Characterizing and influencing differentiation of Retinal Progenitor Cells

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Characterizing and influencing differentiation of Retinal Progenitor Cells

by

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in partial fulfillment of the requirements for the degree of

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<td>RPCs</td>
<td>Retinal Progenitor Cells</td>
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<td>BPCs</td>
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<td>AHPCs</td>
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<td>Hsp60</td>
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<td>Hsp70</td>
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<td>Cu-Zn SOD</td>
<td>Copper-Zinc Superoxide Dismutase</td>
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<td>E</td>
<td>Embryonic</td>
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<td>P</td>
<td>Postnatal</td>
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<td>GCL</td>
<td>Ganglion Cell Layer</td>
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<td>INL</td>
<td>Inner Nuclear Layer</td>
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<td>ONL</td>
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<td>NBL</td>
<td>Neuroblastic Layer</td>
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<td>OFL</td>
<td>Optic Fiber Layer</td>
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<td>IPL</td>
<td>Inner Plexiform Layer</td>
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<td>OPL</td>
<td>Outer Plexiform Layer</td>
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<td>OS</td>
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ABSTRACT

Blinding degenerative retinal diseases including retinitis pigmentosa, macular degeneration and glaucoma are characterized by loss of retinal neurons. At this time there is no way to replace retinal cell loss due to disease or injury since differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal injury, neural progenitor cells have been proposed as a unique source of transplantable cells to replace lost cells in the damaged retina. Previous studies have transplanted a variety of neural stem cells to the eye in hopes of developing a therapy to replace retinal neurons lost to disease. Successful integration, survival and differentiation of the cell types have been variably successful. At the moment little is known about the fundamental biological differences between stem cell or progenitor cell types. Characterization of these differences will not only increase our general understanding of this broadly characterized group of cells, but also lead to development of criteria for sorting cells, evaluating their differentiation and predicting their suitability for transplantation.

In this dissertation we used protein expression profiling to characterize the molecular differences between two populations of in vitro expanded progenitor cells, retinal progenitor cells (RPCs) and brain progenitor cells (BPCs) isolated from mice of the same age and same genetic background. From this study we identified 4 stress-response proteins that were increased in expression in RPCs compared to BPCs. To see if these stress-response proteins were expressed during normal development, we used immunohistochemistry to characterize their expression in the developing retina. Finally, we tested the hypothesis that attenuation of oxidative stress would decrease the expression of stress-response proteins. We found that heat shock 60 (Hsp60), heat shock protein 70 (Hsp70), copper-zinc superoxide dismutase
(Cu-Zn SOD) and catalase (CAT) are dynamically expressed in the developing retina. Further, we report that treatment of cultured progenitors with the antioxidant vitamin E (alpha-tocopherol) decreases expression of these proteins and alters their differentiation. These results are the first to characterize the expression of stress-response proteins during retinal development and demonstrate that reduction of oxidative load on cells can alter their differentiation profile.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

The general introduction is divided into several sections. The first section describes the development of the retina. The second section explains the isolation and expansion of neural progenitor cells. The third section describes the morphology, integration, and survival of progenitor cells once transplanted into normal and diseased retinas. The fourth section focuses on various signaling molecules that can affect differentiation of retinal progenitor cells. The fifth section describes the expression of stress-response proteins during development. The sixth section explains oxidative stress. Sections seven and eight give details on the different type of proteomic approaches and how gene ontologies are used.

RETINAL DEVELOPMENT

The vertebrate neural retina is a complex organ that is well suited for studying development of the central nervous system. The retina is often used for experimental manipulation because of its accessibility. During development the neural retina forms from an outpocketing of the diencephalon called the optic vesicle. The head ectoderm is made competent to form a lens by a series of inductive interactions during early development (Purves et al., 2004). When the optic vesicle contacts the head ectoderm, it sends inductive signals that cause the epithelium to form a lens placode. The lens placode invaginates, pinches off from the surface and differentiates into the lens (Purves et al., 2004). As a result the optic vesicle bends around the lens and forms a two-layered structure, the optic cup. The inner layer of the optic cup gives rise to the neural retina, while the outer layer gives rise to the retinal pigment epithelium (RPE) (Purves et al., 2004). Once the retinal epithelium is
specified retinal cell types are generated from a common precursor, the retinal progenitor cell, which gives rise to all retinal cell types (Levine and Green, 2004). These multipotent retinal progenitor cells are able to proliferate, exit the cell cycle and commit to a specific cell fate in response to both intrinsic and extrinsic signals (Cepko et al., 1996; Donovan and Dyer, 2005). Retinal cell types are generated in a conserved sequence with the early born cells exiting the cell cycle first followed by later born cells. The early-generated cohort of cells includes ganglion, amacrine, horizontal and cone photoreceptor cells, while the later cohort consists of bipolar, Mueller glia and rod photoreceptor cells. Various studies have given considerable insight into cell type specification in the vertebrate retina. R.W. Young (1985) did one of the first studies of birth dating of retinal cell types using triturated ([³H]) thymidine labeling. Young’s study demonstrated that retinal cell types were born in cohorts that overlapped in the times at which they were produced. Subsequently, lineage analysis using intracellular injection of tracers or retroviruses has shown that retinal cells, including both neurons and glia can arise from common progenitors (Turner and Cepko, 1987; Wetts and Fraser, 1988).

Once progenitor cells leave the cell cycle they migrate to their appropriate nuclear layer within the retina. The retina is composed of three layers of nerve cell bodies separated by two synaptic layers. The ganglion cell layer (GCL) contains cell bodies of ganglion cells and displaced amacrine cells. The inner nuclear cell layer (INL) contains cells bodies of the horizontal, bipolar, and amacrine cells. Mueller glia also has their cell bodies located in the INL. The outer nuclear cell layer (ONL) contains cell bodies of the photoreceptors, which are the rods and cones. Between each nerve cell layer is a synaptic layer called a plexiform layer. The synaptic layer between the ganglion cell layer and inner nuclear layer is the inner
plexiform layer (IPL) where connections between bipolar, amacrine, and ganglion cells occur. The outer synaptic layer is called the outer plexiform layer (OPL) and is located between the inner nuclear and outer nuclear layers. This OPL is made up of synaptic contacts between photoreceptors, bipolar and horizontal cells.

**ISOLATION AND EXPANSION OF NEURAL STEM CELLS**

Studies have shown that mitotically active progenitor cells from developing and adult rodents can be successfully isolated and expanded *in vitro* by addition of the growth factors basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) in the media. These cells are multipotent and have the ability to differentiate into neurons, astrocytes and oligodendrocytes. Shatos and colleagues (2001) isolated and expanded retinal and brain progenitor cells from the mouse neonatal neuroretina and brain respectively. These cells were able to proliferate into neurospheres in the presence of EGF and bFGF. Upon removal of growth factors the retinal progenitor cells (RPCs) began to differentiate and express neuronal markers, whereas the brain progenitor cells (BPCs) began to express neuronal and glial markers (Shatos et al., 2001). Gage and colleagues (1995) cultured and expanded adult rat hippocampal progenitor cells (AHPCs) in the presence of the mitogen FGF-2. These cells proliferated and formed neurospheres, which expressed glial and neuronal markers upon differentiation by the removal of FGF-2. Ciliary body cells have also been isolated, cultured and expanded *in vitro* in the presence of mitogens EGF/FGF2 (Tropepe et al., 2000). These cells can also be differentiated into cells that express neuronal and glia markers (Ahmad et al., 2000; Tropepe et al., 2000; Ahmad et al., 2004).
progenitor cells were able to express retina specific markers, this suggested that they possess the potential to differentiate into different retinal cell types (Ahmad et al., 2000).

**TRANSPLANTATION OF PROGENITOR CELLS**

Blinding degenerative retinal diseases including retinitis pigmentosa, macular degeneration and glaucoma are characterized by loss of retinal neurons. At this time there is no way to replace retinal cell loss due to disease or injury since differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal injury, neural progenitor cells have been proposed as a unique source of transplantable cells to replace lost cells in the damaged retina. Generally, single-cell suspensions of cultured neural progenitor cells are transplanted into the subretinal space (space between the photoreceptors and retinal pigmented epithelium) or the vitreous of the eye. There are various cultured progenitor cell types that have been transplanted into the retina including retinal progenitor cells (RPCs) (Qiu et al., 2005) brain progenitor cells (BPCs) (Sakaguchi et al., 2003), adult hippocampal progenitor cells (Takahashi et al., 1998) (AHPCs) and ciliary-derived cells (Tropepe et al., 2000). Transplantation of different progenitor cell types have been variably successful; however, little is known about the fundamental biology of these cell types and how that may influence their successful transplantation.

Neural progenitor cells isolated from the brain (BPCs) and neuroretina (RPCs) of mice have been transplanted subretinally and intravitreally into normal retinas and diseased retinas (Lu et al., 2002; Mizumoto et al., 2003; Sakaguchi et al., 2003; Van Hoffelen et al., 2003; Sakaguchi et al., 2004). These cells express GFP, have the ability to migrate into specific laminar layers and differentiate into various cell types. Van Hoffelen and colleagues
(2003) transplanted mBPCs intravitreally into developing retinas of Brazilian opossums. One week after transplantation the GFP positive cells were observed throughout the posterior segment of the eye, though there was no cell integration of the cells within the neural retina. However, at four weeks post-transplantation, the GFP-expressing cells were incorporated throughout the retinal layers and morphological differentiation and integration was observed (Van Hoffelen et al., 2003). This study suggested that BPCs were capable of surviving, integrating and differentiating in the host retina. Mizumoto and colleagues (2003) transplanted GFP-BPCs subretinally into normal retinas. They showed that one-week after transplantation of BPCs into the normal retina, the GFP positive cells had migrated to the outer and inner plexiform layers and the inner nuclear layer. After 2-3 weeks the GFP positive cells migrated throughout all the retinal layers and showed positive expression of nestin, GFAP, MAP2 and NF200 (Mizumoto et al., 2003) suggesting that the cells were capable of differentiating into neurons and astrocytes, as well as integrating and surviving within the normal host retina. Sakaguchi and colleagues (2004) transplanted GFP-RPCs intravitreally into developing retinas of Brazilian opossums ages postnatal (PN) 5, 10, and 15. Three weeks after the transplantation the RPCs were located near the inner retina and along the inner limiting membrane (ILM) or vitreous. However after three weeks the RPCs were found within the retina and observed in the GCL, IPL, INL and occasionally in the ONL (Sakaguchi et al., 2004). Further, the cells had morphological characteristics of some retinal cell types. This suggested that the RPCs were also capable of survival, differentiation and integration within the host retina.

Neural progenitor cells have also been transplanted into diseased retinas. Lu and colleagues (2002) transplanted GFP positive BPCs subretinally into rd mice and observed
that approximately 50% of the cells had migrated into the host retina 2 weeks after transplantation. These migrated cells showed morphological characteristics of neurons and glia. Less than 1% of the neurons looked like mature retinal cells, however four weeks post-transplantation approximately 10% of the cells assumed the general morphology of amacrine, horizontal and ganglion cells (Lu et al., 2002). Their cell bodies were seen in the ganglion cell layer, inner cell layer, or outer nuclear layer and their processes were distributed into the corresponding plexiform layers. However, after four weeks the cells failed to express any markers characteristic of retinal cells (Lu et al., 2002). Mizumoto and colleagues (2003) also transplanted GFP-BPCs into the subretinal space of rd mice. Once this occurred the GFP positive cells migrated to the ganglion cell layer and inner plexiform layer where they extended processes. These GFP positive cells not only migrated but also expressed nestin and GFAP (Mizumoto et al., 2003). This suggests that diseased retinas usually show better results with transplantation.

