Chapter I

Introduction

Galen correctly identified the brain as ‘the source of the nerves, of all sensation, and of voluntary motion’ nearly 2000 years ago (May, 1968). Although he was mistaken about fluid flowing from the ventricles through nerves to muscles, he correctly identified nerves as conductors of information. It is clear today that the central nervous system is an extremely complex and elaborate organ system consisting of millions of different cells intimately connected to each other. This remarkable system allows us to interact with the world around us through the five classic senses: sight, touch, smell, hearing, and taste. We are able to perceive our existence, carry a conversation with a friend, and adapt to and change our environment because of the nervous system. When problems affecting the nervous system occur, it has a major impact on everyday life. Memories are forgotten, mobility is limited, and vision is lost. Unfortunately, once the central nervous system is damaged, there is no way to preserve or restore function. Intense research efforts are directed at finding strategies to reverse the devastating effects of central nervous system injury. Stem cell based therapies are being pursued by scientists around the world. Another approach to identify potential therapies is to observe successful regeneration and elucidate the underlying mechanisms. Then, principles of successful regeneration may be applied to human conditions where regeneration would be
beneficial. This work describes progress toward understanding the mechanisms of successful regeneration in the zebrafish retina.

**Development of the central nervous system**

The nervous system is divided into central and peripheral components based primarily on anatomical location in the body. The brain, spinal cord, and eyes are part of the central nervous system, whereas the peripheral nervous system is made up of sensory nerves in the limbs and trunk of the body which relay information to the central nervous system. The central and peripheral nervous systems are both made up of neurons and glia, the two major cell types of the nervous system. A wide variety of specialized neurons perform different functions, much like sensors or wires in an electrical circuit. Photoreceptors in the eye sense light; hair cells lining the cochlea detect sound waves; taste buds in the tongue identify specific molecules; motor neurons instruct muscles to contract; and other neurons transmit information from one neuron to another. In the brain, neurons can function as groups to interpret and process sensory input into what is perceived as sight, sound, smell, touch and taste. Historically, glia were thought to only provide structural support for neurons. In the peripheral nervous system, Schwann cells, a specialized type of glia, myelinate axons to increase action potential transmission speed along the length of the axon. In the central nervous system, Ependymal cells line the cavities of the brain and secrete cerebrospinal fluid. Recently however, glia have been found to act as stem cells in the developing brain (Noctor et al., 2001), and participate in signal transmission (reviewed in Schipke and Kettenmann, 2004).
It is remarkable to think that the various cells in a mature organism are all derived from a single cell. Early development is highly conserved among vertebrate species. After fertilization, a cell undergoes numerous divisions to become a relatively uniform ball of cells known as a blastocyst. The blastocyst is transformed into an embryo with three distinct layers, or types of tissue, through a process known as gastrulation. These three layers will each give rise to specific tissues within the body. The endoderm, or inner layer, will become the gut and lungs. The mesoderm, or middle layer, will give rise to blood vessels, muscles, bones, and connective tissues. The ectoderm, or outer layer, will develop into the skin and nervous system. As gastrulation occurs, the cells of the blastocyst are fundamentally changed from being capable of producing any and all the cells of the organism, to the more restricted fate of one of the three germ layers. This process of cell fate restriction is also known as differentiation.

How does the nervous system develop? It would be impossible to explain the complete sequence of events that occur during nervous system development in a single paragraph; however, a great deal is known about how the nervous system is formed. Neural stem cells are derived from the ectoderm, where cells will either become neural or epidermal tissue. What instructs the neural stem cells to become neural cells and not epidermal cells? This question may also be posed as a general question of how cell fate decisions are made. If the process of cell fate determination could be reduced to a simple choice between one of two fates, it might be relatively easy to control cell fate. Certainly not all cell fate decisions are this simple, but neural stem cell differentiation from the surrounding ectoderm is an example of this type of cell fate determination. This process has been elegantly characterized in the fruit fly, *Drosophila melanogaster* (reviewed in
Within the ectoderm, some cells are singled out to become neuroblasts by expressing a specific proneural gene (the term neural stem cell is not used here because stem cells are self-renewing and neuroblasts do not self-renew). Four genes which are now known as the achaete-scute complex (asc), achaete, scute, asense, and lethal of scute, promote neuroblast formation among ectodermal cells, and were therefore dubbed proneural genes. Mutation of proneural genes causes a lack of neural structures, while ectopic expression produces extra neural tissue (Jimenez and Campos-Ortega, 1990). Expression of these proneural asc genes is necessary and sufficient for neuroblast formation. How do these genes determine the neuroblast fate?

The proneural genes belong to a large family of basic helix-loop-helix transcription factors (see below) which regulate expression of target genes. One of these target genes, delta, encodes a transmembrane protein which acts as a ligand for notch receptor proteins (reviewed in Fleming, 1998). When notch receptors are activated, they suppress proneural gene expression. The delta and notch families are known as neurogenic genes because mutations of these genes in Drosophila neuralizes the fly; epidermal tissue assumes a neural fate, resulting in excess neural tissue (Hartenstein et al., 1992). Asc, delta and notch genes each play a part in an intercellular feedback loop which is responsible for many cell fate decisions (Fig 1.1). These three proteins are initially present in the same cell, and can be thought of as existing in a precarious equilibrium. Minor variations in gene expression can disrupt the balance and cause cells to adopt one fate or the other.

