservation of previous cluster and plasticity is the ability to add a new cluster (Aleshunas et al. 1994). ART2 does this by creating a new cluster when a data point does not fit with the previous ones. A main problem with ART2 is the potential to become proliferative, that is, continuing to add new clusters without stopping, also known as overfitting.

SOM takes data that is in high dimensional space and attempts to put it into lower dimensional space while preserving the topography. It uses competition, cooperation and adaptation (Aleshunas et al. 1994; Kohonen 2001). Competition means that given certain inputs some clusters become activated. Those that are activated the most “win”. Cooperation means that the winner spreads this activation to create the network. Finally, adaptation means that the winner and the network with its neighbors adapt themselves in order to best suit the inputs.

SOM is competitive like human neural networks. Nodes are determined by use or disuse and by a series of interactions including lateral interactions and inputs (Aleshunas et al. 1994; Kohonen 2001; Tamayo et al. 1999). Those training networks that are not
being used decrease over time. The result is a trained network where nodes that are close
are similar and those that are farther away are different. One limitation to SOM is that it
should not be used for pattern recognition (Aleshunas et al. 1994; Kohonen 2001). It
takes data points through a series of iterations and ends up putting them in a lower
dimensional grid while always retaining the topology (Tamayo et al. 1999).

1.3.11 Summary

The environment during retinal development and differentiation is of utmost
importance. Understanding this environment and its interactions will be key to
uncovering the mechanism of cellular differentiation in the retina. This information will
allow for a better understanding of the developmental process and also will begin to
address controlling cell fate decisions made by retinal stem cells transplanted to treat
retinal degeneration. Proteomics is a powerful tool to accomplish this goal.
1.4 References


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2.1 Abstract

Purpose: The process of rod photoreceptor genesis, cell fate determination and differentiation is complex and multi-factorial. Previous studies have defined a model of photoreceptor differentiation that relies on intrinsic changes within the presumptive photoreceptor cells as well as changes in surrounding tissue that are extrinsic to the cell. We have used a proteomics approach to identify proteins that are dynamically expressed in the mouse retina during rod genesis and differentiation.

Methods: A series of six developmental ages from E13 to P5 were used to define changes in retinal protein expression during rod photoreceptor genesis and early differentiation. Retinal proteins were separated by isoelectric focus point and molecular weight. Gels were analyzed for changes in protein spot intensity across developmental time. Protein spots that peaked in expression at E17, P0 and P5 were picked from gels for identification.

Results: There were 239 spots that were picked for identification based on their dynamic expression during the developmental period of maximal rod photoreceptor genesis and...
differentiation. Of the 239 spots, 170 of them returned reliable identities. Twenty-three proteins were represented by multiple spots, suggesting they were post-translationally modified. Of the 107 distinct proteins identified, only 11 had been previously reported to be associated with the developing retina.

**Conclusions:** Our results represent the first proteomics study of the developing mouse retina that includes prenatal development. We identified 23 proteins with dynamic post-translational modifications and 96 proteins with dynamic expression in the developing retina whose dynamic expression had not yet been reported in the developing retina.

### 2.2 Introduction

Retinal diseases involving degeneration of photoreceptors are an increasing cause of blindness in this country, particularly among the aging population. Advances in stem cell research may someday make replacement of photoreceptors a feasible therapy for the treatment of retinal degeneration. MacLearen and colleagues recently reported that only post-mitotic rod precursors were able to successfully and functionally integrate into the mature retina. Currently we are not able to reliably bias stem cells to adopt a photoreceptor fate. In this regard, it will be crucial that we have a clear understanding of the retinal environment during normal photoreceptor genesis as well as the combination of factors both intrinsic and extrinsic to developing retinal cells that influence their decision to adopt a photoreceptor cell fate. To this end we have characterized the developmental proteome of the mouse retina during late embryonic and early postnatal development, the time when the vast majority of rod photoreceptors are born, commit to their cell fate and begin to differentiate. Retinal progenitor cells pass through a
characteristic set of competency states during which extrinsic cues can influence what type of retinal neurons they will become. Progenitors in the developing retinal environment during later development are strongly biased towards rod photoreceptor differentiation. Thus, if we are to learn how to bias stem cells to adopt at first a retinal and then a photoreceptor cell fate, characterizing protein expression during later stages of retinal neurogenesis seems a reasonable starting point.

We have used two-dimensional gel electrophoresis to profile protein expression in developing mouse retinas during late stages of retinal neurogenesis. Self-organizing mapping (SOM) was used to cluster protein spots into groups based on their changing levels of expression across developmental time. From this we identified clusters of proteins that peaked in expression at embryonic day 17 (E17; prior to the peak of rod genesis); birth (P0; during the peak of rod genesis) and postnatal day 5 (P5; a time when rods are making irreversible cell fate commitment decisions and have begun to differentiate).

In this analysis we separated 474 distinct protein spots. Of those spots, 60 peaked in expression at E17, 56 peaked at P0 and 123 peaked at P5 and were picked for identification. One hundred and seventy protein spots (71.1%), representing 107 distinct proteins returned identities that could be confirmed based on molecular weight by manual inspection of the gel images. An analysis of the literature revealed that 11 of the dynamically expressed proteins had a previously published link to retinal development. Further, 23 of the identified proteins were represented by multiple spots, indicating dynamic changes in their migration characteristics during retinal development, likely due to post-translational modification. This analysis has identified proteins that are
dynamically expressed or modified in the retina during rod genesis and differentiation, and has therefore generated a list of candidate proteins for further investigation.

2.3 Materials and Methods

Sample Preparation: Pups were taken from timed pregnant C57BL/6 mice at ages E13, E15, E17, E18, day of birth (P0) and P5. Eyes were enucleated and retinas immediately placed in ice cold Phosphate Buffered Saline (PBS, 0.14M NaCl, 2.68mM KCl, 10.14mM Na2HPO4, 1.76mM KH2PO4, pH 7.2). The tissue was suspended in rehydration buffer (8M Urea, 2% CHAPS, 0.5% ZOOM Carrier Ampholytes (Invitrogen, Carlsbad, CA), 0.002% bromophenol blue and 20mM DTT), sonicated for 30 seconds and spun at 4,000 rpm for 10 minutes at 4°C. The pellet was re-suspended in rehydration buffer (RHB). The sample was spun again at 4,000 rpm for 10 minutes at 4°C. The remaining supernatant was collected and frozen at -80°C. The total protein concentration was determined using the EZQ protein assay (Invitrogen). The sample was diluted to a final concentration of 35µg per 165µl (0.212 µg/µl). All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Two-dimensional separation of protein spots: Proteins were separated on the basis of their isoelectric focus poing (pI) using a ZOOM IPGRunner 7cm strip pH 3-10 (Invitrogen). The total protein loaded on the strip was 35µg. The first dimension running conditions were as follows: 20 minutes at 200V, 15 minutes at 450V, 15 minutes at 750V and 45 minutes at 2000V. Proteins were separated by molecular weight using a 7cm Bis
Tris 3-12% pre-cast gel (Invitrogen). The gels were subjected to a continuous voltage of 200V for 50 minutes.

The gels were fixed with 50% Methanol, 10% Trichloroacetic acid overnight, washed in ddH$_2$O followed by a wash in 10% methanol, 7% acetic acid for 30 minutes. The gels were stained with SYPRO Ruby (Invitrogen) overnight and washed in 10% methanol, 7% acetic acid for 60 minutes followed by dH20 the next morning. They were imaged on a Typhoon 9410 fluorescent scanner (GE Healthcare Life Sciences, Piscataway, NJ) for quantitative analysis and then stained with Simply Blue Coomassie (Invitrogen) overnight to allow hand picking of spots.