Adult rat hippocampal progenitor cells (AHPCs) are multipotent cells that are able to self-renew (Palmer et al., 1997). These cells have been isolated (Palmer et al., 1997), cultured (Gage et al., 1998) and transplanted into the retina (Takahashi et al., 1998; Young et al., 2000; Sakaguchi et al., 2004). Previous studies have shown that AHPCs are able to integrate and migrate into specific neuronal layers of normal neonatal rat retinas (Takahashi et al., 1998) as well as damaged retinas from adult rats (Nishida et al., 2000; Young et al., 2000). Takahashi and colleagues (1998) found that within four weeks of transplanting AHPCs into the normal neonatal rat retina, integration of transplanted cells were observed within most layers with appropriate cell morphologies though expression of appropriate cell-specific antigens was not observed. Other studies have also shown that within four weeks post-
transplantation AHPCs had migrated, integrated and differentiated into the various layers of
the retina (Gage et al., 1998; Young et al., 2000; Sakaguchi et al., 2004). However, when
these cells were injected into the eyes of normal (no injury or damage) adult rats there was no
integration or neuronal differentiation of the cells (Takahashi et al., 1998; Nishida et al.,
2000; Young et al., 2000). This suggests that AHPCs are able to migrate, integrate and
survive better within neonatal retinas than normal adult retinas.

However, when AHPCs were transplanted into dystrophic retinas of immature and
mature rats at least half of the cells survived and started to migrate into the retinal layers
following transplantation (Young et al., 2000). There was neuronal differentiation, migration
and integration observed within the retina suggesting that AHPCs may be appropriate for
transplantation into damaged or injured retinas (Nishida et al., 2000; Young et al., 2000).

FACTORS THAT AFFECT DIFFERENTIATION OF RETINAL CELLS

There are various signaling molecules that can affect the in vitro differentiation of
retinal cells, in particular to differentiate into rod photoreceptors. Examples of these
molecules are: taurine, retinoic acid (RA), epidermal growth factor (EGF), transforming
growth factor-alpha (TGF-α), basic fibroblast growth factor (bFGF), and ciliary neurotrophic
factor (CNTF). They can either stimulate or inhibit the in vitro development of retinal
progenitor cells. Taurine is a stimulatory factor for rod photoreceptors and has been shown to
promote differentiation of rat rod photoreceptors. Altshuler and colleagues (1993) used low-
density cell cultures of P0 rat retinas and found a low-molecular weight fraction from
conditioned medium could satisfy the requirement for high cell density in rod differentiation.
Since taurine was found to be present in the medium and when added to the culture caused an
increase in the number of rhodopsin-expressing cells (Altshuler et al., 1993). Even though, there was an increase in the number of rhodopsin-expressing cells, taurine did not significantly affect the overall cell number in the cultures (Altshuler et al., 1993).

Retinoic acid (RA) is also a stimulatory factor for rod photoreceptors. Cell culture studies have shown that the addition of RA to dissociated embryonic mammalian retinal cells causes an increase in the number of rod photoreceptors in a dose-dependent manner (Levine et al., 2000). Kelley and colleagues (1994) used photoreceptor-specific antibody markers (anti-recoverin and anti-rhodopsin) to assess the effects of RA. They found an increased in the number of cells expressing these markers after 2-8 days in vitro. The effect on photoreceptor differentiation was specific since other cell types produced at this point in development, like amacrine cells, did not increase with RA treatment but were inhibited instead. This shows that RA increases the number of rod photoreceptors at the expense of other cell types.

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are both inhibitory factors that decrease differentiation of rod photoreceptors (Lillien and Cepko, 1992). Anchan and colleagues (1991) first reported that these factors were mitogens for retinal RPCs and they were subsequently shown to be mitogenic for progenitor cells in many areas of the CNS. The activation of the EGF receptor (EGFR) by these factors causes RPCs to proliferate in vitro (Levine et al., 2000). Activation of the EGFR by these factors suppresses the differentiation of rod photoreceptors however; removal of these factors in culture medium allows at least some of the RPCs to differentiate into rod photoreceptors.

Hicks and colleagues (1988) showed that addition of partially purified FGF fraction (containing both FGF1 and FGF2) stimulated rhodopsin expression levels in dissociated P0
rat retinal cells grown as monolayers. Another study by Hicks and colleagues (1992) demonstrated that addition of FGF2 to these cultures caused an increase in the number of rhodopsin-expressing photoreceptors. This effect was specific to FGF2, since neither EGF nor nerve growth factor elicited a similar response (Hicks and Courtois, 1992). In their assays, other cell types were largely unaffected and the increase in rhodospin-expressing photoreceptors did not appear to be linked to enhanced proliferation or survival, suggesting that FGF2 is a differentiation factor for immature rods that is limiting in monolayer culture (Hicks and Courtois, 1992).

Ciliary neurotrophic factor (CNTF) can support survival in certain types of developing neurons and promote the expression of phenotypic properties in other neurons (Sendtner et al., 1994). CNTF can inhibit the proliferation of progenitor cells and influence the choice of their developmental fate (Ezzeddine et al., 1997). In the vertebrate retina, CNTF regulates the differentiation of rod photoreceptors during a transient period of development however; the effects are the opposite in chick and rat retina using rhodopsin as the marker (Levine et al., 2000). CNTF expression has been noted in the embryonic retina and in Mueller glia postnatal retina. Treatment of CNTF inhibits rod differentiation in the rodent retina in vitro (Ezzeddine et al., 1997). Ezzeddine and colleagues (1997) found that more rods develop in retinal explant cultures of mice lacking a functional ciliary neurotrophic factor receptor. Therefore, CNTF appears to act as a specific negative regulator of rod development in rodents.
EXPRESSION OF STRESS-RESPONSE PROTEINS DURING DEVELOPMENT

Heat shock proteins (Hsp) are a group of proteins that are present in all cells. These proteins are needed for normal cell growth and maintenance and have been detected during embryogenesis in various organisms (Calabrese et al., 2002). Developmental profiles of Hsps have suggested that these proteins have a role in neural cell differentiation (Calabrese et al., 2002). Hsps are believed to function as molecular chaperones (i.e., proteins that assist other proteins to fold properly) under normal and stressful conditions (Kaarniranta et al., 2002). Under stress, cellular proteins may misfold and Hsp chaperones facilitate refolding of the misfolded proteins; therefore promoting cell survival. A majority of heat shock proteins fold, unfold, bind, transport, assemble and chaperone other proteins in the cell thereby influencing their function.

Heat shock protein 60 (Hsp60) is present in the cytoplasm as well as in dendritic processes of neurons (D'Souza and Brown, 1998; Calabrese et al., 2002). Functions of Hsp60 are dependent on Hsp10, which binds to Hsp60 and regulates its substrate binding and ATPase activity (Richter-Landsberg and Goldbaum, 2003). Hsp60 also participates in the folding, assembly and transport of proteins into the mitochondria (Richter-Landsberg and Goldbaum, 2003). Developmental profiles have shown that Hsp60 increases during postnatal development in the rat brain stem and the cerebral hemispheres; thus Hsp60 levels are significantly higher at P20 and in adult compared to P1 in these regions (Calabrese et al., 2002).

Heat shock protein 70 (Hsp70) is synthesized at high levels and is present in the cytosol, nucleus and endoplasmic reticulum (Calabrese et al., 2002). Expression of the gene encoding Hsp70 has been found in numerous cell populations within the nervous system,
including neurons, glia and endothelial cells (Takeda et al., 2000; Calabrese et al., 2002). Hsp70 is expressed in the external plexiform layer in the olfactory bulb, ventrally in the septum and dorsally in the neocortex before embryonic (E) 15.5 in mice (D'Souza and Brown, 1998; Loones et al., 2000; Calabrese et al., 2002). By E15.5 in mice, Hsp70 is detectable in the central nervous system and at E17.5 all Hsps are expressed in the hippocampus (Loones et al., 2000; Calabrese et al., 2002). Developmental analysis in the postnatal rat brain has shown that basal levels increased in the cerebral hemisphere until postnatal (P) 20 and then decreased in the adult, whereas there was little change observed in the cerebellum during postnatal development (D'Souza and Brown, 1998; Calabrese et al., 2002).

Thioredoxin (TRX) is small protein (12-13kDa) known to function as an antioxidant protein catalyzing thiol-disulfide oxido-reductions (Holmgren, 1985). TRX has the conserved amino acid sequence Cys-Gly-Pro-Cys in its active site (Holmgren, 1985; Carvalho et al., 2006) where the two cysteines are able to participate in reversible oxidation-reduction reactions (Holmgren, 1985). TRX appears to play biologically important roles in hormone secretion, cell signaling, regulation of the intracellular apoptotic pathway and cell proliferation (Saitoh et al., 1998; Kobayashi-Miura et al., 2002; Tanito et al., 2002). TRX has also been shown to regulate the DNA binding activity of various transcriptional factors such as nuclear factor (NF-kB), activator protein 1 (AP-1), myb, redox factor 1 and mitogen-activated kinase (Okamoto et al., 1992; Hirota et al., 1997). Meyer and colleagues (1993) have reported that Trx induces AP-1 through de novo transcription of c-Fos and c-Jun therefore leading to a possible neuroprotective function (Takagi et al., 1999). A study by Saitoh et al (1998) has shown that Trx is a physiological inhibitor of the apoptosis signal-
regulating kinase (ASK) 1. Here the Trx binds to the N-terminal of the ASK1 and inhibits the kinase activity as well as the ASK1 dependent apoptosis (Saitoh et al., 1998). While many of the functions attributed to Trx are known to be important developmental processes, we are not aware of any specific studies of the role of Trx during neural development.

Copper-Zinc superoxide dismutase (Cu-Zn SOD) is a key cytosolic enzyme present throughout the CNS and the PNS (Przedborski et al., 1996). Cu-Zn SOD is considered to be one of the key enzymes used by cells to protect themselves against oxygen free radicals (Fridovich, 1995; Przedborski et al., 1996). The main function of Cu-Zn SOD is to catalyze the conversion of superoxide anion to hydrogen peroxide (Przedborski et al., 1996; Jaarsma et al., 2000; Selverstone Valentine et al., 2005). The removal of hydrogen peroxide is then facilitated by catalase and glutathione peroxidase, which are two other free radical scavenging enzymes (Przedborski et al., 1996; Khan and Black, 2003). In mouse brain, Cu-Zn SOD immunoreactivity is found in neurons, the cerebral cortex and hippocampus as well as in anterior horn of the spinal cord and substantia nigra (Pardo et al., 1995). A study using mRNA profiles reported an increase in Cu-Zn SOD in the brains of late-gestation and neonatal mouse fetuses (de Haan et al., 1994). Developmental studies on the brains of mice found that protein levels were low at embryonic (E) 18 and postnatal (P) 1 compared to P21 (Khan and Black, 2003).

Catalase (CAT) is a common enzyme located in the peroxisome. Its functions include catalyzing the decomposition of hydrogen peroxide to water (Przedborski et al., 1996; Khan and Black, 2003). This catalytic reaction protects the cell from the toxic effects of hydrogen peroxide (Oh et al., 2005). Developmental changes in embryonic and postnatal brains of mice showed that protein levels for catalase were high at E18 and remained elevated throughout
development (Khan and Black, 2003). In rats, CAT activity decreased in the cerebral cortex, striatum, cerebellum and brainstem between 11 and 40 weeks of age (Del Maestro and McDonald, 1987).

**OXIDATIVE STRESS**

We have described an increase in expression of stress-response proteins when comparing proliferating retinal progenitor cells to brain progenitor cells. This raised the possibility that the RPCs were under some type of cellular stress. Oxidative stress is a common cellular stress and can occur in cells at any time when there is an increase in oxidant generation, a decrease in antioxidant protection or a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species (ROS). ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. ROS can damage a cell’s membrane, genetic material and cause cell death. Glaucoma and retinitis pigmentosa are 2 diseases in which oxidative stress is a component of the pathogenesis. Glaucoma is a degenerative disease, which affects at least 2.5 million Americans and can lead to blindness. Vision is lost because of damage to the optic nerve, which carries information from the eye to the brain (Izzotti et al., 2006). Oxidative damage in glaucoma is caused by free radicals that lead to the production of toxic substances that interact with oxygen (Izzotti et al., 2006). Retinitis pigmentosa is a genetic condition of the eye that affects the photoreceptors (rods) and can lead to progressive vision loss. Once the rods begin to die the cones will progressively follow. Rods consume a high level of oxygen therefore, as the rods die the tissue level of oxygen in the retina increases (Shen et al., 2005). This oxygen increase is very
damaging to the photoreceptors and results in the generation of reactive oxygen species, which causes the cells to die (Shen et al., 2005). Using antioxidants like alpha-tocopherol, also known as vitamin E, is a way to attenuate the damage done by ROS. Alpha-tocopherol is one of the most active forms of vitamin E in humans and is a very powerful and useful antioxidant. This antioxidant protects the cells against the effect of free radicals, which can be potentially damaging to the body.