To illustrate how this process can occur, consider the developing Drosophila ectoderm. It is made up of a relatively homogeneous population of quiescent cells which
have the potential to become epidermal or neural tissue. Then, expression of a proneural gene in one of the nascent ectodermal cells becomes slightly higher compared to the surrounding cells. More abundant proneural protein in this cell increases the amount of $\delta$ protein on the cell surface, leading to an increase in $\text{notch}$ receptor activation on the surrounding cells. Increased $\text{notch}$ receptor activation suppresses proneural gene expression, preventing these cells from becoming neuroblasts. Proneural gene expression in neuroblasts then accomplishes two things. First, it causes neuroblasts to be formed. Second, it inhibits adjacent cells from assuming the same fate by activating notch signaling. This phenomenon is known as lateral inhibition. Proneural gene expression can be thought of as a cell fate switch. Expression in one cell commits that cell to a particular lineage, and maintains surrounding cell in an uncommitted state. This is an example of how proneural gene expression can control binary cell fate decisions.

In the developing nervous system, there must be an appropriate balance of differentiation and cell division in order to produce the correct number of neurons and glia. Proneural gene expression and lateral inhibition via notch signaling is one way to maintain this balance. One population of cells commits to a particular lineage and simultaneously signals to surrounding cells that they should remain uncommitted, thereby maintaining the population of multipotent cells. Here the term committed does not imply anything about whether the cell has the ability to continue dividing, only that it is more restricted in its ability to generate different cells. Neural progenitors are committed to a specific fate compared to ectodermal cells because neural progenitors only give rise to neural tissue, while ectoderm can give rise to both neural and epidermal tissue. As cells become more and more committed however, they eventually lose the ability to divide and
produce cells. These cells ultimately adopt physical and biological properties that are crucial to their function in the nervous system. This process is known as terminal differentiation.

For illustrative purposes, consider two simplified extremes: if every cell divides or maintains the ability to divide and never differentiates, there would be an infinite number of undifferentiated cells and no mature organism. On the other hand, if cells commit to particular fates too early, development would end before all of the needed cells are produced. The reality in the developing nervous system must lie somewhere between these two extremes. If one were to propose a strategy to ensure a balance between differentiation and maintaining the ability to continue dividing, one could imagine a network of cell communication to instruct some cells to maintain their potential, and other cells to limit their potential and differentiate. Proneural gene expression and lateral inhibition are part of this network of cell communication which helps coordinate development of the nervous system.

**Neural basic helix-loop-helix transcription factors**

The proneural genes mentioned above belong to a large family of basic helix-loop-helix (bHLH) transcription factors that exert their effect by regulating target gene expression. All bHLH family members have a conserved helix-loop-helix motif that allows dimerization to other proteins with a hlh domain. Because there are hundreds of members of this family of transcription factors, they have been divided into seven classes according to DNA binding specificity, tissue specific expression, the ability to form homo- or heterodimers, and transcriptional activity (Massari and Murre, 2000, Murre et
al., 1994). Each class has features which distinguish it from the other bHLH family members. The E proteins $E_{12}$ and $E_{47}$ are representative of Class I bHLH proteins. These proteins bind E-boxes (CANNTG), are expressed ubiquitously, form homo- and heterodimers, and activate transcription (Murre, McCaw and Baltimore, 1989). The proneural genes described above are Class II bHLH proteins. In contrast to Class I, Class II family members are expressed in specific cells or specific tissues and do not form homodimers. Class II proteins usually form heterodimers with the E proteins to bind E-boxes and activate transcription (Johnson et al., 1992). Class III family members, including $Myc$, have a leucine zipper domain in addition to the bHLH domain. Class IV proteins form heterodimers with myc or each other (Blackwood, Luscher and Eisenman, 1992). Class V proteins, also known as id proteins, lack the basic portion of the bHLH domain, which renders them incapable of binding DNA, but allows them to sequester Class I and II proteins away from DNA, thereby repressing transcription (Benezra et al., 1990). Class VI proteins such as $Hes-1$ act as transcriptional repressors (Chen et al., 1997), are distinguished by a proline amino acid residue in their basic region. Class VII proteins have a PAS domain in addition to the bHLH domain, which also acts as a dimerization motif.

This diverse group of related transcription factors is involved in many aspects of development including neurogenesis, myogenesis, sex determination and hematopoeisis. One feature which all these transcription factors share is their involvement in cell fate determination and differentiation. Several bHLH genes are expressed in the developing nervous system including family members from Class I, II, V, and VI. Class II proneural genes are expressed in neural progenitors. Class VI proteins such as $Hes-1$ participate in
notch-mediated suppression of the proneural genes by blocking expression of Class II proteins, and are often called neurogenic bHLH factors because of their relationship to the neurogenic gene notch.