**Software Analysis:** For the protein spot detection Phoretix 2D Expression software (Nonlinear Dynamics; Nonlinear USA, Durham, NC) was used. Gels were warped and spots matched automatically by the program but matching was manually checked on all gels and adjusted to correct for incorrect matches. All gels were scrutinized to ensure accurate spot detection, matching and that artifacts were not counted as actual spots. Three replicates of each age were grouped together to make an average gel for that age. Spots present on at least two of the three gels were included on the average gel for that age group. Expression values for each spot were expressed as protein spot volumes. Background subtraction was employed using the Mode of Non-Spot (default) at a margin of 45 (default). The spot volume was normalized to total spot volume on its average gel.

**Clustering of Data:** To cluster the data, we used the SOM (Self-Organizing Maps) method provided by the GeneCluster 2.0. Available at [http://www.broad.mit.edu/cancer/software/genecluster2/gc2.html](http://www.broad.mit.edu/cancer/software/genecluster2/gc2.html).
data, we replaced missing expression values with 0s, interpreting a missing expression value as an absence of a signal, and normalized the data to mean of 0 and variance of 1. The SOM algorithm was executed with the desired cluster range of 6 and the rest of the parameters left unchanged (50000 iterations, seed range of 42, initialization of centroids to random vectors, bubble neighborhood, initial and final learning weights of .1 and .005, and initial and final sigmas determining the size of the update neighborhood of a centroid set to 5 and .5, respectively). This produced 6 clusters with the peak at each time point.

**Spot Picking and Identification of Proteins:** For protein identification, gels were stained with SimplyBlue (Invitrogen). Spots of interest were hand picked based on clustering results and maps from Phoretix software analysis. Trypsin digestion and deposition to a target for MALDI were performed using an Ettan Spot Handling Workstation (Amersham Biosciences, Newark, NJ, USA). For MALDI analysis, the tryptic peptides dissolved in 50% CH3CN/0.1% TFA were mixed with a matrix solution (CHCA 10 mg/mL in 50% CH3CN/0.1% TFA) and applied on a target plate. For ESI experiments, protein digest solution was taken out after trypsin digestion, extracted and dried to needed volume.

MALDI-TOF MS/MS MS analyses were performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an MALDI ion source. The mass spectrometer was operated in the positive ion mode. Mass spectra for MS analysis were acquired over m/z 500 to 4000. After every regular MS acquisition, MS/MS acquisition was performed against most intensive ions. The molecular ions were selected by information dependent acquiring in the quadrupole analyzer and fragmented in the collision cell. For ESI Mass Spectrometry the peptide
digest samples were introduced to the QSTAR XL quadrupole TOF mass spectrometer with a Switchos LC pump and a FAMOS autosampler (LC Packings, San Francisco, USA). Other parameters of the mass spectrometer were the same as MALDI analysis.

All spectra were processed by MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches. Typical search parameters were as follows: Max missing cleavage is one, fixed modification carboxyamidomethyl cysteine, variable modification oxidation of Methionine. Peptide mass tolerances were +/- 100 ppm. Fragment mass tolerances were +/- 1 Da. No restrictions on protein molecular weight were applied. Protein identification was based on the probability based Mowse Score. The significance threshold $p$ was set to less than 0.05.

**Gene Ontology (GO analysis):** GO Tree Machine (GOTM; 4) was used to return the GO categories for each of the clusters. If any level 3 category appeared more than once, the additional copies were removed.

### 2.4 Results

As an initial step to better understand rod photoreceptor development we profiled the proteome of the developing mouse retina during the time of maximal rod photoreceptor genesis and cell fate determination. To make the expression analysis more robust, we analyzed retinas from ages E13, E15, E17 E18 P0 and P5. Representative gels from each age are shown in figure 1. Quantitatively, each age gel is a composite gel made up of three replicate gels. Spots on composite gels from each age were matched across ages. To control for slight loading, staining or scanning differences, expression
levels for each spot were normalized and expressed as a percentage of the total signal (spot volume) for each gel. Expression values were used to cluster spots based on their changing levels of expression from E13 to P5. Figure 2 shows the results from Gene Cluster when 6 clusters were pre-specified. The resulting clusters contained groups of proteins that had their peak in expression at each of the ages examined. For this analysis, we were most interested in the clusters that contained proteins that peaked at E17, which is just prior to the peak of rod photoreceptor genesis, P0 which is at the peak of rod photoreceptor genesis and P5, which is past the time of rod genesis, but the time when early, irreversible rod differentiation is occurring.

Based on the clustering analysis, spots in cluster 1 (c1; expression peaked at E17), c4 (expression peaked at P0) and c0 (expression peaked at P5) were hand-picked for identification. Of the spots that were picked for analysis, 71.1% (170/239) returned high probability IDs that could be confirmed based on known or predicted molecular weights and isoelectric focus points (pIs). Twenty-three proteins were represented by more than one protein spot. Tables 1, 2 and 3 list the 107 distinct proteins that peaked at E17, P1 and P5 respectively. Gene ontology (GO) annotations for the 107 proteins were retrieved using GO Tree Machine 4. Ontologies at level 3, that is, three branch points in from the top-level ontologies of cellular localization, molecular function and biological process, were tallied. The most highly represented categories in the cellular component branch were intracellular compartment and intracellular organelle. The most highly represented molecular function categories were protein binding, nucleic acid binding, nucleotide binding and ion binding. The most highly represented biological process categories were metabolism, cellular physiological process, localization and cell communication (data
To better understand the proteins that were identified in this analysis, we did a manual literature search to look for published links between each protein and retinal development, retina, CNS development, CNS and finally any published link to cancer. Figure 3a illustrates the number of proteins in our analysis with previously published links to the indicated criteria and that peaked at E17. Of 60 proteins in the cluster, 44 were positively identified. Based on a search of the literature, 3 proteins had been previously linked to retinal development, 10 to retina, 5 to CNS development, 13 to CNS, and 13 had been previously linked to cancer. Fifteen of the proteins had no published link to any of the search criteria. These data are also represented in table 1. Figure 3b summarizes the published links between criteria terms and proteins in our analysis that peaked at P0. Of 56 protein spots in the cluster, 21 were identified. Based on a search of the literature, 1 had been previously linked to retinal development, 6 to retina, 1 to CNS development, 2 to CNS, and 10 to cancer. Seven proteins had no published link to criteria terms. These data are also represented in table 2. Figure 3c summarizes the published links between criteria terms and proteins in our analysis that peaked at P5. Of 123 protein spots in the cluster 102 were identified. Based on a search of the literature, 9 had been previously linked to retinal development, 17 to retina, 11 to CNS development, 16 to CNS, and 35 to cancer. Twenty-four of the proteins had no previously published link to the criteria terms. These data are also represented in table 3.

This analysis identified 107 distinct proteins that are dynamically expressed in the retina during rod photoreceptor development. Of these proteins, 23 were represented by more than one protein spot, suggesting they are dynamically post-translationally
modified. Finally, a manual search of the published literature identified prior published reports had already linked 11 of the 107 proteins to retinal development in some way. Thus, we have identified dynamic expression and/or modification of 96 proteins that had not been previously described in the developing retina.

2.5 Discussion

We have used protein expression profiling to identify proteins that are dynamically expressed during rod genesis. Our analysis of the developing mouse retina focused on proteins that peaked in expression at E17, immediately prior to the peak of rod genesis, P0 during the peak of rod genesis and at P5, when the majority of newly born rods are differentiating. Our analysis identified proteins spots that peaked at each of these three ages. Of these dynamically expressed proteins, some of them have been described previously in the developing retina.