PROTEOMICS

Proteomics is an emerging approach that involves identifying, characterizing and quantifying proteins in whole tissues, cells or body fluids (Guo et al., 2007). Protein characterization includes post-translational modifications, amino acid sequence analysis and the identification of its binding partners and cellular localization (Guo et al., 2007). Proteins are often expressed in quantities and physical forms that cannot be predicted from DNA and mRNA analysis; therefore proteomics is useful in these contexts. Quantitative analysis of global protein expression is important for the system-based understanding of the molecular function of each protein component and is expected to provide insights into molecular mechanisms of various biological processes and systems (Yan and Chen, 2005). There are several proteomic technologies used in protein profiling.

The classical proteomics technology is two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS) (Monteoliva and Albar, 2004; Yan and Chen, 2005; Guo et al., 2007). Two-dimensional gel electrophoresis is a simple and easy visual method used for mapping differences in protein expression (Monteoliva and Albar, 2004). In this method, proteins of interest are separated by 2-DE (isoelectric focusing (IEF) and molecular
weight). Two-dimensional gel electrophoresis images are analyzed by specialized software and differentially expressed protein spots are picked from gels, proteolytically digested and analyzed by matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI TOF-MS). The peptide mass fingerprint (PMF) is matched against genomic or protein databases to obtain candidate proteins. Tandem mass spectrometry (MS/MS) and peptide sequencing by analysis of fragmentation spectra can assist in identifying peptides when ambiguity remains after MALDI-TOF analysis. There are limitations to using 2-DE technology such as poor solubility of membrane proteins, low detection sensitivity, limited loading capacity of gradient pH strips, high cost and reproducibility of gels (Monteoliva and Albar, 2004; White et al., 2004; Yan and Chen, 2005; Guo et al., 2007).

Another proteomic technology is differential in-gel electrophoresis (DIGE). This technology uses fluorescent labeling of cell extracts with one of three fluorescent dyes (Cy2, Cy3, and Cy5) prior to gel separation therefore cellular protein levels of two different samples can be compared within a single gel. The three dyes are matched in mass and charge (so they won’t differentially effect in-gel migration) and each has a different emission wavelength. The labeled samples are then combined and subjected to 2-DE (Monteoliva and Albar, 2004; Guo et al., 2007). The gel is imaged at different emission wavelengths and multiple images corresponding to a set of samples are generated and overlaid. DIGE allows the differentially regulated proteins to be viewed as changed in color (Monteoliva and Albar, 2004; Guo et al., 2007). DIGE significantly improves gel reproducibility and minimizes alignment issues some issues regarding quantification and translating gel maps to allow protein spot excising for downstream mass spectrometry identification remain (Guo et al., 2007).
GENE ONTOLOGY

Since the completion of the human genome-sequencing project there has been an accumulation of vast amounts of biological data (Lei and Dai, 2006). A way to integrate that information for biologists is through the development and use of annotation standards such as ontologies (Harris et al., 2004). In order to help manage the vast amount of current biological data that are difficult to tie together gene ontologies (GO) have been developed. GO contains predefined terms that have already been pre-selected to describe genes and their gene product attributes (Harris et al., 2004). GO can be split into ontologies and annotations. Ontologies cover the areas of Molecular Function (MF), Biological Process (BP), and Cellular Component (CC).

Molecular Function describes activities, such as catalytic or binding activities, at the molecular level. GO molecular function corresponds to activities rather than the entities that perform the actions and do not specify where, when, or in what context the action takes place. Biological Processes describe biological goals accomplished by one or more ordered assemblies of molecular functions. Cellular Component describes locations at the levels of subcellular structures (Harris et al., 2004; Lei and Dai, 2006).

Annotations are used to characterize the gene product with supporting documentation. Annotations are attributed to a source, for example another database or literature reference and must indicate the type of evidence the cited source provides to support the association between the gene product and the GO term (Harris et al., 2004). Evidence codes are usually used to qualify annotations with respect to different types of experimental determinations. For example, inferred from direct assay (IDA) is more reliable than inferred from physical interaction (ISS). This dissertation explores the comparison of 2 different populations of
expanded cells, characterization of stress-response proteins in the developing retina and influencing differentiation of retinal progenitor cells \textit{in vitro}. 
DISSERTATION ORGANIZATION

Chapter one of this dissertation gives a general overview of topics related to this research. Chapter two describes the work I did using protein expression profiling to characterize the molecular differences between two populations of in vitro expanded progenitor cells, retinal progenitor cells (RPCs) and brain progenitor cells (BPCs) isolated from mice of the same age and same genetic background. In this work I identified 4 stress-response proteins that were increased in expression for RPCs when compared to BPCs. In chapter three I characterized the developmental expression of stress-response proteins Hsp60, Hsp70, Cu-Zn SOD, and catalase in the mouse retina at embryonic (E) 15, E17, postnatal (P) 1, P5, P10, and adult. Chapter four describes the affects vitamin E has on differentiation of cultured retinal progenitor cells in vitro. The final chapter, chapter five contains general discussion and conclusions that can be obtained from this work as well as future experiments that can be used to further our understanding of this broadly characterized group of cells in hopes of predicting their suitability for transplantation.

In general, each chapter is organized as a paper ready for submission to a scientific journal. Chapter two has already been submitted to Stem Cells and Development and chapters three and four are ready for submission. Each paper begins with an abstract, introduction, materials and methods, results, discussion/conclusion, and references. The figures and tables for the papers appear at the end.

REFERENCES


CHAPTER 2. PROTEOMIC DIFFERENTIATION BETWEEN MURINE RETINAL AND BRAIN DERIVED PROGENITOR CELLS

A paper submitted to *Stem Cells and Development*

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ABSTRACT

Previous studies have transplanted a variety of neural stem cells to the eye in hopes of developing a therapy to replace retinal neurons lost to disease. Successful integration, survival and differentiation of the cell types have been variably successful. At the moment little is known about the fundamental biological differences between stem cell or progenitor cell types. Characterization of these differences will not only increase our general understanding of this broadly characterized group of cells, but also lead to development of criteria for sorting cells, evaluating their differentiation and predicting their suitability for transplantation. We have used two-dimensional gel electrophoresis protein expression profiling to characterize the molecular differences between two populations of murine progenitor cells; retinal progenitor cells and brain progenitor cells isolated from mice of the same age and same genetic background. Our protein expression profiling identified 22 proteins that are differentially expressed in retinal progenitor cells when compared to brain progenitor cells. Four of the differentially expressed proteins correspond to proteins known to be involved in a cellular response to stress, and analysis of potential transcription factor
binding sites in the promoter regions of their genes suggests these proteins could be co-regulated at the transcriptional level.

Further investigation of differences between multiple populations of retinal progenitor cells and brain progenitor cells during their maintenance and differentiation will further identify fundamental differences that define ‘retinal-like’ characteristics, and provide tools to assay the success of efforts to bias many populations of stem cells to adopt a retinal cell fate.

INTRODUCTION

The vertebrate retina is a well-studied model of neural development and has facilitated a better understanding of neural progenitor cell fate determination (Cepko et al., 1996). All retinal cell types are all generated from a common precursor (Levine and Green, 2004). Multipotent retinal progenitor cells are able to proliferate, exit the cell cycle and commit to a specific cell fate in response both intrinsic and extrinsic signals (Cepko et al., 1996; Donovan and Dyer, 2005). Retinal cell types are generated in a conserved sequence with the early born cells exiting the cell cycle first followed by later born cells. Early generated cohort of cells consists of ganglion, amacrine, horizontal and cone photoreceptor cells, while the later cohort consists of bipolar, Muller glia and rod photoreceptor cells. Once progenitor cells leave the cell cycle they migrate to appropriate nuclear layers within the retina, differentiate and establish synaptic connections.

Blinding degenerative retinal diseases including retinitis pigmentosa and macular degeneration are characterized by a loss of photoreceptors, whereas glaucoma is defined by a loss of retinal ganglion cells. At this time there is no way to replace retinal cell loss due to
disease or injury because differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal injury, neural progenitor cells have been proposed as a unique source of transplantable cells to replace lost cells in the damaged retina/eye. Various neural progenitor cell types have been transplanted into the retina including retinal progenitor cells (RPCs) (Klassen et al., 2004b; 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) (Takahashi et al., 1998; Young et al., 2000; Nishida et al., 2000) and iris-derived/ciliary body cells (Tropepe et al., 2000; Ahmad et al., 2000; Ahmad et al., 2004). Although transplantation of different progenitor cell types has been variably successful; retinal and ciliary body derived cells have shown the most promise. However, little is known about the fundamental biology of these cell types and how that may influence their successful transplantation.

In this study, we utilized protein expression profiling to compare retinal progenitor cells (RPCs) with brain progenitor cells (BPCs) isolated from neonatal mice. Characterizing the molecular differences among these cells using a proteomics approach is important because different populations of progenitor cells display differential differentiation, integration, and migration after transplantation. It is generally accepted that progenitor cells derived from the retina are more appropriate for retinal transplantation; however, beyond expression of a few basic transcription factors fundamental differences in RPCs and BPCs have not been well characterized. A powerful aspect of this study is that protein expression patterns were compared in RPCs and BPCs isolated from mice of the same age, in the same laboratory and from the same genetic background (Lu et al., 2002; Klassen et al., 2004a). Thus, differences in protein expression profiles are more likely to represent actual differences
between the cell populations as opposed to artifact introduced by differences in culture methods.

In this analysis, significant differences in expression levels between RPC and BPC protein spots were identified. Here, we focus on 22 proteins that were successfully identified by tandem mass spectrometry and manually verified by known or predicted molecular weights and pIs. Notably, four out of the 22 proteins were stress-response proteins encoded by genes that share at least 11 potential transcription factor-binding sites in common, suggesting that their expression could be co-regulated. This is the first study in which protein expression profiling has identified differences between two populations of murine progenitor cells (BPCs, and RPCs) isolated from genetically similar mice. Our results suggest that protein expression profiling offers a useful approach to understanding the fundamental differences between different populations of stem cells.

MATERIALS AND METHODS

Cell Culture

Retinal progenitor cells (RPC) and brain progenitor cells (BPC) used in this study were isolated and expanded in vitro as previously reported (Klassen et al., 2004a) from postnatal enhanced green fluorescent protein (GFP) – expressing transgenic mice (TgN (β-act-eGFP) 040bs). The progenitor cells were maintained as neurospheres in neurobasal medium (Invitrogen, Carlsbad, CA) containing 2 mM of L-glutamine (Invitrogen), and 10,000 units/mL of penicillin/streptomycin solution (Sigma, St Louis, MO), 1% B-27 supplement (Invitrogen), 20 ng/mL of human recombinant epidermal growth factor
(Invitrogen), 10,000 units/mL of Nystatin (Sigma). Cells were fed every two days and maintained in a 37°C incubator containing 95% CO2 and 5% O2.

**Cell Fractionation**

For protein extraction, RPCs and BPCs were rinsed in Earl’s balanced salt solution (EBSS; Invitrogen), resuspended in EBSS, and placed on ice. The cells were rinsed with phosphate buffered saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.2), resuspended in 1 mL of hypo buffer (1 mM Tris, 1 mM MgCl₂) with Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and placed on ice for 20 minutes. Cells were sonicated for 30 seconds and centrifuged at 4000 rpm for 10 minutes at 4°C. The protein extract was acetone precipitated from the supernatant overnight at -20°C. The protein sample was resuspended in 1X sample rehydration buffer containing urea (8M), CHAPS (2%), and bromophenol blue (0.002%) and stored at 80°C. A protein assay was done using the EZQ Protein Quantitation Kit (Invitrogen) to determine the total protein concentration. The final concentration of the diluted sample was 35 µg per 165 µl (0.212 µg/µl).