In vertebrates, proneural bHLH genes regulate various processes such as formation of neural progenitors, neuronal differentiation, and cell cycle exit. Proneural bHLH genes also seem to promote neural properties while inhibiting glial properties. Mutations in Ascl1 for example, disrupt telencephalic progenitor formation (Casarosa, Fode and Guillemot, 1999). Mice lacking the proneural homolog Math1 lack a specific type of cerebellar neuron known as a granule neuron, although other neurons and glia within the cerebellum are normal (Ben-Arie et al., 1997). These examples illustrate how proneural gene expression can be involved in cell fate specification at both early and late stages. Although the complete story of development is complicated, one could imagine that multiple iterations of cell fate decisions, each using different combinations of bHLH genes, could easily create the incredible diversity that is seen in the developed nervous system.

bHLH genes and vertebrate eye development

Because of the relatively simple structure of the vertebrate retina, it has been used as a model to investigate the role bHLH factors play in the complex process of central nervous system development. The retina is composed of six neuronal types and one type of glia. This simple arrangement makes questions about cell fate determination relatively less complicated. These seven cell types are organized into three nuclear layers (Fig 1.2). Mature retinal cells are derived from retinal progenitors (Turner and Cepko, 1987) and
are generated in a specific histologic order with ganglion cells appearing first, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and finally, Müller glia; although production of these cell types overlaps considerably (Young, 1985). Rod and cone photoreceptors detect light which enters the eye through the lens. These light sensing cells relay information to bipolar cells which transmit their signals to ganglion cells. Retinal ganglion cells send information to the brain via axons which travel within the optic nerve. Horizontal and amacrine cells modulate signal transmission between photoreceptor, bipolar and ganglion cells.

The mouse retina is formed during a three week period that starts before birth and ends by postnatal day 12 (Young, 1985). A sheet of neuroepithelial tissue known as the optic cup is populated with numerous retinal progenitors that will become mature neurons and glia. These retinal progenitors express a variety of homeodomain (HD) and bHLH transcription factors in the developing retina. Pax6 in particular, is a very important HD transcription factor that is required for retina development. Mice lacking Pax6 do not have eyes (Hill et al., 1991, Hogan et al., 1988) and ectopic pax6 expression is sufficient to induce formation of ectopic eyes in frogs (Chow et al., 1999), demonstrating an essential role for Pax6 in eye development. How does pax6 control eye development? Part of the answer lies in its ability to regulate proneural bHLH expression in retinal progenitors. In an elegantly designed experiment, pax6 was shown to regulate Ascl1, Ngn2, and Math5 expression in retinal progenitors (Marquardt et al., 2001). Normally these genes are expressed in different subsets of retinal progenitors (see below). However, when Pax6 was removed from the distal part of the developing optic cup, retinal progenitors no longer expressed these genes, and only amacrine cells were
produced instead of the normal complement of seven retinal cell types. These experiments indicate that _Pax6_ is required for proneural gene expression in retinal progenitors. The observation that different proneural genes are expressed in different retinal progenitors suggests that these genes play a role in cell fate specification (Alexiades and Cepko, 1997). _Math5_ is required for retinal ganglion cell production in mice and fish (Brown et al., 2001, Kay et al., 2001). _Ascl1_ is expressed in retinal progenitors (Jasoni and Reh, 1996) and is required in combination with _Ath3_ for bipolar cell specification (Hatakeyama et al., 2001, Tomita et al., 1996). Mutations in _NeuroD_ affect amacrine and photoreceptor production (Akagi et al., 2004, Morrow et al., 1999). These experiments suggest that proneural genes are involved in specifying distinct cell types. Indeed, when _Ascl1, Ngn2_ and _Math5_ are absent from retinal progenitors, only amacrine cells are produced (Marquardt et al., 2001). When other proneural bHLH genes are mutated, other cell types are formed at the expense of the missing cell type (Akagi et al., 2004, Brown et al., 2001, Inoue et al., 2002, Wang et al., 2001), suggesting the progenitors of the missing cell types assumed a different fate. Proneural genes then appear to play similar roles in _Drosophila_ and vertebrates to specify neural cell fate.

The observation that retinal cell types are born in a specific order led to the proposal that retinal progenitors go through ‘competence states’ where early retinal progenitors produce early retinal cell types, and late retinal progenitors produce later born cell types (Cepko et al., 1996). As mentioned above, bHLH transcription factors are expressed in subsets of retinal progenitors and predispose them toward specific cell fates. This could be accomplished by inhibiting other progenitors from adopting the same fate by activating the notch signaling pathway (Belliveau and Cepko, 1999, Harris and
expression seems to inhibit expression of the other proneural genes in retinal ganglion cell progenitors (Mu et al., 2005). Notch signaling is required to instruct fate choices in the developing Drosophila eye (Cagan and Ready, 1989, Fortini et al., 1993). In the absence of notch signaling, lateral inhibition is impaired, and retinal precursor cells adopt incorrect cell fates, disrupting the highly regular pattern of the Drosophila eye. A similar process could act in the developing vertebrate eye. Notch signaling could be activated by proneural bHLH gene expression in different types of retinal progenitors, thereby inhibiting the undifferentiated cells from adopting the same fate.

While it is clear that proneural genes are required for cell fate specification, it is not entirely clear how all of these proneural genes work at the molecular level. It is presumed that proneural genes in vertebrates activate a neuronal differentiation program by inducing expression of target genes which regulate cell type specification. Indeed, overexpression of proneural bHLH genes in cell culture caused progenitors to exit the cell cycle and differentiate into neurons (Farah et al., 2000), suggesting these genes function as differentiation factors. Perhaps the best documented example of this occurring in vivo is Math5 mediated differentiation of retinal ganglion cells. As mentioned earlier, Math5 is required for ganglion cell specification (Brown et al., 2001, Kay et al., 2001). It initiates differentiation of ganglion cells by inducing expression of the POU transcription factor Brn3b (Brown et al., 2001, Liu, Mo and Xiang, 2001, Wang et al., 2001). Brn3b is an early marker of retinal ganglion cell differentiation (Gan et al., 1996), and is required for differentiation to proceed (Gan et al., 1996). A network of
genes is activated by *Brn3b* which ultimately leads to the terminal differentiation of ganglion cells (Mu et al., 2005).