At age E17, an age before the majority of rods are born, we identified 3 proteins that peaked in expression at E17 that have been previously described in the developing retina. These proteins are fructose-bisphosphate aldolase A, ras-related protein Rab-11A and stathmin.

Fructose-bisphosphate aldolase A, is a member of the class I fructose-bisphosphate aldolase family and is involved in pathways including glycolysis and carbohydrate degradation. This protein has been putatively linked to retinoblastoma in fetal retina. It should be noted that we identified 4 fructose-bisphosphate aldolase A spots in our analysis, one spot peaked in expression at E17, and three others that peaked at P5. This can be explained by the presence of post-translational modifications (PTMs)
to the protein. We did not identify which PTMs are present in the developing retina, however it has been reported that one PTM, tyrosine nitration, impairs mammalian aldolase A activity.\(^7\)

Stathmin (phosphoprotein p19) is a phosphoprotein located in the cytoplasm and is ubiquitously expressed.\(^8\) It functions to destabilize the microtubule system.\(^8\) In a study of the developing rat retina stathmin mRNA was highly expressed in retinal ganglion cells and interneurons.\(^9\) We identified 4 stathmin spots in our analysis. Two spots peaked at E17 and two peaked at P0. Beretta and colleagues identified four potential phosphorylation sites and have observed 14 molecular forms of stathmin on 2D gels. Of the 14 stathmin spots they identified 2 were unphosphorylated and 12 were phosphorylated on one or more sites, however the functional significance of these phosphorylation sites is not clear.

Ras-related protein Rab-11A is a peripheral membrane protein that modulates endosome trafficking and is a GTP binding protein belonging to the GTPase superfamily. Rab-11, has been shown to be required for proper *Drosophila* eye development.\(^11\) Rab11 is synthesized during larval development by the photoreceptors and becomes localized to rhabdomeres and lamina neuropil in the adult. In one Rab 11 mutant strain the photoreceptors and bristles failed to form. Its dynamic expression in our analysis suggests it may be important for mammalian retinal development as well.

Rod genesis in the mouse retina peaks near P0.\(^12\) In our analysis of the 22 identified protein spots that peaked at P0, only one, stathmin, has been previously described in developing retina. As previously discussed, stathmin is shown to be highly expressed in developing ganglion cells and interneurons. Two stathmin spots were
identified as peaking at P0 representing post-translational modifications suggesting that
the role of this protein during retinal development may be dependent on its modification.

Of the ages examined in this analysis, P5 is after the peak of rod photoreceptor
genesis and at a time when we would expect many rod photoreceptors to be
differentiating. Of the 103 identified protein spots that peaked at P5, we were able to find
published reports previously linking 10 of them to retinal development. These proteins
include fructose bisphosphate aldolase A which has been discussed previously. In
addition, calretinin, eukaryotic translation initiation factor 4B, fatty acid-binding protein
(epidermal), myosin light polypeptide 6, phosphoglycerate kinase 1, retinol-binding
protein I (cellular), SET protein and vimentin were proteins that peaked at P5 in our
analysis and have been previously reported in the developing retina.

Calretinin (calbindin 2) is a calcium binding protein that is widely expressed in
the retina and developing CNS \(^{13}\). Its increased expression likely represents the
maturation of the retina in this analysis. Vimentin is expressed in retinal progenitor cells
early in development \(^{14}\) and only in Müller glia later in the mature retina \(^{15}\). Our
identification of the presence of vimentin spots, is perhaps more confirmatory and does
not necessarily add to the body of knowledge of retinal development.

Eukaryotic translation initiation factor 4B is needed to bind mRNA to ribosomes
\(^{16}\). When expressed in a cultured \textit{Drosophila} cells, eIF4B is required for cell survival and
over expression in eye imaginal discs promotes cell proliferation \(^{17}\). Our observation of
the increasing expression in the P5 retina may suggest it is acting as a survival factor as
cells differentiate.

Fatty acid-binding protein (epidermal) is localized to the cytoplasm and has a high
affinity for fatty-acids\textsuperscript{18}. In the rat retina, the protein is involved in axonal elongation of retinal ganglion cells during development.\textsuperscript{19} Previous studies have also suggested that FABP is involved in synapse formation\textsuperscript{20}, which would be consistent with an increasing expression in the P5 retina, at which time the retinal cells are forming synapses.

Fatty-acid binding protein, brain (B-FABP) is a member of a family of small cytoplasmic proteins that bind hydrophobic ligands. Expression patterns of B-FABP in the developing nervous system suggest it may play a role in neurogenesis or neural migration\textsuperscript{21}. In addition to the developing brain B-FABP expression has been reported in the human fetal retina\textsuperscript{22}.

Myosin light polypeptide 6 plays a role in retinal ganglion cell growth cone motility in *Xenopus*\textsuperscript{23}. Presumably there is little RGC growth cone motility in the P5 retina, suggesting it is perhaps involved in other remodeling events occurring in the P5 mouse retina.

SET protein is found in the cytoplasm, nucleus and endoplasmic reticulum. It is a multitasking protein involved in functions such as transcription, apoptosis and nucleosome assembly\textsuperscript{24}. High levels of SET have been described in the developing mouse retina, particularly in the nucleus suggesting that it may play a role in cell proliferation\textsuperscript{25}. However, in our analysis, we observed a peak in the expression of this protein spot when cell division is almost complete, perhaps suggesting an additional role for the protein during retinal development.

Retinol-binding protein (cellular) functions to transport retinol intracellularly\textsuperscript{26}. Its expression has been described in the developing retina and RPE in mouse\textsuperscript{27}. Expression of retinol-binding protein has also been reported in the embryonic mouse
brain suggesting a role in CNS development.  

A number of important studies have used expression analysis to identify genes or proteins expressed during retinal development. The motivation behind this approach is two-fold. Firstly, molecules important for particular events during retinal development, may be expected to change at the time that said event is occurring. Secondly, profiling genes that change in relation to one another may help investigators to identify pathways or groups of genes that work together during retinal development. 

Protein expression profiling can be a powerful compliment to mRNA expression analysis. Changes in protein expression are a more definitive measure of how much gene product is present in cells. However, the most powerful compliment that 2D gel expression analysis offers is the ability to capture not only changes in expression but also changes in post-translational modification. The existence of post-translational modifications can be discovered by differences in pI or molecular weight. In our analysis alone, we identified 23 proteins with dynamic post-translational modifications. In future experiments specific dyes for phosphorylation and glycosylation may be useful to identify and quantify specific post-translational modifications.  

A recently published complementary study used 2D-gel electrophoresis to profile dynamic changes in protein expression in the postnatal mouse retina. In this study they identified 174 total protein spots. Of the 170 total protein spots identified in this study (E17, P0 and P5), 47 of them were in common with the previous study. Differences may be due to the different ages profiled as the previous study used strictly postnatal ages. Protein expression profiling has also been successfully applied in the post-hatch chick retina. Even though these studies may have profiled different ages and/or species it
still may be useful to integrate the information from these and other studies to generate a more comprehensive profile of changes in protein expression during vertebrate retinal development.

We have used protein expression profiling to characterize dynamic changes in protein expression during rod photoreceptor genesis. In this analysis we identified proteins whose expression pattern peaked at E17, P0 or P5 (the oldest age profiled). We identified a number of proteins known to be important for rod development. We also identified several proteins that have a published role in CNS development but no other link to the retina, making them potential candidates for further investigation. Proteins identified as dynamically expressed in the developing retina, that had have previously reported to be expressed in various forms of cancer may also be an interesting group of candidate proteins to pursue in relation to retinal cell differentiation. Finally, there were 47 protein spots that have no previous published link to retina or CNS development or cancer. This group may also include a number of interesting candidate proteins that would have otherwise, not likely ever been investigated.