**Two-Dimensional Separation of Proteins**

Two-dimensional gel electrophoresis (2D), isoelectric focusing (IEF) Zoom strips (Invitrogen; pH 3-10NL 7.7 cm) were rehydrated by adding protein sample, dithiothreitol (DTT; Invitrogen; 20 mM), ampholytes (Bio-Rad, Hercules, CA; 0.5% vol/vol), and iodoacetamide (Bio-Rad; 116 mg) for 35 µg of total protein per strip. The strips rehydrated overnight (8-16 hours) at room temperature. The voltage protocol used for isoelectric focusing (IEF) was 200 V for 20 minutes, 450 V for 15 minutes, 750 V for 15 minutes and 2000 V for 45 minutes.
After isoelectric focusing proteins were separated by molecular weight using pre-cast Nupage 4-12% Bis-Tris Zoom® Gels (Invitrogen). The voltage used for the gels was continuous at 200 V for 50 minutes. Proteins were visualized with an overnight incubation of SYPRO Ruby fluorescent protein stain (Molecular Probes). After destaining for three hours in 10% methanol and 7% acetic acid, gels were imaged at the proteomics facility at Iowa State University using a Typhoon 9410 fluorescent scanner (GE Biosciences, Piscataway, NJ). Once imaged the gels were stained with Simply Blue Comassie (Invitrogen) protein stain overnight.

**Protein Expression and Identification**

Gels were analyzed for significant changes in expression levels using Phoretix 2D software (Nonlinear Dynamics, Durham, North Carolina). Proteins that were differentially expressed in RPCs were hand picked from the gels. Trypsin digestion and deposit 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% CH$_3$CN/0.1% TFA were mixed with a matrix solution (CHCA 10 mg/mL in 50% CH$_3$CN/0.1% TFA) and applied on a target plate. For Electrospray Ionization (ESI), protein digest solution was taken out after trypsin digestion, extracted and dried to needed volume.

MALDI-TOF 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 used for MS/MS ion searches were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada). The search parameters were as follows: Max-missing cleavage of one, fixed modification of carboxyamidomethyl cysteine, variable modification oxidation of Methionine. Peptide mass tolerances were set to +/- 100 ppm and fragment mass tolerances were set to +/- 1 Da. No restrictions on protein molecular weight were applied. Protein identification was based on the probability based Mowse Score (Savitski et al., 2005). The significance threshold $p$ was set to less than 0.05.

**Database Analysis**

For each gene analyzed, DNA sequences for a 1 kb promoter region (from 700 bp upstream to 300 bp downstream from the transcript start site) were retrieved from Cold Spring Harbor Laboratory *Mus musculus* Promoter Database version 2.33 (MmPD; [http://rulai.cshl.edu/cgi-bin/CSHLmpd2/mmpd.pl](http://rulai.cshl.edu/cgi-bin/CSHLmpd2/mmpd.pl); (Xuan et al., 2005)).

Potential transcription factor binding sites (TFBS) were identified within each promoter region using TRANSFAC version 7.0 ([www.gene-regulation.com](http://www.gene-regulation.com); (Matys et al., 2003)) P-Match version 1.0, (Chekmenev et al., 2005), a tool that combines pattern matching and weight matrix approaches.

For each identified protein, gene ontology (GO) annotations were manually retrieved from Swiss-prot ([http://ca.expasy.org](http://ca.expasy.org); (Gasteiger et al., 2001)).
RESULTS

Proteomics analyses were used to examine differences in protein expression between retinal and brain progenitor cells, isolated from mice of the same age, in the same laboratory and from the same genetic background. The samples were separated first by their isoelectric point (pI) and second by their molecular weight. Figure 1 shows representative gels from RPCs and BPCs. Phoretix 2D Expression (Nonlinear Dynamics, North Carolina) software was used for analysis. Data from three replicates were combined to generate an average gel for each cell type. After matching like protein spots between average gels, normalized expression levels were determined for each protein spot.

A total of 323 distinct protein spots were separated from RPC samples and 233 distinct protein spots from BPC samples. There were significant differences in the expression levels of 136 distinct protein spots. Ninety out of these 136 spots were unique to RPC gels, 32 showed an increase of at least two-fold in RPC gels and 14 showed a decrease of at least two-fold in RPC gels. Based on their differential expression in RPCs, these protein spots were hand picked and analyzed using tandem-mass spectrometry (MALDI or ESI MS/MS). Protein identifications were manually verified by comparing known or predicted molecular weights and pIs with their approximate molecular weight and pI on gels. Twenty-two proteins (16%) were confirmed based on these criteria. Table 1 shows a list of protein accession numbers along with their protein identifications and their expression in RPCs.

As a first step in characterizing these RPC proteins, gene ontology (GO) annotations were retrieved for each protein. Table 2 lists GO annotations for biological process, molecular function, and cellular localization, respectively. Analysis of the biological processes annotations (Table 2) indicated that four out of the 22 proteins were involved in
transport, tow were involved in protein folding, two were involved in actin cytoskeleton organization, two were cell adhesion proteins, one in glycolysis, three in protein biosynthesis and three in metabolism. Biological process annotations were not available for five proteins. The GO molecular function annotations (Table 2) showed that 13 out of the 22 proteins identified were involved in protein, actin, nucleic, lipid, RNA or DNA binding whereas eight were identified as to being involved in catalytic, elongation, or structural activity. One protein had an unknown molecular function. GO cellular localization annotations revealed that seven out of the 22 proteins localized to the nucleus, eight were localized to the mitochondria, two were localized to the cytosol, two were translation elongation factors, one F-actin capping protein and 1 membrane protein. There was one protein with no GO localization annotation. This initial, relatively high-level GO annotation analysis failed to reveal any obvious functional relationships among the identified proteins.

Analysis of annotations at a more detailed level of the GO hierarchy revealed that 4 of the differentially expressed proteins were known to be involved in the cellular response to stress. These four proteins were: heat shock protein 60, heat shock protein 70, thioredoxin, and Cu-Zn superoxide dismutase. Heat shock 60, thioredoxin, and Cu-Zn superoxide dismutase showed at least a two-fold or greater increase in RPCs relative to BPCs whereas, heat shock 70 was unique to RPC cells.

To investigate the mechanism of this observed differential expression, we examined the genes encoding all four proteins and compared the DNA sequences of their promoter regions. Sequences within a region including 700 bp upstream and 300 bp downstream from the transcript start site for each gene were queried for the presence of known transcription factor binding sites (TFBS). This analysis revealed that potential TFBS for 11 transcription
factors are located within the promoters regions of all 4 genes (Table 3). Potential binding sites for several additional transcription factors were shared among two or three, but not all four genes (data not shown). Most of the shared TBFS common to all four genes were present in multiple copies (e.g., all had 8-10 copies of the CREB motif). Although the significance of this finding requires further investigation, the presence of sets of shared TFBS in the promoters of all 4 genes suggests that they could be co-regulated at the transcriptional level.

DISCUSSION

Our protein expression profiling identified 22 proteins that are differentially expressed in RPCs when compared to BPCs isolated from genetically similar mice. Four of the differentially expressed proteins correspond to proteins known to be involved in a cellular response to stress, and analysis of potential transcription factor binding sites in the promoter regions of their genes suggests these proteins could be co-regulated at the transcriptional level.

Potential Developmental Role of Stress-Response Proteins Expressed by RPCs

Heat shock proteins (Hsp) are a group of proteins that are present in all cells at all biological levels. These proteins are needed for normal cell growth and maintenance and have been detected during embryogenesis in various organisms (Calabrese et al., 2002). Different developmental profiles of Hsps have suggested that these proteins have a role in neural cell differentiation (Calabrese et al., 2002). Stress can cause alterations in protein structure and function leading to misfolding of proteins in cells (Kaarniranta et al., 2002). One role of Hsps is to function as molecular chaperones (i.e., proteins that assist other
proteins in attaining their functional three-dimensional structures) under both normal and stressful conditions (Kaarniranta et al., 2002). Under stress induced by heat shock, Hsp chaperones promote survival by facilitating the refolding of misfolded proteins.

Heat shock protein 70 is found in the cytosol, nucleus, and endoplasmic reticulum and its expression has been detected in numerous cell populations within the nervous system, including neurons, glia and endothelial cells (Takeda et al., 2000; Calabrese et al., 2002). By embryonic (E) 15.5 in mice, Hsp70 is detectable in the central nervous system and at E17.5 all Hsps are expressed in the hippocampus (Loones et al., 2000; Calabrese et al., 2002). Developmental analysis in the postnatal rat brain has shown that basal levels increased in the cerebral hemisphere until postnatal (P) 20 and then decreased in the adult, whereas there was little change observed in the cerebellum during postnatal development (Calabrese et al., 2002). Heat shock protein 60 (Hsp60) has also been described in neurons (Calabrese et al., 2002). Developmental profiles have shown that Hsp60 increases during postnatal development in the rat brain stem and the cerebral hemispheres; therefore Hsp60 levels are significantly higher at P20 and in adult compared to P1 in these regions (Calabrese et al., 2002).

Thioredoxin (TRX) is small protein (12-13kDa) known to function as an antioxidant protein (Holmgren, 1985). TRX has the conserved amino acid sequence Cys-Gly-Pro-Cys in its active site (Holmgren, 1985; Carvalho et al., 2006) where the two redox-active cysteines participate in reversible oxidation-reduction reactions (Holmgren, 1985). TRX appears to play biologically important roles in hormone secretion, cell signaling, regulation of the intracellular apoptotic pathway, and cell proliferation (Saitoh et al., 1998; Kobayashi-Miura et al., 2002; Tanito et al., 2002). TRX has also been shown to regulate the DNA binding
activity of various transcriptional factors such as nuclear factor (NF-kB), activator protein 1 (AP-1), myb, redox factor 1, and mitogen-activated kinase (Okamoto et al., 1992; Hirota et al., 1997). Meyer et al have reported that TRX induces AP-1 through *de novo* transcription of c-Fos and c-Jun, therefore leading to a possible neuroprotective function (Takagi et al., 1999). A study by Saitoh et al (1998) has shown that TRX is a physiological inhibitor of the apoptosis signal-regulating kinase (ASK) 1. TRX binds to the N-terminal region of ASK1 and inhibits the kinase activity and ASK1 dependent apoptosis (Saitoh et al., 1998). While many of the functions attributed to TRX are known to be important developmental processes, we are not aware of any specific studies of the role of TRX during neural or retinal development.

Copper-Zinc superoxide dismutase (Cu-Zn SOD1) is an antioxidant enzyme found in the nucleus, mitochondria, and cytosol. It is abundantly expressed in all vertebrate tissue and catalyzes the conversion of superoxide anion to hydrogen peroxide (Jaarsma et al., 2000; Selverstone Valentine et al., 2005). SOD1 is considered a key enzyme to helping a cell protect itself against oxygen free radicals (Fridovich, 1995; Jaarsma et al., 2000). A mutation in the SOD1 gene causes amyotrophic lateral sclerosis (ALS), a neurodegenerative disease. To our knowledge, however, there are no published reports suggesting a potential role for SOD1 during neural or retinal development.

It is not yet clear what developmental role these stress-response proteins may have in retinal progenitor cells. However, expression of these proteins does clearly differentiate RPCs from BPCs which were generated and maintained under the same conditions and may well represent fundamental biological differences between these two cell populations.
Shared Transcriptional Regulatory Motifs in Stress-Response Genes Expressed in RPCs

Co-regulation of genes by sets of shared transcription factors is a common theme in the developmental regulation of eukaryotic gene expression (Nelander et al., 2005). Analysis of the promoter regions of the genes encoding the four stress response proteins that were over-expressed in RPCs relative to BPCs revealed 11 transcription factor-binding site motifs in common. At least 10 copies of the motifs for three of these (c-Rel, Evi-1 and Elk-1) were found within a 1 kb promoter region of each gene. Thus, transcriptional co-regulation of the genes for these four stress-related proteins is potentially responsible for their elevated expression in RPCs relative to BPCs.