Once the eye is formed and the retinal progenitors have differentiated, the retina stops growing. A similar process is thought to occur during human development. Human eyes grow an average of 6mm between birth and age 8 (Fledelius and Christensen, 1996), although it is not clear whether there are any new cells added to the retina, or whether this growth represents a change in eye shape or size only. Regardless of how this growth occurs, when retinal cells are destroyed, they are not replaced. Any disease or injury which kills retinal cells often results in partial or complete loss of vision. Understanding how to replace diseased retinal cells is a major goal of vision research today. Such knowledge would not only restore sight to patients with eye disease, it may provide insight into how to repair other parts of the central nervous system.

**Regenerative capacity of the teleost retina**

The teleost retina is histologically identical to the mammalian retina and is formed from retinal progenitor cells in much the same way, although retinogenesis occurs much more rapidly; fish are able to see and catch prey within 72 hours post fertilization. In addition to more rapid development, there is another major distinction between the fish and mammalian retinas; new retinal tissue is formed throughout the life of fish by a population of retinal stem cells. After the larval retina is formed, retinal progenitors become confined to the periphery of the eye. They form an annulus of proliferating cells at the edge of the mature retina called the circumferential germinal zone (CGZ). The
CGZ is a reservoir of retinal stem cells that continually adds new retinal cells as the eye grows. All retinal cell types are produced by the CGZ except rod photoreceptors.

The eye of teleost fish not only grows by adding new cells at the retinal margin, it also expands in circumference, reducing the density of cells. With a decrease in density, one might expect a loss in visual sensitivity; however, rod photoreceptors are generated in the central retina, possibly as a compensatory mechanism. These new rods are apparently integrated into the existing retinal circuitry, as visual sensitivity is retained even as the retina expands (Powers et al., 1988). In order for rods to be continually added, there must be a population of cells in the central retina which can respond to the decreased cell density and initiate rod production. Indeed, rod precursors were found to reside in the outer nuclear layer and give rise to new rods (Johns and Fernald, 1981). Later, a population of stem cells in the inner nuclear layer was identified as the source of the rod precursors (Julian, Ennis and Korenbrot, 1998, Otteson, D'Costa and Hitchcock, 2001) and a model of rod genesis was developed (Otteson, D'Costa and Hitchcock, 2001). The inner nuclear layer stem cells were identified by long term BrdU labeling and expression of the master eye regulator *Pax6* (Otteson, D'Costa and Hitchcock, 2001). These cells are thought to divide to produce a rod precursor and a retinal stem cell. This population of stem cells in the central retina is very intriguing; cells such as these might be used to repair a damaged retina.

The regenerative capability of the fish retina was discovered in the late 1960s, long before the identification of rod precursors and inner nuclear layer stem cells (reviewed in Hitchcock et al., 2004). The earliest experiments induced retina regeneration by surgically removing a portion of the central retina. Following injury,
mitotic cells line the edge of the incision and proliferate to give rise to new retinal tissue. Because the INL stem cells had not been identified yet, it was postulated that the CGZ was the source of these regenerated cells. Regeneration restores the original architecture and histology of the retina (Cameron and Easter, 1995, Cameron, Vafai and White, 1999, Hitchcock et al., 1992, Vihtelic and Hyde, 2000), as well as function (Hitchcock and Cirenza, 1994, Mensinger and Powers, 1999). When rod precursors were identified, they were originally thought to be the source of new neurons and glia during regeneration (Raymond, Reifler and Rivlin, 1988). Identification of stem cells in the inner nuclear layer immediately raised the possibility that these cells could be the source of retina regeneration (Otteson, D'Costa and Hitchcock, 2001). Indeed, after injury, clusters of proliferating cells known as ‘neurogenic clusters’ appear in the inner nuclear layer (Cameron, 2000, Vihtelic and Hyde, 2000, Wu et al., 2001). These proliferating cells express genes that are associated with retinal stem cells such as pax6, vsx1, n-cadherin and notch (Hitchcock et al., 1996, Levine et al., 1994, Sullivan et al., 1997, Wu et al., 2001). Newborn cells derived from neurogenic clusters are closely associated with Müller glia and appear to migrate along their radial processes (Vihtelic and Hyde, 2000). However, these newborn cells could also come from Müller glia, since Müller glia also re-enter the cell cycle after retinal injury (Wu et al., 2001). Evidence from the developing chick indicated that Müller glia could generate new neurons following retinal injury (Fischer and Reh, 2001), further enhancing speculation that Müller glia could generate multipotent cells that contribute to retina regeneration in fish, and that Müller glia were the previously uncharacterized inner nuclear layer stem cells.
**Alpha1-tubulin transgenic zebrafish to study central nervous system regeneration**

Although substantial progress was being made toward understanding nervous system development and regeneration, there were not any existing models which took full advantage of zebrafish as a model system for studying regeneration. Zebrafish development is very rapid; larvae swim and eat within three days post fertilization. Developing embryos are transparent, making it very easy to visualize the eye, brain and spinal cord. Single cell embryos are easily injected with DNA or RNA for transient expression analysis and creation of stable transgenic lines. In addition, zebrafish are amenable to forward genetic screens. Finally, zebrafish, like other teleost fish, are able to successfully regenerate their central nervous system. Therefore, our lab set out to create transgenic zebrafish to facilitate studies of gene regulation during central nervous system development and regeneration.