**Acknowledgements:** The authors wish to thank Dr. William Lewis at the Iowa State University Proteomics Facility for technical assistance. Funding: NIH EY014931
2.6 References


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2.7 Figure Legends

**Figure 1:** Representative images of gels from embryonic and postnatal retinal protein samples. Proteins were separated first by isoelectric focus point (pH 3-10) then by molecular weight.

**Figure 2:** Changes in protein expression across developmental time were used to cluster protein spots into six groups. Each group contained protein spots whose expression peaked at a particular developmental age. In each panel the y-axis represents relative expression levels and the x-axis represents the ages analyzed. Black dots represent ages E13, E15, E17, E18, P0 and P5 from left to right respectively. Gray lines represent one standard deviation on either side of the mean expression pattern for each group of proteins.

**Figure 3:** Identities of dynamically expressed proteins whose expression peaked at E17 (A), P0 (B) and P5 (C) were used to search previously published literature. Search criteria terms were retina, retinal development, CNS, CNS development and cancer.
2.8 Figures

Table 1. Dynamically expressed retinal proteins that peaked at E17.

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* Protein that was represented on a gel by more than one spot.

Numbers indicate references used to link the protein to a particular search criteria.
Table 2. Dynamically expressed retinal proteins that peaked at P0.

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Table 2 (cont)

| P17751 | Triosephosphate isomerase (EC 5.3.1.1) |   |   | 58 |

* Protein that was represented on a gel by more than one spot. Numbers indicate references used to link the protein to a particular search criteria.
Table 3. Dynamically expressed retinal proteins that peaked at P5.

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* Protein that was represented on a gel by more than one spot.
Numbers indicate references used to link the protein to a particular search criteria.
Figure 1: Representative 2D Gels From Retinal Samples
Figure 2. Clustering results using SOM.
CHAPTER 3: THE ROLE OF MAMMALIAN TARGET OF RAPAMYCIN (mTOR) IN PHOTORECEPTOR DEVELOPMENT IN THE RETINA

A paper to be submitted to Vision Research

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3.1 Abstract

Currently the exact mechanism by which a photoreceptor chooses its cell fate is not completely understood. Biasing cells to a rod photoreceptor cell fate has the potential to improve vision in patients afflicted with photoreceptor degenerative diseases. We have previously observed that insulin-like growth factor-1 (IGF-1) promoted rod differentiation. Mammalian target of rapamycin (mTOR) is a molecule downstream of IGF-1 signaling. In order to investigate the potential role of (mTOR) signaling during rod photoreceptor differentiation we examined the developmental expression of mTOR during retinal development. We characterized the developmental distribution of mTOR and its binding partners, raptor and rictor in the developing mouse retina using immunohistochemistry. mTOR is present throughout the developing and mature retina as are raptor and rictor. Next we tested the functional significance of mTOR during development. We cultured developing murine retinal explants with an mTOR inhibitor, rapamycin for 10 days. Inhibition of mTOR in E17, but not P0 explants increased rod
photoreceptor differentiation by approximately 20%. Our results suggest that mTOR is important in modulating differentiation of rod photoreceptor cells.

*Keywords*: Development, Retina, Mouse, Photoreceptor, mTOR

### 3.2 Introduction

Diseases of the retina cause loss of vision and can lead to blindness. Diseases such as macular degeneration and retinitis pigmentosa are the result of photoreceptor degeneration. Age related macular degeneration (AMD) is the number one cause of blindness in retirement aged Americans (Ambati et al. 2003) and is a growing concern as the population ages. The loss of sight will hinder the ability to be self sufficient and live independently as well as have an economic impact with increased dependent care situations. Since photoreceptors cannot be regenerated, cell transplantation to replace degenerate photoreceptors is a potential approach to regaining loss of vision in these individuals.

Successful cell transplantations with appropriate spatial and functional integration into the host retina are necessary if we hope to restore vision to those afflicted with blinding retinal diseases. Various neural progenitor cell types have been transplanted into the retina including retinal progenitor cells (RPCs) (Klassen et al. 2004; Qiu et al. 2005) brain progenitor cells (BPCs) (Lu et al. 2002; Mizumoto et al. 2003; Sakaguchi et al. 2003), adult hippocampal progenitor cells (AHPCs) (Nishida et al. 2000; Takahashi et al. 1998; Young et al. 2000) and iris-derived/ciliary body cells (Ahmad et al. 2004; Ahmad et al. 2000; Tropepe et al. 2000).
The only example to date of successful photoreceptor transplantation with differentiation and functional integration into the host retina utilized cells that had already begun to express *Nrl*, an early marker of photoreceptor cell fate commitment (MacLaren et al. 2006). Cells transplanted prior to rod cell fate commitment did not successfully integrate suggesting that in order to achieve success cells must first be biased to the desired cell fate. Thus, based on current information it appears that in order to effectively transplant cells and have them incorporate into the retina it is necessary to be able to bias them to a photoreceptor cell fate. Therefore, studies to better understand the mechanisms controlling cell fate determination in the developing retina may provide valuable information towards this end.

A Target of Rapamycin (TOR) molecule, present in *Drosophila* (dTOR) has been reported to have an effect on photoreceptor development in the metazoan eye via the Tsc-1 protein (Bateman and McNeill 2004). A mutagenesis screen revealed mutated Tsc-1 protein in abnormal eye patterning in *Drosophila*. Tsc-1 protein is downstream in insulin and TOR signaling pathways.

Mammalian TOR (mTOR) molecules are 235kDa Phosphatidylinositol kinase (PIK)-related kinases with multiple functions in cell growth and proliferation (Sarbassov et al. 2005a). There are two binding complexes for mTOR. mTORC1’s (mTOR/Raptor complex) major role is in controlling cell growth (cell size) (Schmelzle and Hall 2000). mTORC2’s (mTOR/riCTOR complex) role is controlling cellular proliferation, cytoskeletal organization and microtubule growth (Swiech et al. 2008).

mTOR can be activated by IGF-1 signaling (Oldham and Hafen 2003). Previous studies have demonstrated that
application of IGF-1 to cultured retinal explants increases rod photoreceptor
differentiation (Hecker and Greenlee, Personal Communication). To investigate the
potential role for mTOR in retinal development, we characterized the expression of
mTOR and two of its binding partners, raptor and rictor in the developing mouse retina.
To determine if mTOR has a role specifically in photoreceptor development, we inhibited
mTOR in developing retina using rapamycin, a specific inhibitor of mTOR/raptor
complex and assayed rod photoreceptor differentiation. Our results demonstrate that
mTOR and its binding partner rapamycin is expressed in developing and mature
photoreceptors. Further, our results demonstrate that inhibition of mTORC1
(mTOR/raptor) decreases rod photoreceptor differentiation.