C-Rel transcription factor is a member of the nuclear factor κB (NF-κB) transcription factor family. NF-κB transcription factors are composed of dimeric combinations of members of the Rel transcription factor family (Janssens and Tschopp, 2006). Five different Rel family members have been identified in mammals: RelA, RelB, c-Rel, NF-κB1 (p50 and p105) and NF-κB2 (p52 and p100). The NF-κB/Rel proteins share a 300 amino acid long highly conserved N-terminal called the Rel homology domain (RHD) which is responsible for DNA binding and dimerization (Ravi and Bedi, 2004; Janssens and Tschopp, 2006). These transcription factors play an important role in cell proliferation, apoptosis, stress response, and inflammation.

Evi-1 is a transcription factor that encodes a 145-kDa nuclear protein of the zinc-finger family, which contains two domains of zinc fingers (Morishita et al., 1988; Matsugi et al., 1995). This transcription factor possesses seven and three repeats of Cys2His2-type zinc-finger motifs separated into two domains (Morishita et al., 1988; Hirai et al., 2001). Evi-1 also contains proline rich and acidic domains, which may function as transcriptional
activation, or repression domains (Matsugi et al., 1995). Evi-1 is thought to promote growth, cell proliferation or block differentiation in some cell types (Hirai et al., 2001).

Elk-1 is an ETS-domain transcription factor involved in the MAP (mitogen-activated protein) kinase pathway (Yang et al., 2003; Yang and Sharrocks, 2006). Phosphorylation of Elk-1 by MAP kinases triggers its activation (Yang et al., 2003). Once activated Elk-1 is converted from a transcriptional silent state to a highly active state and then back to a basal level (Yang and Sharrocks, 2006). Ternary complex factors (TCF) also represent a subclass of ETS-domain transcription factors for which Elk-1 is a member (Yang et al., 2003; Cesari et al., 2004; Yang and Sharrocks, 2006). Three TCFs have been identified in mammals, Elk-1, SAP-1 (SRF accessory protein 1; also called Elk-4), and SAP-2 (SRF accessory protein 2; also called Elk-3) (Yang et al., 2003; Cesari et al., 2004). Cesari et al demonstrated that Elk-1 did not fulfill an essential function in mouse development, though in rats Elk-1 was expressed selectively in neurons.

Co-regulation of preferentially RPC-expressed cellular stress-response proteins by c-Rel, Evi-1 or Elk-1 would be consistent with a role during proliferation of RPCs (a neuronal cell population). Future studies to assay the expression of these transcription factors in RPCs compared to BPCs may address this question. Certainly identification of transcription factors that underlie the differential expression of proteins between populations of cells is an important aspect of understanding fundamental biological differences between them.

Proteomics is becoming a powerful approach to identify differences in protein expression and post-translational modification between cell types (Guo et al., 2007; Ahn et al., 2007). Profiling the expression of proteins, rather than relying on analysis of differential RNA expression, is critical for understanding the many post-transcriptional mechanisms that
regulate the ultimate function of proteins in cellular processes because changes in cellular mRNA levels often do not directly correlate with changes in their protein levels (Ahn et al., 2007). Furthermore, protein expression profiling allows detection of many protein post-translational modifications and can provide valuable snapshots of cellular metabolism that complement the results of RNA-based assays.

In conclusion, we have provided the first systematic proteomics comparison of expression patterns in retinal and brain progenitor cells from genetically similar mice. The identification of four stress-response proteins among those differentially expressed in RPCs relative to BPCs is intriguing. Perhaps proliferation or differentiation of RPCs is inherently more stressful for RPCs than for BPCs, or perhaps RPCs normally have a higher baseline level of these proteins. Although there are no direct data to support either of these possibilities at present, two of the four stress-response proteins have been implicated in neural development by other studies, leading us to propose that they are somehow involved in RPC development. Finally, the shared potential transcriptional regulatory sites in the genes for these four stress response proteins, suggesting their transcriptional co-regulated in RPCs, makes these cells potentially useful as biomarkers to differentiate ‘retinal-like’ progenitor cells from ‘brain-like’ progenitor cells. Further investigation of differences between multiple populations of RPCs and BPCs during their maintenance and differentiation will further identify fundamental differences that define ‘retinal-like’ characteristics, and provide tools to assay the success of efforts to bias many populations of stem cells to adopt a retinal cell fate.
REFERENCES


### Identified Protein Spots Over-expressed in Retinal Progenitor Cells When Compared to Brain Progenitor Cells

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Protein Identifications</th>
<th>Expression Fold Increased in RPCs compared to BPCs</th>
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<tr>
<td>P63017</td>
<td>Heat shock cognate 71 kDa protein</td>
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<tr>
<td>P14733</td>
<td>Lamin B1</td>
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<td>60 kDa heat shock protein, mitochondrial precursor (Hsp60)</td>
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Table 1. List of protein identifications and expression levels between retinal progenitor cells (RPCs) and brain progenitor cells (BPCs).
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<th>Protein</th>
<th>GO: Biological Process</th>
<th>GO: Molecular Function</th>
<th>GO: Cellular Localization</th>
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<td>(Endozepine)</td>
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<td>Protein</td>
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<td>GO: Molecular Function</td>
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Table 2. (two pages). List of identified proteins with their corresponding gene ontology (GO) annotations for biological process, molecular function and cellular localization.
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<td>Elk-1 *</td>
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<tr>
<td>Zid</td>
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Table 3. List of transcription factor-binding sites and the number of times they are seen in the promoter region of each stress-response gene. * Represents the three transcription factor-binding sites that were seen more than ten times on their promoter region.
Figure 1. Representative gels after two-dimensional gel electrophoresis of samples from retinal progenitor cells (left) and brain progenitor cells (right).
CHAPTER 3. CHARACTERIZATION OF STRESS-RESPONSE PROTEINS IN THE DEVELOPING MOUSE RETINA

A paper to be submitted to *Brain Research*

Tyra Dunn-Thomas¹ and M. Heather West Greenlee¹,².

Bioinformatics and Computational Biology Program¹ and, Biomedical Sciences², Iowa State University.

ABSTRACT

We have characterized the developmental expression of proteins heat shock 60 (Hsp60), heat shock 70 (Hsp70), copper-zinc superoxide dismutase (Cu-Zn SOD) and catalase (CAT) in the developing mouse retina at embryonic (E) ages 15, E17, postnatal (P) ages 1, P5, P10, and adult. Here, we describe the dynamic expression of these proteins in the developing retina. Antibody labeling for each protein generally increased around P5 in the presumptive developing photoreceptors but then decreased by maturity. Despite the developmental decrease in expression of much of the retina, the outer segments remained immunoreactive with antibodies against each of these proteins. These results are the first to describe developmental expression of these proteins in a well-defined tissue and represent our initial efforts to understand their role in the developing retina.

INTRODUCTION

The vertebrate neural retina is a complex organ that is well suited for studying development of the central nervous system. All vertebrate retinas develop from a neural
epithelium into a laminar structure composed of three layers of nerve cell bodies separated by two synaptic layers. Retinal cell types are all generated from a common precursor, the retinal progenitor cell, which gives rise to six neural cell types and one glial cell type (Levine and Green, 2004). Despite the extensive characterization of the development of this organ, the complex series of events including cell proliferation, migration, differentiation and how these events work in concert with process outgrowth and remodeling is not well understood. Further, the physiological cost (oxidative and metabolic) of these processes to a tissue is also not well understood.

Our previous study has described higher levels of expression of several stress response proteins in expanded murine retinal progenitor cells (RPCs) compared to expanded brain progenitor cells (BPCs) [Dunn-Thomas et al; submitted]. Proteins that were more highly expressed in RPCs included: heat shock 60 (Hsp60), heat shock protein 70 (Hsp70) and copper-zinc superoxide dismutase (Cu-Zn SOD).

To begin to understand the role that stress-response proteins may play in the context of a developing tissue we have characterized the developmental expression of Hsp60, Hsp70, Cu-Zn SOD, and catalase in the mouse retina at embryonic (E) ages 15, E17, postnatal (P) ages 1, P5, P10, and adult. We describe the differential expression of some but not all of these proteins in the developing retina. These results are the first to describe the developmental expression of these proteins in a well-defined tissue and represent our initial efforts to understand their role in the developing retina.
METHODS AND MATERIALS

Tissue Preparation

C57BL/6 mice ages E15 to P5 were euthanized and their heads were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.2). Postnatal day 10 and adult mice were euthanized, their eyes enucleated and immersion fixed in 4% paraformaldehyde in PBS. The tissue was cryoprotected in a 30% sucrose solution in PBS and embedded in OCT mounting media (Fisher, Pittsburgh, PA). Twenty-micrometer-thick cryosections were cut and thaw mounted to colorfrost microscope slides (Fisher, Pittsburgh, PA) and stored at -20°C. All animal procedures had the approval of the Iowa State University committee on animal care.

Immunohistochemistry

Standard procedures were used for immunostaining cryosectioned tissue. Frozen tissue sections labeled with antibodies against heat shock 60 and 70 were pre-treated by boiling in 0.1 M citrate buffer (0.1 M citric acid, 0.1 M Na₃ citrate, pH 5.5) for 10 minutes and cooled to room temperature. All tissue sections were rinsed in 0.5 M potassium phosphate buffered saline (KPBS; 0.15 M NaCl, 0.034 M K₂HPO₄, 0.017 M KH₂PO₄, pH 7.4) and incubated in blocking solution consisting of KPBS, 1% bovine serum albumin (BSA; Fisher), 0.4% Triton-X 100 (TrX-100, Sigma, St. Louis, MO) and 1.5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratory, West Grove, PA) at room temperature for two hours. Tissue sections were incubated in primary antibodies diluted in blocking solution overnight at room temperature. The primary antibodies used were: anti-heat shock protein 60 (Hsp60, mouse monoclonal IgG; Chemicon, Temecula, CA; 1:500); anti-heat shock protein 70 (Hsp70, rabbit polyclonal IgG; Chemicon, Temecula, CA; 1:200);
anti-catalase (CAT, mouse monoclonal IgG; Sigma, St. Louis, MO; 1:1000) and anti-copper-
zinc superoxide dismutase (Cu-Zn SOD, rabbit polyclonal IgG; Stressgen, BC, Canada;
1:1000). The following day the slides were washed in KPBS containing 0.02% TrX-100 and
incubated in secondary antibody for 2 hours. Secondary antibodies (Alexa Fluor 488 donkey
anti-mouse or donkey anti-rabbit, Molecular Probes, Carlsbad, CA; 1:500) were diluted in
KPBS with 1% BSA, 1.5% NDS and 0.4% Triton X-100. The slides were washed in KPBS
containing 0.02% TrX-100. Slides were then incubated for 5 minutes in 300 µM DAPI (100
mM) diluted in KPBS at room temperature. Again the slides were washed in KPBS and
coverslipped with Vectashield fluorescence mounting medium (Vector Laboratories,
Burlingame, CA).

**Imaging**

A Nikon E800 (Melville, NY) microscope equipped with a Retiga digital camera
pixel 1300 (Qimaging Burnaby, BC, Canada) was used to capture images. Images were
Macromedia Freehand 8 (Macromedia Incorporated, San Francisco, CA) was used to prepare
figures.