*Alpha1-tubulin* (α1T) is a microtubule protein subunit which is expressed in the developing and regenerating peripheral nervous system of mice (Gloster et al., 1994). This gene is also induced in the regenerating zebrafish optic nerve (Hieber et al., 1998). By investigating how expression of an early marker of regeneration such as α1T is regulated, our lab hoped to identify genes involved in successful regeneration. A fragment of the α1T promoter was cloned upstream of the coding sequence for green fluorescent protein (GFP) to create an α1T:GFP reporter. Then, transgenic zebrafish harboring this α1T:GFP reporter were created (Goldman and Ding, 2000, Goldman et al., 2001). Transgene expression in these fish faithfully recapitulates endogenous α1T expression during development and axon regeneration (Goldman and Ding, 2000, Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004). These transgenic fish
could be used as a probe to investigate mechanisms of successful CNS regeneration. The basic premise of this model is to identify DNA elements that mediate transgene expression by deleting or mutating portions of the α1T promoter. Once DNA elements are identified, potential transcription factors which act at these DNA elements can be tested for their ability to regulate transgene expression in cell culture or in vivo. Transcription factors which regulate α1T likely regulate other genes involved in successful regeneration. Therefore, identifying transcription factors which regulate α1T transgene expression may provide insight into the mechanisms underlying successful regeneration. Using this transgenic model, we set out to test whether Müller glia function as injury induced retinal stem cells and found that they can generate all neurons and glia in the regenerating retina. Because α1T expression is induced in proliferating Müller glia following retinal injury, we searched for DNA elements that mediate α1T transgene expression in these cells. Transcription factors which regulate α1T expression during regeneration are likely involved in regulating other aspects of successful retina regeneration. Indeed, we found that the proneural bHLH gene ascl1a not only regulates α1T promoter activity, but also regulates transformation of Müller glia into multipotent retinal progenitors which are required for successful retina regeneration in zebrafish.
Figure 1.1 Proneural gene expression and lateral inhibition. The tan cell expresses higher levels of proneural bHLH protein than the pink cell, which induces delta expression and leads to higher levels of delta on the surface of the tan cell. Notch receptors are activated in the pink cell, which leads to inhibition of bHLH gene expression. Adapted from Marquardt and Gruss, 2002.
Figure 1.2 Schematic diagram of the retina. The seven retinal cell types are shown in their respective nuclear layers. Nuclei are represented as blue ovals. Light entering the eye reaches the photoreceptors by traveling through the layers of the retina. ONL – outer nuclear layer, INL – inner nuclear layer, GCL – ganglion cell layer.
References


Chapter II

A Role for α1 Tubulin-Expressing Müller Glia in Regeneration of the Injured Zebrafish Retina

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Summary

α1 tubulin (α1T) is a neuron-specific microtubule protein whose expression is induced in the developing and regenerating CNS. In the adult CNS, α1T expression remains high in neural progenitors. Transgenic zebrafish harboring a 1.7kb α1T promoter fragment along with the first exon and intron express the transgene in a manner that recapitulates expression of the endogenous gene. We recently showed that this promoter mediates gene induction in retinal ganglion cells during optic nerve regeneration and in a subset of Müller glia that proliferate following retinal injury (Senut, Gulati-Leekha and Goldman, 2004). To further characterize these Müller glia we generated transgenic fish harboring an α1T promoter fragment that is specifically induced in these cells following retinal damage. Transgene expression, BrdU-labeling and stem cell marker expression suggested that α1T-expressing Müller glia dedifferentiate and become multipotent in response to injury. In addition, GFP and BrdU-mediated lineage tracing combined with retinal gene expression analysis indicated that α1T-expressing Müller glia were capable of generating retinal neurons and glia.
These data strongly suggest α1T-expressing Müller glia dedifferentiate and mediate regeneration of the injured zebrafish retina.

**Introduction**

The capacity of fish to regenerate an injured retina has been recognized for decades (for review, see Hitchcock et al., 2004). Early work demonstrated that after removal of a patch of retina, new cells were added at the margins of the lesion to repair the damaged retina (Hitchcock et al., 1992). Subsequently, other injury models, including mechanical, chemical and light lesions have also been shown to induce a regenerative response which is capable of restoring retinal architecture and function (Braisted, Essman and Raymond, 1994, Cameron, 2000, Maier and Wolburg, 1979, Vihtelic and Hyde, 2000). One commonality in all these models is that the injury produces columns of proliferating cells know as ‘neurogenic clusters’. These neurogenic clusters express markers of retinal stem cells such as *Pax6, Vsx1, Notch-3*, and *N-cadherin* (Hitchcock et al., 1996, Levine et al., 1994, Sullivan et al., 1997, Wu et al., 2001). It was originally speculated that these clusters were derived from rod precursors (Raymond, Reifler and Rivlin, 1988). However, with the identification of putative stem cells in the inner nuclear layer (INL) of the goldfish retina (Otteson, D'Costa and Hitchcock, 2001), and evidence that INL cell proliferation precedes retina regeneration (Braisted, Essman and Raymond, 1994, Vihtelic and Hyde, 2000, Wu et al., 2001, Yurco and Cameron, 2005), it seems likely that putative stem cells in the INL are the predominant source of progenitors for retinal regeneration. Interestingly, Müller glia, whose cell bodies are also located in the INL, show a proliferative response following
retinal injury and have not been eliminated as a source of retinal progenitors (Braisted, Essman and Raymond, 1994, Wu et al., 2001). In addition, Müller glia have been suggested as a source of retina regeneration in zebrafish (Yurco and Cameron, 2005). Recent reports that Müller glia in postnatal chicks (Fischer and Reh, 2001) and rodents (Ooto et al., 2004) have a limited capacity to generate neurons following retinal injury further implicates them as a potential source of retina repair in the damaged fish retina. However, the lack of suitable markers for retinal stem cells and the inability to perform lineage tracing in adult fish make the identification of the cellular source of retina regeneration difficult.