3.3 Materials and Methods

Animals and Tissue Preparation
C57BL/6 mice were obtained from a colony maintained at Iowa State University. The
date of birth is designated as postnatal day 0 (P0). Mice ages E15 to P5 were euthanized,
their heads were removed and immersion fixed in 4% paraformaldehyde in 0.1M PO4
buffer (pH 7.5). Postnatal day 10 and adult mice were euthanized and their globes were
immersion fixed in 4% paraformaldehyde. The tissue was cryoprotected in a 30%
sucrose solution in 0.1 M PO4 buffer (pH 7.4), embedded in OCT and sectioned at a
thickness of 20 µm. For retinal explant culture, eyes were removed from E17 and P0
heads for dissection. All animal procedures were in adherence to the ARVO statement
for the use of Animals in Ophthalmic and Vision Research had the approval of the ISU
committee on animal care.
Antibodies

All primary antibodies were diluted in blocking solution (potassium phosphate buffered saline (KPBS; 0.15 M NaCl, 0.034 M K₂HPO₄, 0.017 M KH₂PO₄, pH 7.4) containing 1% BSA, 0.4% Triton-X 100, and 1.5% Normal Donkey Serum (NDS, Jackson ImmunoResearch Laboratory, West Grove, PA). Rabbit polyclonal anti-mTOR antibody (Bethyl Laboratories, Inc., Montgomery, TX) was diluted 1:20. Mouse monoclonal antirhodopsin antibody (a generous gift from Dr. Colin Barnstable, Yale University) was diluted 1:250. Rabbit polyclonal anti-raptor (Bethyl Laboratories, Inc., Montgomery, TX) was diluted 1:50. Rabbit polyclonal anti-riCTOR (Bethyl Laboratories, Inc., Montgomery, TX) was diluted 1:100. The fluorescent secondary antibodies Alexa Fluor® 594 donkey-anti mouse and Alexa Fluor® 594 donkey-anti rabbit (Molecular Probes, Eugene, OR) were diluted 1:500. Secondary antibodies were diluted in KPBS containing 1% BSA, 1.5% NDS, and 0.02% Triton-X 100.

Immunohistochemistry

Frozen tissue sections or dissociated retinal cells (adhered to slides and fixed) were rinsed in 0.5M KPBS and incubated in blocking solution. Slides were incubated in primary antibody overnight at 4°C. The following day slides were washed in KPBS containing 0.02% Triton-X 100 after which fluorescent secondary antibody was applied. Prior to cover-slipping, the slides were incubated in 300 µM DAPI diluted in KPBS.

Retinal Explant Culture

The explant culture procedure was adapted from Zhao and Barnstable (Zhao and Barnstable 1996). Briefly, eyes from E17 and P0 pups were dissected in culture media and whole retinas with lenses intact were isolated away from the RPE. One retina, lens
face up, was added to each well of a 24 well plate with 1 mL of culture media (UltraCulture (Cambrex, East Rutherford, New Jersey) with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) and 10 µg/mL of gentamycin (Invitrogen). After 10 days in culture the explants were harvested. Rapamycin (Calbiochem, San Diego, CA), an mTOR inhibitor was added to the UltraCulture media before use at a concentration of 0.1, 1 or 5 µM for E17 explants and 1µM for P0 explants. An equal amount of vehicle (dimethyl sulfoxide) was added to control explants.

**Cell Dissociation**

At the termination of the experiment, lenses were dissected from the eyes. Following lens removal, 2 retinas per experimental condition were collected and washed in sterile phosphate buffered saline (PBS; 14 mM NaCl, 2.7 mM KCl, 5.37 mM Na₂HPO₄, 1.76 mM KH₂PO₄). Tissue was incubated at 37°C for 5 minutes in PBS containing 0.25% trypsin (Invitrogen) and titrated with a glass pipette to generate a single cell suspension. The cells were centrifuged and re-suspended in PBS with 0.0025% trypsin inhibitor (Invitrogen) and incubated at 37°C for 5 minutes. Cells were re-suspended in explant culture media. The cells were transferred to 0.01% poly-L-lysine (Sigma, St. Louis, MO) coated 8 well chamber slides (Nalge Nunc International, Rochester, NY) and incubated at 37°C for 30 minutes prior to fixation and analysis.

**Cell counting**

For each experimental condition, 2 explanted retinas were placed in a centrifuge tube containing 2 mls of UltraCulture media. The number of cells in each explant culture condition was approximated following dissociation of the retinal explants, by determining density of cells (from known number of explants) in a known volume of
media. For obtaining percentages of cells expressing rod photoreceptor markers, cells were counted using a fluorescence microscope with a 20x objective. Ten fields in each chamber of each 8 well chamber slide were counted.

Statistical analysis

Analysis of the statistical significance of the observed differences between treatments in retinal explant culture was done using SUPERANOVA software (Abacus Concepts, Berkeley, CA). Tukey-Kramer (All means) and Bonferroni-Dunn (all means) tests were performed at a significance level of 0.05. Values are expressed as means ± SEM.

Microscopy

Tissue sections were imaged with a Nikon fluorescence microscope (Nikon Instruments, Melville, NY) equipped with a Retiga 1300 digital camera (QIImaging, Burnaby, BC, Canada). Adobe Photoshop version 9 (Adobe Systems Incorporated, San Jose, CA) was used to crop images and Macromedia Freehand 11 (Macromedia Incorporated, San Francisco, CA) software was used to prepare figures.

Hyperspectral microscope

The Spectral DV (Optical Insights) hyperspectral microscope attached to a Nikon TE2000-E equipped with a Roper Scientific Cascade 512B camera at the Roy J. Carver laboratory for Ultrahigh Resolution Biological Microscopy (Iowa State University, Ames, Iowa) was used to image immunolabeled slides. All fluorescent antibodies, GFP tissue sections, wild type tissue sections and DAPI were imaged to determine their
spectral fingerprints for inclusion in the software’s database. The Tri Filter was used for all experiments. The experimental slides were analyzed and the dark offset removed. The wavelength signatures on the corrected data were plotted and extracted. The plots were then added to the database.

Images analysis was done using Melange Spectral Imaging Software, version 3.4.0 (Optical Insights, LLC). After the dark offset was removed, the corrected data was spectrally unmixed using the spectral definition supplied and each individual fluorophore contribution to the image was extracted. Files were separated and saved. A color overlay was done and colors were assigned for the antibodies of interest. Any fluorophore can be included or removed as desired. Autofluorescence was removed to facilitate visualization of the antibodies of interest.

### 3.4 Results

To determine the developmental distribution of mTOR in the retina, we used immunohistochemistry to examine mTOR immunoreactivity in retinal tissue sections from animals, ages embryonic day (E) 15, E17, postnatal day (P) 0, P5, P10 and adult.

**mTOR immunoreactivity in the retina**

At E15 (Figure 1A) the retina is still largely unlaminated consisting primarily of neuroepithelial cells. At this age, mTOR-immunoreactivity (mTOR-IR) was diffuse throughout the retina, and was more intense in the developing ganglion cell layer (GCL). Similarly, mTOR-IR in the E17 (Figure 1B) retina was diffuse throughout the retina, with more intense mTOR-IR in the inner one-third of the retina. In the P0 mouse retina (Figure 1C), the inner plexiform layer (IPL) is morphologically recognizable. mTOR-IR
was diffusely distributed throughout the retina. In addition, more intense mTOR-IR in cells adjacent to the RPE was visible (asterisks). Diffuse mTOR-IR persisted in the postnatal day 5 retina (Figure 1D). The most intense mTOR-IR was present in the GCL and the inner portion of the inner nuclear layer (INL). The outer one-half of the retina, the presumptive outer nuclear layer (ONL) was also moderately immunoreactive, while the IPL and outer plexiform layer (OPL) were devoid of immunoreactivity. The intense mTOR-IR adjacent to the RPE also seen in P0 persisted in the P5 retina (asterisks). In the P10 retina (Figure 1E), the diffuse mTOR-IR present throughout the retina was less intense than at earlier ages. There were intense immunoreactive puncta in the GCL and inner INL. The developing OPL had fewer mTOR-IR puncta, and the ONL was relatively devoid of mTOR-IR. However, the intense mTOR-IR adjacent to the RPE, presumably developing outer segments, was still present. mTOR-IR in the mature retina (Figure 1F) was most intense in the IPL, OPL and photoreceptor outer segments. Relatively light mTOR-IR was observed in the OFL, GCL and INL, as were faint, distinct, immunoreactive puncta were observed in the ONL.