**Western Blots**

Protein extracts were prepared from P1 and adult C57BL/6 mouse retinas and brains.
Retinas were dissected from the eyes and placed in 1X SDS sample buffer (62.5 mM Tris-
HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue) and sonicated
on ice. Samples were centrifuged 10 minutes at 4000 RPM and the supernatant was collected.
EZQ Protein Quantitation Kit (Molecular Probes) was used to determine the protein
concentration of the sample. A total of 15 µg of protein was loaded in each well of a 4-12%
Bis-Tris Zoom® gel (Invitrogen, Carlsbad, CA). Gel electrophoresis was performed at 200V for 50 minutes and the proteins were transferred to a nitrocellulose membrane (Bio-rad, Hercules, CA). The membrane was incubated for one hour in blocking solution (5% BSA, 0.05% NDS in tris-buffered saline with 0.1% Tween (TBST)) and incubated overnight in primary antibodies: Hsp60 - 1:100, Hsp70 – 1:200, CAT 1:4000, and Cu-Zn SOD 1:3750. The following day the membrane was washed in TBST. The membrane was incubated for two hours in biotinylated secondary antibody (biotinylated anti-mouse or biotinylated anti-rabbit, Jackson; 1:500) followed by a 1-hour incubation in Vectastain ABC solution (Vector Laboratories, Burlingame, CA). To aid in visualizing bands, membranes were incubated in a diaminobenzidine (DAB) solution (0.05% DAB, 0.04% NH₄Cl; Sigma) diluted in deionized water and PBS with 1% nickel sulfate. After completion of the reaction, the membrane was air dried and scanned on a Hewlett-Packard scanjet 3500c.

RESULTS

To investigate the expression of stress-response proteins in the developing retina, we used antibodies to characterize the expression patterns of heat shock 60 (Hsp60), heat shock 70 (Hsp70), copper-zinc superoxide dismutase (Cu-Zn SOD) and catalase (CAT) in the developing mouse retina. To determine if there were any differences in global expression of these proteins between retina and brain, we used protein extracts from retinas and brains taken from P1 and adult. Figure 1 summarizes these findings. Anti-Hsp60 recognized a band at approximately 60 kDa in brain and retina in both P1 (Figure 1A) and adult samples (Figure 1B). Anti-Hsp70 recognized a band at approximately 70 kDa in brain and retina in both P1 (Figure 1A) and adult samples (Figure 1B). Anti-Cu-Zn SOD recognized a band at
approximately 19 kDa in brain and retina in both P1 (Figure 1A) and adult samples (Figure 1B). Anti-CAT recognized a band at approximately 60 kDa in brain and retina in both P1 (Figure 1A) and adult samples (Figure 1B). We did not observe differences in expression between brain and retina for any of the proteins assayed.

Developmental expression of heat shock 60-immunoreactivity (Hsp60-IR) is shown in Figure 2. Hsp60-IR was uniformly diffuse throughout the developing cellular layers of the retina at ages E15 (2A), E17 (B) and P1 (2C). At P5, hsp60-IR expression was diffuse throughout the retina (2D). At P10 (2E) Hsp60-IR expression was most intense in the GCL, INL (inner nuclear layer), throughout the ONL (outer nuclear layer) and developing photoreceptor outer segments (arrow). In the adult (2F) retina, expression of Hsp60-IR was diffuse throughout the tissue and more intense in the outer segments of the photoreceptors.

Developmental expression of heat shock 70-immunoreactivity (Hsp70-IR) is illustrated in Figure 3. Hsp70-IR in retinas from mice aged E15 (3A), E17 (3B), and P1 (3C) was diffuse throughout the retina. In the P5 retina Hsp70-IR was observed diffusely throughout the retina (3D) but was more intense in the optic fiber layer (OFL) and neuroblastic layer (NBL) compared to the rest of the retina. Hsp70-IR in the P10 (3E) retina was most intense in the GCL, INL and ONL including the developing outer segments of photoreceptors (arrow). Hsp70-IR in the adult (3F) retina was detected primarily in the OFL, INL, OPL and photoreceptor outer segments (OS).

Figure 4 characterizes the developmental expression of copper-zinc superoxide dismutase immunoreactivity (Cu-Zn SOD-IR). Cu-Zn SOD-IR was diffuse throughout the developing retina at ages E15 (4A), E17 (4B) and P1 (4C). Cu-Zn SOD-IR in the P5 (4D) retina was detected in the OFL, IPL, and NBL whereas there was little difference between
the GCL and INL. At P10 (4E), expression of Cu-Zn SOD-IR was most intense in the ONL and developing outer segments and more diffuse in the rest of the tissue. Conversely, in the adult (4F) Cu-Zn SOD-IR was diffuse throughout the developing retina but more intense in the INL and photoreceptor outer segments.

Developmental expression of catalase immunoreactivity (CAT-IR) is summarized in Figure 5. Catalase-IR was diffuse throughout the developing retina and more intense in the OFL at ages E15 (5A) and E17 (5B). CAT-IR expression in P1 (5C) was more intense in the OFL, GCL, IPL and INL and diffuse throughout the rest of the retina. At age P5 (5D) CAT-IR was again diffuse throughout the retina however it was more intense in the OFL, GCL and IPL. CAT-IR in the retina at P10 (5E) was diffuse throughout the retina and more intense in the developing photoreceptor outer segments (arrow). CAT-IR in the adult (5F) was diffuse throughout the retina but more intense in the OFL, photoreceptor outer segments, and blood vessels (double arrows).

**DISCUSSION**

In our previous study we identified higher levels of expression of several stress response proteins (Hsp60, Hsp70 and Cu-Zn SOD) in growth-factor expanded retinal progenitor cells (RPCs) compared to expanded brain progenitor cells (BPCs) isolated from neonatal retina and brain, respectively (Dunn-Thomas et al; submitted). Western blotting for these proteins suggests that there is not differential expression of these proteins between whole retina and whole brain either at birth (P1) or in adult tissue. To better understand the role these proteins may play during retinal development, we characterized their expression patterns during cytogenesis and histogenesis of this organ. In all of the ages (E15, E17, P1,
P5, P10, adult) there was dynamic expression of these proteins. Antibody labeling for each protein, generally increased around P5 in the outer retina but then decreased by maturity. Despite the decreased expression in much of the retina, the outer segments remained immunoreactive with antibodies against each of these proteins. The dynamic and differential expression of these proteins in the developing retina suggests they play a role in development of this organ.

Expression of Stress-Response Proteins in the Developing ONL

Immunoreactivity for Hsp60, Hsp70, and Cu-Zn SOD increased in the ONL around P5 and P10, the time at which rod photoreceptors are differentiating (Morrow et al., 1998). Expression for these proteins decreased in the ONL but remained in the outer segments, suggesting they also play a role in photoreceptor maintenance. The transient increase in expression of Hsp60, Hsp70, and Cu-Zn SOD during rod differentiation suggests these proteins may be important for their proper development.

Heat shock proteins (Hsp) are a group of proteins that are present in all cells. These proteins are needed for normal cell growth and maintenance and have been detected during embryogenesis in various organisms (Calabrese et al., 2002). Developmental profiles of Hsps have suggested that these proteins have a role in neural cell differentiation (Calabrese et al., 2002).

Heat shock protein 60 (Hsp60) is present in the cytoplasm as well as in dendritic processes of neurons (D'Souza and Brown, 1998),(Calabrese et al., 2002). Functions of Hsp60 are dependent on Hsp10, which binds to Hsp60 and regulates its substrate binding and ATPase activity (Richter-Landsberg and Goldbaum, 2003). Hsp60 also participates in the folding and assembly of transporting proteins into the mitochondria (Richter-Landsberg and
Goldbaum, 2003). Developmental profiles have shown that Hsp60 increases during postnatal development in the rat brain stem and the cerebral hemispheres (Calabrese et al., 2002). Our study demonstrates differential expression of Hsp60 as the retina matures.

Heat shock protein 70 (Hsp70) is synthesized at high levels and is present in the cytosol, nucleus, and endoplasmic reticulum (Calabrese et al., 2002). Expression of the gene encoding Hsp70 has been found in numerous cell populations within the nervous system, including neurons, glia and endothelial cells (Takeda et al., 2000),(Calabrese et al., 2002). Hsp70 is expressed in the external plexiform layer in the olfactory bulb, ventrally in the septum and dorsally in the neocortex before embryonic (E) 15.5 in mice (D'Souza and Brown, 1998),(Loones et al., 2000),(Calabrese et al., 2002). By E15.5 in mice, Hsp70 is detectable in the central nervous system and at E17.5 all Hsps are expressed in the hippocampus (Loones et al., 2000),(Calabrese et al., 2002). Developmental analysis of the postnatal rat brain has shown that basal levels of Hsp70 increased in the cerebral hemisphere until postnatal (P) 20 and then decreased in the adult, whereas there was little change observed in expression of Hsp70 in the cerebellum during postnatal development (D'Souza and Brown, 1998),(Calabrese et al., 2002). In our analysis, expression of Hsp70 appeared to peak in cellular layers of the retina around P10, though expression was still observed in the adult retina.

Copper-Zinc superoxide dismutase (Cu-Zn SOD) is a key cytosolic enzyme present throughout the CNS and the PNS (Przedborski et al., 1996). Cu-Zn SOD is considered to be one of the key enzymes used by cells to protect themselves against oxygen free radicals (Fridovich, 1995),(Przedborski et al., 1996). The main function of Cu-Zn SOD is to catalyze the conversion of superoxide anion to hydrogen peroxide (Przedborski et al., 1996),(Jaarsma
et al., 2000), (Selverstone Valentine et al., 2005). In mouse brain, Cu-Zn SOD immunoreactivity is found in neurons, the cerebral cortex and hippocampus as well as in anterior horn of the spinal cord and substantia nigra (Pardo et al., 1995). A study using mRNA profiles reported an increase in Cu-Zn SOD in the brains of late-gestational and neonatal mouse fetuses (de Haan et al., 1994). However, developmental studies on the brains of mice found that protein levels were low at embryonic (E) 18 and postnatal (P) 1 ages (Khan and Black, 2003). The results presented here suggest increasing expression of Cu-Zn SOD in the developing retina. The most striking immunoreactivity for Cu-Zn SOD was in the developing photoreceptors at P10.

Catalase (CAT) is a common enzyme located in the peroxisome. Its functions include catalyzing the decomposition of hydrogen peroxide to water (Przedborski et al., 1996), (Khan and Black, 2003). This catalytic reaction protects the cell from the toxic effects of hydrogen peroxide (Oh et al., 2005). Developmental changes in embryonic and postnatal brains of mice showed that catalase protein levels were high at E18 and remained elevated throughout development (Khan and Black, 2003). In our study CAT-IR appeared to peak around P1, despite the fact that it was expressed throughout development.

In conclusion, we have characterized the dynamic expression patterns of Hsp60, Hsp70, Cu-Zn SOD and CAT in the developing retina. This study is the first to examine expression of these proteins during retinal development. We report the dynamic expression patterns for these proteins, particularly in differentiating photoreceptors, suggesting their expression is key for proper photoreceptor development.
REFERENCES


**LEGENDS**

Figure 1. Western blots of brain and retina for P1 (A) and adult (B) samples for stress-response proteins Hsp60, Hsp70, SOD, and CAT. P = Postnatal, Hsp60 = Heat shock 60, Hsp70 = Heat shock 70, SOD = Copper-Zinc superoxide dismutase, CAT= Catalase.

Figure 2. Developmental expression of heat shock 60 in retinas from mice aged E15 (A), E17 (B), P1 (C), P5 (D), P10 (E), and Adult (F). E = Embryonic P = Postnatal

Figure 3. Developmental expression of heat shock 70 in retinas from mice aged E15 (A), E17 (B), P1 (C), P5 (D), P10 (E), and Adult (F). E = Embryonic P = Postnatal

Figure 4. Developmental expression of copper-zinc superoxide dismutase in retinas from mice aged E15 (A), E17 (B), P1 (C), P5 (D), P10 (E), and Adult (F). E = Embryonic P = Postnatal

Figure 5. Developmental expression of catalase in retinas from mice aged E15 (A), E17 (B), P1 (C), P5 (D), P10 (E), and Adult (F). E = Embryonic P = Postnatal
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CHAPTER 4. DIFFERENTIATION OF RETINAL PROGENITOR CELLS USING THE ANTIOXIDANT VITAMIN E

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ABSTRACT

Understanding the factors that influence retinal progenitor cell fate is key to the development of effective cell replacement therapies for the treatment of retinal degeneration. Our previous study has described higher levels of expression of several stress-response proteins in expanded murine retinal progenitor cells (RPCs) compared to expanded brain progenitor cells (BPCs).