We previously demonstrated that a 1.7 kb $\alpha_1$ tubulin ($\alpha_1T$) promoter fragment along with its first exon and intron directed GFP transgene expression to the developing and regenerating zebrafish CNS (Goldman and Ding, 2000, Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004). In the adult CNS, this promoter directs transgene expression to neural progenitors (Goldman et al., 2001). Interestingly, retinal injury causes induction of the $\alpha_1T$ promoter in a sub-population of Müller glia that are proliferative (Senut, Gulati-Leekha and Goldman, 2004, Vihtelic et al., 2006) and exhibit certain characteristics that are shared with stem cells (Yurco and Cameron, 2005). In light of these observations and because Müller glia have been reported to generate retinal neurons in other systems (Fischer and Reh, 2001, Ooto et al., 2004), we were interested in determining if the $\alpha_1T$-expressing Müller glia function as neural progenitors in the injured zebrafish retina. To facilitate these studies we identified a truncated $\alpha_1$ tubulin promoter fragment (approximately 1 kb) that was specifically induced in proliferating Müller glia following retinal injury. Transgenic fish harboring this promoter fragment
driving GFP expression were used in conjunction with BrdU labeling to identify and follow \( \alpha_{1T} \)-expressing Müller glia after retinal injury. Our data suggest that these particular Müller glia are able to dedifferentiate, proliferate, and generate new neurons to repair the damaged retina.

**Results**

-1016\( \alpha_{1T} \) transgene expression is induced in Müller Glia following retinal injury

We previously reported that transgenic fish harboring the wild-type 1.7 kb \( \alpha_{1T} \) promoter predominantly express GFP in proliferating Müller glia within three days of retinal injury (Senut, Gulati-Leekha and Goldman, 2004). We hypothesized that \( \alpha_{1T} \)-expressing Müller glia may function as retinal stem cells based on four observations. First, Müller glia exhibit proliferative properties and survival typical of stem cells (Yurco and Cameron, 2005). Second, the \( \alpha_{1T} \) transgene is predominantly expressed in stem cells of the adult CNS (Goldman et al., 2001) suggesting its expression in the retina may identify stem cells there. Third, transgene expressing Müller glia begin to express the neuronal marker \textit{HuC/D} at 1 week following light-induced retinal injury (Vihtelic et al., 2006), suggesting cells derived from transgene-expressing Müller glia may differentiate into neurons. Fourth, Müller glia in postnatal birds and rodents can be induced to generate new neurons (Fischer and Reh, 2001, Ooto et al., 2004).

To pursue these studies we thought it was important to identify an \( \alpha_{1T} \) promoter fragment that specifically directed transgene expression to Müller glia that proliferate following retinal injury. Because the 1.7 kb promoter fragment is induced in cells that are regenerating their damaged axons (Goldman et al., 2001, Senut, Gulati-Leekha and
Goldman, 2004), it is not a suitable promoter for these studies. Therefore, we assayed various promoter deletions in transgenic fish for expression following retinal injury and identified deletion -1016α1T that is missing 680 bp from the 5’ end of the α1T promoter. Three independent lines of transgenic fish harboring the -1016α1T:GFP transgene in their germ line were identified and all exhibited expression in Müller glia following injury, but not in injured retinal ganglion cells (Fig 2.1). To confirm -1016α1T:GFP transgene expression was not induced in injured retinal ganglion cells, we first performed \textit{in situ} hybridizations on sections from 4 days post-injury (4dpi) retinas and found that the -1016α1T transgene was expressed in endogenous α1 tubulin expressing cells of the INL, but not in α1 tubulin expressing retinal ganglion cells (data not shown). Next, we examined transgene expression following optic nerve crush. As expected, the injured ganglion cells expressed GAP43, which is induced during axon regeneration (Bormann et al., 1998, Perry, Burmeister and Grafstein, 1987), but not GFP (Fig 2.2), confirming our initial observations that retinal ganglion cells harboring the -1016α1T:GFP transgene do not induce GFP in response to injury. In uninjured control retinas, endogenous α1T is only expressed in progenitors at the circumferential germinal zone (CGZ), and the -1016α1T transgene retains this expression pattern (Fig 2.3).