In mature photoreceptors outer segments are often brightly autoflourescent. We utilized the hyperspectral microscope in order to distinguish mTOR immunoreactivity in the photoreceptors from autofluorescence. The hyperspectral microscope allows us to separate spectra by spectral definition. In the mature retina, with auto-fluorescence removed, mTOR-IR was intense in the inner part of the photoreceptor outer segments (Figure 2).

To determine which downstream targets of mTOR might be expressed in the developing retina, we used immunohistochemistry to characterize the expression of two
major binding partners of mTOR. We used immunohistochemistry to characterize immunoreactivity for raptor and rictor the two major binding partners of mTOR comprising the mTORC1 and mTORC2 complexes, respectively.

**Raptor immunoreactivity in the retina**

At E15 in the undifferentiated retina (Figure 3A), raptor-IR was diffusely distributed but appeared more intense in the developing ganglion cell layer. Raptor-IR at E17 (Figure 3B) was similar to that observed at E15 with the most intense immunoreactivity in the inner one third of the retina. At birth (Figure 3C), the raptor-IR was diffusely distributed throughout the retina with a more intense concentration in the inner third of the retina. Occasional intense immunoreactivity was observed in the GCL. Raptor-IR in the retina at P5 (Figure 3D) was limited to the outer two thirds of the retina, the location of differentiating photoreceptors. By P10 (Figure 3D), however, raptor-IR could be observed throughout the thickness of the retina, though the most intense immunoreactivity was observed in the outer nuclear layer and the photoreceptor outer segments. In the adult retina (Figure 3E), Raptor-IR was diffusely distributed throughout the retina with the most intense immunoreactivity in the IPL, OPL and photoreceptor outer segments.

**Rictor Immunoreactivity in the retina**

Rictor-IR in the developing E15 (Figure 4A) retina was diffusely distributed but much more intense in the presumptive GCL. At E17 (Figure 4B), however, rictor-IR was limited to the inner 1/4 of the retina, in the developing GCL. At P0 (Figure 4C), once again, rictor-IR was diffusely distributed with increased intensity in the GCL as well as cells adjacent to the RPE (arrows). At P5 (Figure 4D), Rictor-IR was diffuse throughout
the retina but was most intense in the outer 3/4 of the retina including the developing INL and ONL. Rictor-IR at P10 (Figure 4E) was most intense in the GCL. It was diffusely distributed throughout the rest of the retina. However, there was also an area of intense Rictor-IR adjacent to the RPE. In the mature retina (Figure 4F), Rictor-IR was most intense in the GCL and the INL. It was present in the IPL and very faint in the ONL and photoreceptor outer segments.

**Functional role for mTOR in retinal development**

To investigate the functional role of mTOR in mammalian photoreceptor differentiation we inhibited mTOR, using rapamycin, in developing retinal explants for 10 days. After 10 days retinas were dissociated and adhered to slides. The slides were then processed for rhodopsin immunoreactivity. The effect of mTOR inhibition on rod photoreceptor differentiation in retinal explants was assayed by counting cells immuno-positive for rhodopsin (Figure 5). In the E17 retina, inhibition of mTOR with 0.1uM rapamycin decreased rod photoreceptor differentiation by 19.3% when compared to the vehicle control (p=0.0017). Compared to control, 1uM rapamycin decreased rods by 16.2% (p=.0083) and 5uM decreased rods by 28.8% (p=0.0001). Though different from vehicle control the differences between experimental groups did not reach statistical significance. In addition, we assayed cell density and cell death in retinal explants to assess whether the addition of rapamycin might be affecting cellular proliferation or cell survival respectively. There were no significant differences in cell density (p=0.95) or cell death (p=0.29) between groups (Figure 6). To investigate the effect of mTOR inhibition at P0, the peak of rod genesis, we assayed the effect of 1uM rapamycin on P0 retinal explants.
However, inhibition of mTOR for 10 days did not significantly affect rod photoreceptor differentiation in P0 explants (p=0.125; data not shown).

3.5 Discussion

Our results demonstrate that mTOR-immunoreactivity is present in developing and mature photoreceptors suggesting that mTOR signaling may play an important role in photoreceptor differentiation. The binding partners for mTOR, raptor (mTORC1) and rictor (mTORC2) are both expressed in developing photoreceptors but only raptor was expressed in mature photoreceptors.

To investigate the effect of mTOR inhibition on rod photoreceptor differentiation, retinal explants from E17 and P0 mice were cultured in a defined medium and treated with rapamycin for 10 days. Expression of the rod specific photopigment, rhodopsin, was used to assay rod differentiation. In control cultures, 20% of the cells expressed rhodopsin. These values are well within the range of what has been reported by others using a similar model system (Chen and Cepko 2007; Donovan and Dyer 2006; Zhang et al. 2004b). The developmental ages chosen for this study were based on their importance in overall rod photoreceptor development. In the E17 retina, there are progenitors leaving the cell cycle and committing to the rod cell fate. Rod photoreceptor genesis does not peak, however, until P0 in the mouse. Therefore, when rapamycin was applied to E17 explants for 10 days, mTOR was inhibited in the majority of cells destined to become rods prior to their exit from the cell cycle and commitment to a rod cell fate.

When rapamycin is applied to P0 retinal explants, however the majority of cells fated to be rods would have already exited the cell cycle and committed to a rod cell fate before mTOR was inhibited. Inhibition of mTOR in E17 (but not P0) retinal explants for 10
days resulted in an approximately 20% decrease in photoreceptor differentiation. There was no difference in explant cell density, or the number of dead cells in explants immediately after dissociation, suggesting, albeit indirectly, that cell proliferation or cell survival was not affected by rapamycin treatment. This suggests that mTOR signaling may mitigate rod photoreceptor differentiation prior to cell fate determination, as inhibition of mTOR in P0 explants did not significantly affect rod differentiation.

The molecule mTOR has many different roles in cell growth and proliferation. Downregulation of mTOR allows differentiation as shown in certain cell lines, vascular smooth muscle and the CNS (Martin et al. 2007; Que et al. 2007; Swiech et al. 2008). In our experiments, however, mTOR activity appeared to promote photoreceptor differentiation as inhibition of mTOR decreased rod differentiation.

The *Drosophila* homologue, dTOR was shown to have an effect on eye development through the protein Tsc-1 as lack of signaling resulted in abnormal eye patterning (Bateman and McNeill 2004).

The effect we induced is likely via mTORC1 (raptor) signaling since we get an effect by adding rapamycin. However a recent study demonstrates the rictor/mTOR complex may be affected by rapamycin as well. This effect is due to the fact that rapamycin eventually binds all the free mTOR. With all of the free mTOR bound, there is no mTOR available to bind to rictor either (Sarbassov et al. 2006).

The IGF-1 signaling pathway when stimulated will increase photoreceptors (personal communication, Hecker and Greenlee). The mTOR molecule is downstream from IGF-1 so the IGF-1 observed effect is potentially realized through mTOR signaling as we observed a decrease in photoreceptor development with inhibition of mTOR. To
further test this hypothesis we would need to add both IGF-1 and rapamycin to our retinal explant cultures. If the increase in rod photoreceptor differentiation we observed is due to the IGF-1 signaling pathway, we would expect rapamycin to attenuate or cancel out the IGF-1 effect.