In this study we tested the effects of the addition of the antioxidant vitamin E to differentiating retinal progenitor cells. We report that the addition of vitamin E to differentiating RPCs decreases their expression of stress-response proteins compared to the untreated controls. Further, vitamin E treatment altered the differentiation profile of RPCs, increasing the number of cells expressing markers of rod bipolar cells and Mueller glia. This is the first study to report the effects of vitamin E on retinal progenitor cell differentiation and suggests that a cell’s oxidative load during this process can influence its differentiation.

INTRODUCTION

Blinding degenerative retinal diseases including retinitis pigmentosa, macular degeneration and glaucoma are characterized by loss of retinal neurons. At this time there is
no way to replace retinal cell loss due to disease or injury since differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal injury, neural progenitor cells have been proposed as a unique source of transplantable cells to replace lost cells in the damaged retina. There are various cultured progenitor cell types that have been transplanted into the retina including retinal progenitor cells (RPCs) (Qiu et al., 2005), brain progenitor cells (BPCs) (Sakaguchi et al., 2003), adult hippocampal progenitor cells (AHPCs) (Takahashi et al., 1998) and ciliary-derived cells (Tropepe et al., 2000). Transplantation of various progenitor cell types has been variably successful. Despite the success of integration into the host retina, there is yet no way to influence the differentiation of transplanted cells into the needed cell type.

Our previous study, which profiled protein expression in expanded brain (BPCs) and retinal (RPCs) progenitor cells from neonatal mice, reported higher levels of expression of several stress-response proteins in RPCs compared to BPCs (Dunn-Thomas et al; submitted). Proteins that were more highly expressed in RPCs included: heat shock 60 (Hsp60), heat shock protein 70 (Hsp70) and copper-zinc superoxide dismutase (Cu-Zn SOD).

In the present study we tested the hypothesis that addition of the antioxidant vitamin E (alpha-tocopherol) to differentiating RPCs would decrease the expression of stress-response proteins and alter the differentiation profile of RPCs. Our results suggest that the environmental oxidative load can affect RPC differentiation. Thus, oxidative stress is a factor that should be considered when trying to influence RPC differentiation.
METHODS AND MATERIALS

Cell Culture

Retinal progenitor cells (RPC) used in this study were isolated and expanded in vitro as reported by Shatos et al (2001) from neonatal enhanced green fluorescent protein (GFP) – expressing transgenic mice (TgN (β-act-eGFP) 040bs). The progenitor cells were maintained as neurospheres in neurobasal medium (Gibco, Carlsbad, CA) containing 2mM of L-glutamine (Gibco), 10,000 units/mL of penicillin/streptomycin solution (Sigma, St. Louis, MO), 1% B-27 supplement (Gibco), 20 ng/mL of human recombinant epidermal growth factor (EGF; Gibco) and 10,000 units/mL of Nystatin (Sigma). Cells were fed every two days and maintained at 37°C in a 95% CO₂ and 5% O₂ incubator.

Coating of 8 well chamber slides

To induce differentiation, cells were plated in eight well chamber slides (Fisher Scientific, Pittsburgh, PA). Slides were double coated with 0.1% poly-L-lysine (Sigma) and 1mg/ml of laminin (mouse, BD Biosciences, Bedford, MA). Poly-L-lysine was added to 0.1 M of borate buffer (0.1 M H₃BO₃, 0.1 M Na₂B₄O₇, pH 8.5) and sterile filtered with a 0.22 µM millipore filter (Millipore Corporation, Billerica, MA). The solution was added to each chamber and incubated at room temperature. After the three hours the slides were washed with sterile tissue culture water (Sigma) and stored in the refrigerator. Prior to use, the sterile water was removed from the slides and allowed to dry for two hours after which laminin was added to 1X of sterile phosphate buffer saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.2), pipetted into each slide and incubated at 4°C overnight. The next day the laminin was aspirated off the slides and washed three times with sterile
PBS. After the last wash, the slides were washed two times with culture media just prior to cell plating.

**Cell Plating**

Cells growing as neurospheres, were dissociated before plating. Media containing retinal neurospheres was extracted from flasks (Fisher Scientific, Pittsburgh, PA) and centrifuged to form a pellet. The pellet was washed in Earl’s balanced salt solution (EBSS; Invitrogen, Carlsbad, CA) containing penicillin/streptomycin solution. The pellet was then incubated in EBSS containing 0.25% trypsin (Invitrogen, Carlsbad, CA) for 15 minutes and centrifuged for one minute. The supernatant was aspirated off, the pellet washed with EBSS and centrifuged. After the wash, the pellet was incubated in EBSS containing 0.0025% trypsin inhibitor (Invitrogen, Carlsbad, CA) for five minutes and centrifuged. The pellet was washed with EBSS and centrifuged. After the last wash culture media was added to the conical tube. The cells were then dissociated by triturating with a fire polished 1 mL pipette until the suspension became cloudy. Dissociated retinal cells were counted on a hemacytometer and plated at a density of 45,000 cells/cm² or 80,000 cells/cm² on coated 8 well chamber slides.

**Media**

Retinal progenitor cells in 8 well chamber slides were maintained for 10 days. Cells were fed by replacement of half of the culture media every other day. Two different medias were used: ultraculture media (Cambrex, East Rutherford, NJ)) containing 2 mM of L-glutamine (Gibco), 10,000 units/mL of Nystatin (Sigma) and 10 ng/mL of gentamicin (Gibco) or neurobasal media containing 2 mM of L-glutamine (Gibco), 10,000 units/mL of penicillin/streptomycin solution (Sigma), 1% B-27 supplement (Gibco), 10,000 units/mL of
Nystatin (Sigma) and 5% fetal bovine serum (FBS; Invitrogen). Vitamin E (in the form of alpha-tocopherol; Sigma) was diluted in ethanol and added to ultraculture media at 5 µM, 25 µM or 50 µM.

**Immunocytochemistry**

Immunocytochemistry was used to assay protein expression and cellular differentiation. After 10 days in culture the cells were fixed in 4% paraformaldehyde in PBS. Cells were rinsed and incubated in blocking solution consisting of potassium phosphate buffered saline (KPBS; 0.15 M NaCl, 0.034 M K₂HPO₄, 0.017 M KH₂PO₄, pH 7.4), 1% bovine serum albumin (BSA; Fisher, Pittsburgh, PA), 0.4% Triton- X 100 (TrX-100, Sigma, St. Louis, MO), and 1.5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratory, West Grove, PA) at room temperature for two hours. Cells were incubated in primary antibodies at room temperature overnight. The following day the cells were washed in KPBS containing 0.02% TrX-100 and incubated in secondary antibody for two hours. After being washed in KPBS containing TrX-100 the slides were incubated for five minutes in 300 µM DAPI (100 mM) diluted in KPBS at room temperature. Again the slides were washed in KPBS and coverslipped with Vectashield fluorescence mounting medium (Vector Laboratories, Burlingame, CA).

**Antibodies**

Primary antibodies used were anti-nestin (mouse monoclonal IgG; Chemicon, Temecula, CA; 1:10) a marker for neural progenitor cells, anti-beta tubulin III (Tuj1; mouse monoclonal IgG, Chemicon, Temecula, CA; 1:300) a marker for early neurons, anti-glutamine synthetase (GS; rabbit polyclonal IgG; Sigma, Saint Louis, MO; 1:10,000) a marker for Muller glia, anti-protein kinase C alpha (PKCα; rabbit polyclonal IgG; Sigma,
Saint Louis, MO; 1:10,000) a marker for rod bipolar cells and recoverin (mouse monoclonal IgG, Chemicon, Temecula, CA; 1:6000) a marker for photoreceptors. Stress-response primary antibodies anti-heat shock protein 60 (Hsp60, mouse monoclonal IgG; Chemicon, Temecula, CA; 1:500); anti-heat shock protein 70 (Hsp70, rabbit polyclonal IgG; Chemicon, Temecula, CA; 1:200); anti-catalase (CAT, mouse monoclonal IgG; Sigma, St. Louis, MO; 1:1000) and anti-copper-zinc superoxide dismutase (Cu-Zn SOD, rabbit polyclonal IgG; Stressgen, BC, Canada; 1:1000). Secondary antibodies (Alexa Fluor 594 donkey anti-mouse or donkey anti-rabbit, Molecular Probes, Carlsbad, CA; 1:500) were diluted in KPBS with 1% BSA, 1.5% NDS and 0.4% TrX-100.

**Imaging**

A Nikon E800 (Melville, NY) microscope equipped with a Retiga 1300 digital camera (Qimaging Burnaby, BC, Canada) was used to capture images. Adobe Photoshop version 9 (Adobe Systems Incorporated, San Jose, CA) and Macromedia Freehand 8 (Macromedia Incorporated, San Francisco, CA) software were used to crop images and prepare figures.

**Cell Counting**

Quantification of the percentage of cells expressing immunocytochemical markers were done by counting 10 random fields using a 20X objective for each chamber. Total cells in one field were counted using the nuclear counter stain 4', 6-Diamidino-2-phenylindole (DAPI). The number of immunopositive cells for a given antibody was also counted for each field. Cell differentiation was expressed as percentage of total cells and compared to the untreated control.
Statistics

Values were given as the means ±SEM (standard error mean) and where appropriate, significance of differences between mean values were determined by analysis of variance (ANOVA; Super ANOVA, ABACUS, Berkeley, CA). P-values of less than 0.05 were considered significant.

RESULTS

Differentiation of RPCs is Influenced by Culture Media

Previous studies of RPC differentiation have used media supplemented with serum (Qiu et al., 2005). For these studies, we wanted to establish how RPC differentiation would be affected by using a defined serum-free media. To induce differentiation of RPCs, cells were dissociated, plated onto poly-L-lysine/laminin-coated slides and fed neurobasal media containing 5% fetal bovine serum (FBS) or serum-free ultraculture media every other day for 10 days. On the 10th day the cells were fixed and labeled with antibodies against cell-type specific proteins. Antibodies used were nestin (neural progenitor cells), beta tubulin III (early neurons), glutamine synthetase (Mueller glia), protein kinase C alpha (rod bipolar) and recoverin (photoreceptors). There were no cells positive for recoverin or beta tubulin III (data not shown). Figure 1 represents the typical morphology of the cells expressing nestin (A), GS (B) and PKC alpha (C). Table 1 compares the antibody labeling in cells differentiating in the two medias. The number of cells immunoreactive for nestin was similar in both medias (31% ± 3 and 33% ± 2.5 in neurobasal and ultraculture media respectively). However glutamine synthetase immunoreactivity was significantly different (16% ± 2.4 in neurobasal media compared to 42% ± 2.2 in ultraculture media (Table 1). In neurobasal media, 44% ± 5.3 of
cells expressed PKC alpha compared to 20% ± 4 of cells that differentiated in ultraculture media (Table 1). All subsequent experiments were done using serum-free media.

**Vitamin E Influences Differentiating Profiles of RPCs**

To investigate the effects of the antioxidant vitamin E on differentiation of RPCs, cells were differentiated in ultraculture media containing 5 µM, 25 µM or 50 µM of alpha-tocopherol (vitamin E). Table 2 shows the differentiation profiles of RPCs in ultraculture media with the 3 concentrations (5 µM, 25 µM or 50 µM) of vitamin E compared to the untreated controls. The number of cells immunopositive for nestin was significantly increased with the addition of 50 µM vitamin E (48% ± 2.5) compared to the control (33% ± 2.5) but lower concentrations of vitamin E did not have a significant effect. The percentage of cells expressing glutamine synthetase was significantly increased with 25 µM (56% ± 2.4) and 50 µM (51% ± 2.6) of vitamin E compared to the control (42% ± 2.2). Finally, the number of cells immunopositive for PKC alpha was increased at all three vitamin E concentrations but the increase only reached significance at 5 µM (48% ± 2.8) and 50 µM (44% ± 3.1) compared to the control (19% ± 4) (Table 2).