-1016α1T expressing Müller glia proliferate following retinal injury

To more carefully examine cell proliferation following retinal injury and correlate this with transgene expression (see below), we performed a BrdU pulse-labeling experiment. -1016α1T:GFP transgenic fish received retinal lesions at day 0 (0dpi) and were given a single dose of BrdU at 24hr intervals (1dpi, 2dpi, etc.). The fish were then
sacrificed 4hr after BrdU administration and the eyes were isolated, cryoprotected, and serially sectioned. BrdU-labeled (BrdU+) cells adjacent to the injury were quantified to estimate the total number of BrdU+ cells per lesion (Fig. 2.4a). Previous studies had shown that there is no detectable cell proliferation outside the circumferential germinal zone (CGZ) in the retinal margin of adult zebrafish at 0 and 1dpi (Cameron, 2000, Yurco and Cameron, 2005). However by 2dpi, cell proliferation in the outer nuclear layer (ONL) and INL is observed (Yurco and Cameron, 2005). Consistent with these results, at 1dpi we rarely observed BrdU+ cells in the extant retina and those that were labeled were generally found in the ONL where rod progenitors, previously described in goldfish, are found (Johns, 1977, Meyer, 1978), and in the optic fiber layer, where proliferating microglia can be found (Braisted, Essman and Raymond, 1994, Negishi and Shinagawa, 1993, Yurco and Cameron, 2005). BrdU+ cells in the CGZ were observed at all time points, indicating BrdU administration was successful at labeling proliferating cells (data not shown), but were excluded from our cell counts. At 2dpi, there is a dramatic increase in the number of BrdU+ cells in the INL, and 76% of the total BrdU+ cell population resides in the INL (183 out of 238 BrdU+ cells/lesion). Interestingly, this increase precedes the increased number of BrdU+ cells in the ONL at 3dpi, perhaps indicating that cells from the INL give rise to the proliferating cells observed at later time points. The number of BrdU+ cells in the INL and ONL reached a peak at 4dpi before declining and returning to near baseline levels by 7dpi (Fig 2.4a). This data suggests that the cells that are required to repopulate a damaged retina are rapidly produced in response to injury and is consistent with previous work demonstrating a spatiotemporal pattern of cell proliferation following injury (Yurco and Cameron, 2005).
We wondered what portion of the BrdU-labeled cells corresponded to GFP expressing (GFP⁺) cells. To answer this question, we assayed BrdU⁺ cells for GFP expression (Fig 2.4b). At 1dpi, rare BrdU⁺ cells that could be rod progenitors and microglia could be identified based on their nuclear morphology and position within the retina (Fig 2.4c and d). Although there were few BrdU⁺ cells at 1dpi, some of these were also GFP⁺, and probably represent the earliest expression of the transgene in an activated Müller glia (Fig 2.4e-g, see below). Almost all BrdU⁺ cells in the INL at 2-5dpi are also GFP⁺ (Fig 2.4b) and have typical Müller glia morphology (Fig 2.4h-k). At 3dpi and later, we noticed some GFP⁺ Müller cells that were not labeled with BrdU (arrowhead, Fig 2.4j). The BrdU⁻/GFP⁺ cells are always associated with elongated nuclear morphology that is typical of the BrdU⁺ nuclei, and we expect that these cells are also proliferative, but were not in S-phase at the time of BrdU administration (see below). From 6-7dpi, GFP expression declines, and corresponds to reduced BrdU labeling at these time points. These data indicate that proliferating cells also express GFP during the first week following retinal injury.

While most BrdU⁺ cells express GFP at 4dpi (94% of the total BrdU⁺ cells, 1087/1161; and 98% of the INL BrdU⁺ cells, 662/673), and these GFP⁺ cells appeared to be Müller glia based on cell morphology, we wanted to determine whether the -1016α1T promoter drives transgene expression exclusively in Müller glia following injury. We therefore examined whether two Müller glial markers, zrf1, (which recognizes glial fibrillary acidic protein [GFAP]), and glutamine synthetase (GS) co-localized with GFP expression at 4dpi. Confocal microscopy showed that GFP⁺ cells also express Müller glial markers (Fig. 2.5). We next quantified the co-expression of GFP and glial markers
to determine what percentage of the GFP\(^+\) cells could be considered Müller glia. Of 107 GFP\(^+\) cells assayed for GFAP expression, 105 (98\%) were labeled. Of 205 GFP\(^+\) cells assayed for GS expression, 200 (98\%) were labeled. We also carried out immunostaining with the neuronal marker \(HuC/D\) and other retinal cell-type specific markers (zpr1, \(PKC\), \(TH\), data not shown) to determine whether any other retinal cell types expressed GFP at 4dpi. Despite an extensive search, no co-labeling could be identified. While we can not rule out the possibility that a small cell population in the INL other than Müller glia proliferates in response to injury, our data suggest that such a population represents less than 2\% of the total proliferating INL cell population.

**‘Neurogenic clusters’ are derived from proliferating Müller glia**

We noticed that GFP\(^+\) cells are remarkably similar to ‘neurogenic clusters’ of regenerating cells described by others studying retina regeneration in zebrafish and goldfish (Faillace, Julian and Korenbrot, 2002, Raymond, Reifler and Rivlin, 1988, Vihtelic and Hyde, 2000, Yurco and Cameron, 2005). The origin of these clusters has not been identified, but they are formed in response to injury and appear as a group of cells with elongated nuclei that can be identified by BrdU labeling or by staining with antibodies to *proliferating cell nuclear antigen*. In the injured \(-1016\alpha1T:GFP\) transgenic retina, groups of cells with elongated nuclei always correspond to GFP\(^+\) Müller glia, leading us to hypothesize that the GFP\(^+\) cells we observe are neurogenic clusters. In the injured goldfish retina, neurogenic clusters are known to express markers of stem/progenitor cells such as *Pax6*, *Vsx1*, *Notch-3*, and *N-cadherin* (Hitchcock et al., 1996, Levine et al., 1994, Sullivan et al., 1997, Wu et al., 2001). To determine if GFP\(^+\)
Müller glia express markers of stem cells, we assayed for Pax6 expression in 4dpi retinas from -1016α1T transgenic fish (Fig. 2.6). This stem cell marker was induced in the GFP⁺ population of Müller glia (30 out of 33 GFP⁺ Müller cells examined expressed Pax6). While not all of the GFP⁺ cells expressed Pax6, all clusters of Pax6 expressing cells were GFP⁺ (arrowheads, Fig. 2.6). These data suggest GFP⁺ Müller glia or cells derived from GFP⁺ Müller glia are equivalent to the neurogenic clusters that may function as multipotent progenitors to mediate retina regeneration. These data also suggest that the -1016α1T promoter can be used to direct gene expression to neurogenic clusters of the injured retina.