Presently, the mechanism by which a cell’s fate is determined in the retina is not well understood. Many extrinsic factors have been investigated for their effect on retinal development. These include fibroblast growth factors, Sonic hedgehog (Levine et al. 1997), retinoic acid (Kelley et al. 1994), taurine (Altshuler et al. 1993; Wallace and Jensen 1999; Young et al. 2005), 3-isobutyl-1-methylxanthine (Wallace and Jensen 1999), brain derived neurotrophic factor (Rohrer et al. 1999), leukemia inhibitory factor (Neophytou et al. 1997) and ciliary neurotrophic factor (Ezzeddine et al. 1997). However, it is still unclear how these factors interact with each other and at what ages each is most influential (Reviewed in (Levine et al. 2000)).

Signaling of other molecules has been shown to modulate rod differentiation. Examples demonstrated that the addition of, acidic fibroblast growth factor (FGF), basic fibroblast growth factor (bFGF), taurine, Sonic Hedgehog protein (Shh) and retinoic acid (RA) increased rod photoreceptor differentiation individually (Osakada et al. 2008). Other studies have investigated individual factors and their effect on rod photoreceptor differentiation. FGF1 and FGF2 were some of the first identified factors shown to affect rod photoreceptor differentiation. These factors stimulated rhodopsin expression in rat retinal cells cultured in vitro and increased the number of cells positive for rhodopsin (Hicks and Courtois 1988; Hicks and Courtois 1992). Additionally, FGF was found to cause a proliferation in both rat monolayer cell cultures and retinal explants with
progenitor cells from younger ages being more responsive to FGF (Lillien and Cepko 1992).

Similarly, Sonic hedgehog (Shh) was found to have a positive effect on rod differentiation. When added to rat retinal cell cultures Shh resulted in an increase in the number of differentiated rods (Levine et al. 1997). Retinoic acid (RA) is another extrinsic factor that positively affects rod photoreceptor development. Like Shh, when Retinoic acid (RA) was added to dissociated rat retinal cells, the number of differentiated rods increased (Kelley et al. 1994). Recently, another group demonstrated that RA affects rod development by regulating Nrl (Khanna et al. 2006). They found that Nrl protein increased in cultured rat and porcine photoreceptors as well as in Y79 cells, a human retinoblastoma cell (Khanna et al. 2006).

Each of the aforementioned studies exerted an effect on rod differentiation by the addition of an extrinsic factor. Conversely, in this study we inhibited a signaling pathway. A logical next question would be whether or not we can excite this same pathway and produce an opposite effect. It has been previously demonstrated that the addition of IGF-1 (a known activator of mTOR signaling) does promote rod photoreceptor differentiation in developing retinal explants (Hecker and Greenlee, personal communications). Studies to test the hypothesis that the effect of IGF-1 is due to the activation of mTOR signaling are underway.

Finally, it is possible that the increase in rod differentiation is at the expense of the differentiation of other retinal cell types. The most likely cell types that would be affected are predicted to be bipolar cells and Müller glia as they are two other ‘late born’
cell types (Young 1985a). We have not yet assayed the effect of mTOR inhibition on
differentiation of these alternate cell types.

Our findings indicate that mTOR is important in rod photoreceptor differentiation.
This study is the first study to demonstrate a modulatory role of mammalian TOR in
retinal development. These results contribute to our understanding of rod cell fate
determination and differentiation. Such an understanding may ultimately facilitate the
development of transplantation therapies to treat blinding diseases in which the ability to
influence retinal cell fate is crucial.

Acknowledgements:
The authors would like to thank Nada Pavlovic and Robert T. Doyle for their technical
assistance. This work was supported in part by a grant from the National Institutes of
Health (EY 014931)
3.6 References


3.7 Figure Legends

Figure 1: mTOR is expressed in the developing and mature mouse retina. Expression of mTOR in the C57/Bl6 wild type mouse at ages E15, E17, P0, P5, P10 and adult (A-F). At E15 (A), mTOR-IR was intense in the developing GCL and there was less IR in the NBL. At E17 (B), mTOR-IR was very similar to that seen in E15. In the P0 retina (C), mTOR-IR was diffuse throughout the layers of the retina with an area adjacent to the RPE with more intense IR (asterisks). At P5 (D), mTOR immunoreactivity was observed in the GCL, IPL, ONL and adjacent to the RPE (asterisks). In the P10 retina (E) mTOR-IR was observed in the GCL, IPL, OPL and the OS. Immunoreactivity for mTOR in the adult (F) retina was similar to P10 with intense labeling in the GCL, IPL, OPL and the OS. Scale bars = 10 µM.

Abbreviations: mTOR, Mammalian Target of Rapamycin; E, embryonic; P, post natal; IR, immunoreactivity; GCL, Ganglion cell layer; RPE, retinal pigmented epithelium; IPL, inner plexiform layer; OPL, outer plexiform layer; NBL, neuroblastic layer; OS, outer segment; INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 2: Wild type adult mice double labeled with mTOR (green) and rhodopsin (red). In A, some autofluorescence was evident (asterisks). In image B, autofluorescence and all other fluorophores were removed except mTOR-IR (green). DAPI nuclear stain is represented in blue. Rhodopsin-IR was observed in the rod photoreceptor outer segments. mTOR-IR is more diffusely distributed and was seen in the inner segment, both plexiform layers and the ganglion cell layer.
Abbreviations: mTOR, Mammalian Target of Rapamycin; E, embryonic; P, post natal; IR, immunoreactivity; GCL, Ganglion cell layer; RPE, retinal pigmented epithelium; IPL, inner plexiform layer; OPL, outer plexiform layer; NBL, neuroblastic layer; OS, outer segment; INL, inner nuclear layer; ONL, outer nuclear layer; DAPI, 4’,6-diamidino-2-phenylindole.

Figure 3: Raptor, a binding partner of mTOR is expressed in the developing and mature mouse retina. Expression of Raptor in the C57/Bl6 wild type mouse at ages E15, E17, P0, P5, P10 and adult (A-F). In the E15 (A) retina, Raptor-IR was observed in the developing GCL and throughout the NBL. At E17 (B), Raptor-IR was present in the developing GCL and the NBL. In the P0 retina (C), Raptor-IR was in the GCL, IPL and NBL. At P5 (D), Raptor-IR decreased in the GCL and was present in the IPL and ONL. In the P10 retina (E), Raptor-IR was diffusely distributed with an increased intensity in the ONL and OS. In the adult retina (F), Raptor-IR was observed in the GCL, IPL, ONL and OS. Scale bars = 10μM.

Abbreviations: mTOR, Mammalian Target of Rapamycin; E, embryonic; P, post natal; IR, immunoreactivity; GCL, Ganglion cell layer; RPE, retinal pigmented epithelium; IPL, inner plexiform layer; OPL, outer plexiform layer; NBL, neuroblastic layer; OS, outer segment; INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 4: Rictor, a binding partner of mTOR is expressed in developing and mature mouse retina. Expression of Rictor in the C57/Bl6 wild type mouse at ages E15, E17, P0, P5, P10 and adult (A-F). In E15 (A) retina, Rictor-IR was intense in the GCL and
moderately present in the NBL. At E17 (B), Rictor-IR was similar to E15 with intense IR in the GCL but decreased IR in the NBL. At P0 (C), Rictor-IR persisted in the GCL and was observed in the IPL and NBL. In the P5 retina (D), IR was decreased in the GCL but was observed in the IPL and ONL. At P10 (E), Rictor-IR was again intense in the GCL and present diffusely throughout the rest of the retina. In the adult (F), Rictor-IR persisted in the GCL with the most intensity and was present in the IPL, INL, ONL and OS. Scale bars = 10 μM.