**Differentiation in Vitamin E Decrease Expression of Stress-Response Proteins**

We used immunocytochemistry to determine if expression of stress-response proteins decreased by addition of vitamin E to culture media. Cells differentiated in ultraculture containing 50µM vitamin E were fixed after 10 days in culture and labeled with antibodies against Hsp60, Hsp70, Cu-Zn SOD and CAT. Two independent, blinded investigators then sorted the images of cells (that had been captured under identical conditions) based on perceived intensity of immunoreactivity with each antibody. Images of cells were easily sorted by both observers based on immunoreactivity for each of the four proteins. Figure 2
demonstrates immunoreactivity for Hsp60, Hsp70, Cu-Zn SOD and CAT in cells differentiated with (B, D, F, H) or without (A, C, E, G) vitamin E. Expression of all four proteins were subjectively decreased when 50 µM vitamin E was added compared to no vitamin E.

DISCUSSION

We have shown that vitamin E alters the differentiation profile of retinal progenitor cells when added to media. There was a significant increase of immunopositive cells for nestin, a marker for neural progenitor cells, glutamine synthetase, a marker for Mueller glia and PKC alpha, a marker for rod bipolar when 50 µM of vitamin E was added to the ultraculture media. We demonstrated that the number of cells expressing markers of Mueller glia (GS) and rod bipolar (PKC alpha) cells were affected. We were still unable to detect recoverin (a marker for photoreceptors) immunoreactivity after vitamin E treatment, demonstrating that we were still unable to bias cells towards a photoreceptor fate. In addition, we saw a decrease in stress-response proteins Hsp60, Hsp70, Cu-Zn SOD and CAT when vitamin E was added to the media compared to no vitamin E being added suggesting their expression was at least in part in response to the oxidative load they were experiencing in culture.

We report that vitamin E results in an increase in cells expressing PKC alpha, a marker of rod bipolar cells in the retina. A previous study by Zahir and colleagues (2005) also reported biasing differentiation of expanded retinal progenitor cells towards PKC alpha expressing cells. They reported that ciliary neurotrophic factor (CNTF) increases the percentage of cells expressing PKC alpha and induces changes in the morphology and rate of
proliferation of RPCs. They observed an increase in expression of PKC alpha but also most of the PKC alpha-positive cells exhibited bipolar morphology once treated with CNTF (Zahir et al., 2005). RPCs in our study also exhibited a bipolar morphology when differentiated in the presence of vitamin E.

We previously described an increase in expression of stress-response proteins when comparing proliferating retinal progenitor cells to brain progenitor cells (Dunn-Thomas et al; submitted). This increase in expression raises the possibility that retinal progenitor cells were under some type of cellular stress. Oxidative stress can occur in cells at any time when there is an increase in oxidant generation, a decrease in antioxidant protection or a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species (ROS). Glaucoma and retinitis pigmentosa are two diseases in which oxidative stress is a component of the pathogenesis. Glaucoma is a degenerative disease that can lead to blindness. Vision is lost because of damage to the optic nerve, which carries information from the eye to the brain (Izzotti et al., 2006). Retinitis pigmentosa is a genetic condition of the eye that affects the photoreceptors (rods) and can lead to progressive vision loss. Once the rods begin to die the cones will progressively follow. Rods consume a high level of oxygen therefore, as the rods die the tissue level of oxygen in the retina increases (Shen et al., 2005). This oxygen increase is very damaging to the photoreceptors and results in the generation of reactive oxygen species, which causes the cells to die (Shen et al., 2005). Degenerative retinal environments may very well impart a significant oxidative burden. The results of our studies suggest that changing the oxidative environment (in our example decreasing the oxidative load by the addition of antioxidant to the culture media) can affect RPC differentiation. Thus, if cells are
to be transplanted into an oxidative environment we must understand how it will affect their differentiation.

In conclusion, we characterized the differentiation of RPCs and discovered that addition of the antioxidant vitamin E affects differentiation of these cells. We observed an increase in expression of nestin, glutamine synthetase and PKC alpha. We saw a decrease in expression of stress-response proteins which many not directly affect differentiation but suggests that the environmental oxidative load does influence RPC differentiation. This is the first study to examine the affects of the oxidative environment on RPC differentiation. Our results suggest that the oxidative environment can significantly affect RPC differentiation and should be considered in the context of developing cell replacement therapies to treat retinal degenerative diseases.

REFERENCES


LEGENDS

Figure 1. Represents the typical morphology of the cells expressing nestin (A), GS (B) and PKC alpha (C). GS = glutamine synthetase

Table 1. Shows the percentages that represent the affects of neurobasal and ultraculture media without vitamin E. The bar represents the standard error.

Table 2. Shows the percentage of positive cells expressing neuronal and glial markers in the ultraculture media with the 3 various concentrations (5µM, 25µM and 50µM) of vitamin E. The bars represent the standard error. * Represents p < 0.05

Figure 2. Immunoreactivity of Hsp60, Hsp70, Cu-Zn SOD and CAT in cells differentiated without (A, C, E, G) and with (B, D, F, H) vitamin E. Hsp60 = Heat shock 60, Hsp70 = Heat shock 70, SOD = Copper-Zinc superoxide dismutase, CAT= Catalase.
Neurobasal and Ultraculture Media without Vitamin E

Percentage of Cells

Nestin  Glutamine synthetase  PKC alpha

Antibodies
Retinal Progenitor Cells in Ultraculture Media containing vitamin E

Percentage of Cells

- Nestin
- Glutamine synthetase
- PKC alpha

Antibodies

Legend:
- Control
- 5uM
- 25uM
- 50uM

* indicates statistical significance
CHAPTER 5. GENERAL CONCLUSION

DISCUSSION

We have used two-dimensional gel electrophoresis protein expression profiling to characterize the molecular differences between two populations of murine progenitor cells; retinal progenitor cells and brain progenitor cells isolated from mice of the same age and same genetic background. There were significant differences in expression levels between RPC and BPC protein spots. Our protein expression profiling identified 22 proteins that are differentially expressed in retinal progenitor cells when compared to brain progenitor cells. Four of the 22 differentially expressed proteins corresponded to proteins known to be involved in a cellular response to stress. These stress-response proteins were: heat shock 60 (Hsp60), heat shock 70 (Hsp70), copper-zinc superoxide dismutase (Cu-Zn SOD), and thioredoxin. The results of this experiment prompted the hypothesis that these proteins may be expressed in the developing retina. To test this hypothesis we characterized the expression of Hsp60, Hsp70, Cu-Zn SOD and catalase (CAT) in the developing retina. We characterized the developmental expression of these stress-response proteins in the developing mouse retina at embryonic (E) 15, E17, postnatal (P) 1, P5, P10, and adult animals using immunohistochemistry and western blots. Our western blots showed no differential expression of these proteins between retina and brain in either P1 or adult. However, in tissue sections, we observed dynamic expression patterns for stress-response proteins Hsp60, Hsp70, Cu-Zn SOD and CAT.

An alternative hypothesis generated by our initial comparison of RPCs to BPCs were that the cultured RPCs were experiencing oxidative stress. We hypothesized that addition of
the antioxidant vitamin E to media would decrease expression of stress-response proteins and potentially alter the differentiation profile of the cells. Decrease in the expression of stress proteins upon the addition of the antioxidant vitamin E suggests that the cells were responding to environmental oxidative stress. Changes in differentiation profiles suggest that the oxidative load experienced by the cells does affect their differentiation.

FUTURE EXPERIMENTS

Effects of Vitamin E on Brain Progenitor Cell Differentiation

Future experiments with retinal and brain progenitor cells would ideally help to identify ways to successfully bias the differentiation of these cells prior to transplantation into the retina. Since we know that vitamin E affects differentiation of retinal progenitor cells it is important to know if the same is true for brain progenitor cells. Vitamin E could be used in a similar way as described in this dissertation but with brain progenitor cells. Instead of using the same concentration of vitamin E like was previously reported in Chapter 4, we could start our concentration at 0.5 µM and go as high as 500 µM of vitamin E in order to see which concentrations, if any, affect the differentiation of the brain progenitor cells. In my previous studies I did not identify increased expression of stress-response proteins in brain progenitor cells when compared to retinal progenitor cells. One reason for this could be that our method of protein identification (MALDI-MS/MS) could not identify the proteins because the total amount of protein wasn’t adequate. Even though stress-response proteins were not identified in the brain progenitor cells, I would assay their expression to determine the relative level of oxidative stress the cells were experiencing. In addition to the antibodies
used in the studies reported in Chapters 3 and 4, an antibody against the stress-response protein thioredoxin would be utilized since it was one of the proteins identified to be increased in retinal progenitor cells but not used in the experiments because the lack of finding a suitable antibody for the cells. Heat shock protein 10 (Hsp10) would also be a good protein to use since it works with heat shock 60 (Hsp60). Since these cells are brain progenitor cells, antibodies to assay differentiation would include: nestin (a marker for neural progenitor cells), anti-beta tubulin III (TuJ1; a marker for early neurons), microtubule-associated protein 2 (MAP2; a marker for neurons), receptor interacting protein (RIP; a marker for Oligodendrocytes) and Glial fibrillary acidic protein (GFAP; a marker for astrocytes).

**Influencing Retinal Progenitor Cell Differentiation**

Blinding degenerative retinal diseases including retinitis pigmentosa, macular degeneration and glaucoma are characterized by loss of retinal neurons. At this time there is no way to replace retinal cell loss due to disease or injury since differentiated retinal cells are unable to regenerate. As a potential approach for treating retinal injury, neural progenitor cells have been proposed as a unique source of transplantable cells to replace lost cells in the damaged retina. By understanding the factors that influence retinal progenitor cell fate we can begin to develop effective cell replacement therapies for the treatment of retinal degeneration.

One future experiment that I would propose for influencing RPC differentiation is co-culturing retinal progenitor cells with retinal explants to see if there is a chance that the retinal explants would secrete factors that might be able to influence the retinal progenitor cells to differentiate the cells into photoreceptors. Since photoreceptors are one of the main
cell types to get damaged by retinal degeneration we need to be able to regenerate these cells in order to restore eyesight. In order to do this experiment I would place a layer of retinal progenitor cells at the bottom of a 24 well plate and culture retinal explants in a culture insert. The culture insert is a way to keep the cells from touching the retinal explants while allowing them access to secreted factors. Hopefully, the explants will secrete extrinsic factors the cells need in order to differentiate into photoreceptors. Since I want to influence the cells to become photoreceptors P1 retinal explants would be a good age to use in this experiment. Another option for this experiment would be using the stimulatory factor taurine in the culture media since it is known to increase the number of cells expressing rhodopsin, a marker for rod photoreceptors. Antibodies for neural progenitor cells (nestin), Mueller glia (GS), amacrine (Syntaxin), rod photoreceptors (Rhodopsin), rod bipolar (PKC alpha), cone and rod photoreceptors (Recoverin), horizontal (Calbindin) and ganglion cells (Brn3.2) could all be used to represent the morphology of the cells.

**Using Vitamin E to Affect Differentiation of “regular” Retinal Progenitor Cells**

Since we know how antioxidant vitamin E affects differentiation of retinal progenitor cells (which are a cell line) it would be nice to know how “regular” retinal progenitor cells are affected by vitamin E.

This experiment to investigate retinal progenitor differentiation would use retinal explants from C57BL/6 mice, cultured in media containing vitamin E. Since we know that at around P1 rod photoreceptors start to differentiate this age would be ideal to use for this experiment. These “regular” cells could either be dissociated or cultured as retinal explants for 10 days. Immunocytochemistry would be used to assay protein expression and cellular differentiation of the cells. Antibodies for neural progenitor cells (nestin), Mueller glia (GS),
amacrine (Syntaxin), rod photoreceptors (Rhodopsin), rod bipolar (PKC alpha), cone and rod photoreceptors (Recoverin) horizontal (Calbindin) and ganglion cells (Brn3.2) could all be used to assay differentiation of the various cell types. Proteomic analysis could also be a way to examine differences in protein expression between the “regular” cells (C57BL/6) and RPCs. As in Chapter 2, using two-dimensional gel electrophoresis along with MALDI-MS/MS would be a way to characterize the molecular differences between the two populations. Eventually co-expression binding sites could be examined between the populations of cells.

Doing additional experiments can one day help in realizing that these cells are and can be viable, useful and successful in retinal transplantations.
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