Abbreviations: mTOR, Mammalian Target of Rapamycin; E, embryonic; P, post natal; IR, immunoreactivity; GCL, Ganglion cell layer; RPE, retinal pigmented epithelium; IPL, inner plexiform layer; OPL, outer plexiform layer; NBL, neuroblastic layer; OS, outer segment; INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 5: Inhibition of mTOR modulates rod photoreceptor differentiation. Addition of rapamycin to E17 retinal explants for 10 days decreases rod photoreceptor differentiation. All concentrations (0.1μM, 1μM and 5 μM) caused a significant decrease in rod photoreceptors assayed by rhodopsin expression. All groups were significantly different from the control but not between concentrations. Asterisks indicate significance from the control (* p=0.0017, **p=0.0083, ***p=0.0001).

Figure 6: mTOR inhibition does not affect cell death in E17 retinal explants. Addition of rapamycin to E17 retinal explants for 10 days did not cause a significant decrease in cell death as compared to control for all concentrations (0.1μM, 1μM and 5 μM).
Figure 1
Figure 2
Figure 3

A, B, C, D, E, F: Developmental stages of the retina, showing different layers such as GCL, INL, IPL, NBL, ONL, OS, and OPL.

E15, E17, P0, P5, P10, Adult: Time points for each stage.
Figure 6

mTOR Inhibition Does not Affect Cell Death in E17 Retinal Explants
CHAPTER 4: GENERAL CONCLUSIONS

4.1 Summary

My long term goal is to understand retinal development. In my dissertation work in particular, I wanted to begin to identify the factors that influence rod photoreceptor development. The answer to the question of how a rod becomes a rod holds potential for understanding cell fate decisions broadly as well as biasing cell fate for successful cell transplantations.

The approach we took for the experiments described in this thesis was twofold. The first was a broad discovery based approach characterizing the proteins present during normal mouse retinal development at 6 different ages, embryonic days 15 and 17, birth (P0), post natal days (P) 5 and P10, using proteins expression profiling, specifically 2-dimensional gel electrophoresis with MALDI-MS/MS protein identification. The total number of protein spots were quantified at each age and compared to the other ages for intensity indicating relative quantities or spot volume. These intensity values were clustered based on normalized values. The protein spots chosen for identification were the ones that peaked at ages E17, P0 and P5 because these are the ages important for rod photoreceptor development. E17 is prior to the peak of rod photoreceptor birth, P0 is the peak of genesis and P5 is when rods are differentiating (Young 1985a). These spots were hand picked and submitted for identification by MALDI-MS/MS. We performed a manual verification of the protein identities, which is not customarily done. Once identities were returned, we compared the identified proteins’ isoelectric focusing point (pI) and the molecular weight (MW) to the spot placement on the gel. If they did not fall within plus or minus one unit in either direction for pI and MW, they were not considered
reliable ID’s. This is because anything outside of this range was thought to be an unreliable identification. Further, we performed an internet search of Pubmed for these proteins associated with the retina, CNS development and/or cancer.

The total number of spots picked was 239. Of these, 170 returned reliable results for a percentage value of 71.1. There were 60 spots picked from E17 gels and 44 were identified. At P0 there were 56 proteins picked and 21 of these were reliably identified. Of 123 protein spots picked at P5, 102 were identified. There were 23 protein identifications present in multiple spots, i.e. with differing MW’s, pI’s or both. This result suggests the possibility of post-translational modifications. Common post-translational modifications include phosphorylation and glycosylation. These proteins could be examined in the future for the presence of post-translational modifications. For example, the use of a phosphoprotein stain could identify proteins that have been phosphorylated post-translationally. These modifications may give a more in depth picture of what these proteins roles are in rod photoreceptor cell fate. There were many proteins identified that had never before associated with the retina or retinal development. Of these proteins some were associated with CNS development while others were associated with neoplastic processes.

We discovered that there are some limitations to this approach. It was more difficult than anticipated to compare the protein database results to existing RNA databases. There could be many reasons for this. For example, the presence of RNA does not necessarily indicate the presence of the translated protein. Also, even at 71% protein identification, which is considered successful in this method, there are obviously proteins present but unaccounted for because they were simply not identified. Additionally, this
broad discovery based approach is costly and time consuming. It was perhaps an overly ambitious endeavor.

4.2 Recommendations for further research

There are many future directions for this work. The proteins represented by multiple spot areas could be examined for post-translational modifications. The most common post-translational modification is phosphorylation (Paradela and Albar 2008). This could be examined using a readily available phosphoprotein gel stain and imaging. The presence of glycosylation could be studied using a glyco stain and imaging (Temporini et al. 2008). The proteins associated with cancer could be looked at for a role in the cell cycle of retinal development. Those proteins associated in the literature with CNS development could be investigated for their role in retinal development. Any of the proteins could be examined as we did for mTOR, first looking at it’s distribution in retinal tissues during development and then performing a functional study by exciting or inhibiting the protein in retinal explants when reagents are available. Molecules best suited to study would be those associated with a signaling pathway.

The second approach was to examine one specific protein, mTOR, and it’s role in rod photoreceptor fate. The insulin-like growth factor-1 (IGF-1) was previously found to promote rod photoreceptor differentiation (personal communications, Hecker and Greenlee). Since mTOR is an important cell growth and proliferation molecule downstream of IGF-1 we hypothesized that the observed IGF-1 effect may be the result of signaling through the mTOR signaling pathway. We characterized mTOR immunoreactivity (mTOR-IR) in retinal tissues during development. We then examined its binding partners, raptor and rictor, in retinal tissues during development. The
function of mTOR in retina has not been described but it has a role in cell growth and proliferation in other tissues. To examine its role in rod photoreceptor development we inhibited it with rapamycin in E17 retinal tissue explants. The embryonic age day 17 was chosen because this is an age prior to the peak of rod photoreceptor genesis. The explants were cultured for 10 days. This effect was not observed in P0 explants.

mTOR immunoreactivity was seen in the developing retina at all ages and was not limited to one layer or cell type. The hyperspectral results confirmed that mTOR was in the rod photoreceptor outer segments. The binding partners for mTOR, raptor and rictor, were also present throughout development.

mTOR inhibition causes a decrease in rod photoreceptors. In the E17 retina, inhibition of mTOR with 0.1uM rapamycin decreased rod photoreceptor differentiation by 19.3% when compared to the control. Compared to control, 1uM rapamycin decreased rods by 16.2% and 5uM decreased rods by 28.8%. Our results suggest that mTOR has a role in modulating rod photoreceptor differentiation. Future directions include determining other molecules that are involved with mTOR in modulating rod differentiation. Another experimental approach could look at what happens to these cells. Rods are downregulated but is there another cell that is upregulated? Is there just a delay in rod differentiation or could they be in a suspended undifferentiated state? Another question that could be asked is what happens to the rod photoreceptors when mTOR is stimulated.
4.3 Concluding remarks

My goals for this PhD study were to examine retinal development focusing on cell fate decisions of rod photoreceptors. By taking a proteomics approach I achieved part of this goal by getting a snapshot of many of the proteins present during the ages important for rod photoreceptor development.

To summarize, I characterized protein expression in developing mouse retina at ages important to rod photoreceptor development. I then identified and characterized a signaling molecule that can modulate rod differentiation. These are contrasting approaches with the first being a very broad method examining all the expressed proteins as opposed to the second approach that characterizes only one molecules’ role in rod differentiation. Both of these approaches served to increase our knowledge of the mechanisms of rod photoreceptor development.

4.4 References