Since we observed an increase in proliferation in the INL at 2dpi that preceded the increased proliferation in the ONL at 3dpi (Fig 2.4a), and BrdU⁺ cells occupy different positions within the INL at 2dpi (Fig 2.4h), we hypothesized that the proliferative INL cells at 2dpi gave rise to cells in other nuclear layers at 3dpi and beyond. We performed a 2dpi pulse-chase experiment by giving a single dose of BrdU at 2dpi and examining retinas at 3, 4, 5, and 7dpi (Fig. 2.7). BrdU labeled cells were present in the ONL and GCL at all time points, suggesting BrdU⁺ cells from the INL had migrated to these layers, or that the few BrdU⁺ cells within the ONL and GCL had divided (Fig. 2.7).

**Neurogenic clusters are closely associated cells with characteristics of Müller glia**

We observed that each neurogenic cluster appeared to correspond to a single, hypertrophied, GFP⁺ Müller glia. Confocal microscopy revealed that BrdU⁺ nuclei reside within what appears to be a single GFP⁺ Müller glia (Fig. 2.8). We captured stacks of images to produce orthogonal projections and found that BrdU⁺ nuclei expressed GFP.
(arrow, top panel, Fig 2.8a). Nuclei within what appears to be a single Müller glia express GFP (white arrows, Fig 2.8b), while nuclei outside the GFP⁺ Müller glia do not (arrowhead, Fig 2.8b). To determine whether the proliferating cells had undergone karyokinesis without cytokinesis or if the cells were clusters of individual GFP⁺ cells, we performed electron microscopy on sections from 4-5dpi retinas (data shown for 4dpi; Fig 2.8c-f). The elongated proliferating nuclei that are faintly stained with DAPI (insets, Fig 2.8a-b) are always GFP⁺ and can be easily identified in the light microscope based on these criteria. These neurogenic clusters are also identifiable in the electron microscope because of their distinct chromatin pattern and elongated nuclear morphology (asterisks, Fig 2.8c). We examined several neurogenic clusters (n=5) and found thin plasma membranes separating individual elongated nuclei (arrowheads, Fig 2.8d), indicating that the clusters are composed of tightly associated cells. Further analysis of the electron micrographs showed that cells within neurogenic clusters shared certain characteristics with Müller glia. First, the cytoplasm of neurogenic clusters is indistinguishable from that of the neighboring Müller glia whose processes are extending around them (Fig 2.8e and f). Müller glia form stereotypical junctions with other Müller glia (Krebs and Krebs, 1991) that are readily identified in the electron microscope (Fig 2.8c, e and f). Second, we identified elongated nuclei in the inner plexiform layer (IPL) surrounded by cytoplasm that extended processes laterally (Fig. 2.9), which is typical of Müller glial processes in this layer (Krebs and Krebs, 1991). These data provide additional evidence to suggest that neurogenic clusters are derived from proliferating Müller glia.
Müller glia give rise to new neurons after injury

The above data suggest that -1016α1T expressing Müller glia respond to retinal injury by dedifferentiating and expressing retinal stem cell markers such as Pax6 and α1T. If these cells function as injury-induced retinal progenitors, they should ultimately generate new neurons. Ideally, we would use GFP expression as a lineage tracer to follow the fate of these cells. However, GFP expression is transient and becomes barely detectable after 14dpi, suggesting GFP expressing cells have differentiated (data not shown). Therefore, we focused our attention on early time points (7-11dpi) when new retinal neurons may be forming and GFP expression would be easily detected. We used two different markers for this experiment. First, we used HuC/D expression to identify newly born neurons because some Müller glia express this marker 7 days following photoreceptor damage (Vihtelic et al., 2006). Next, we used zn5, which is transiently expressed by retinal ganglion cells as they differentiate and extend axons (Trevarrow, Marks and Kimmel, 1990). Consistent with the idea that GFP+ Müller glia generate new retinal neurons, we found GFP+/HuC/D+ cells as early as 7dpi (Fig 2.10a-d). In addition, we identified GFP+/zn5+ retinal ganglion cells in the INL, IPL and ganglion cell layer (GCL) at 7 and 11dpi (11dpi shown, Fig 2.10e-l). The cells in the INL and IPL may represent differentiating retinal ganglion cells (RGCs) that are migrating to the GCL, or misplaced RGCs.

To evaluate the fate of proliferating Müller glia at later time points, we injected fish with a single dose of BrdU at 4dpi and assayed cell fate at 60 and 180dpi. We chose to label dividing cells at 4dpi because it represented the peak of cell proliferation (see Fig 2.4a). At this time point, 98% of the proliferating cells in the INL can be characterized as
Müller glia based on morphology and GFP expression. We observed that cells labeled with BrdU at 4dpi expressed markers of bipolar and amacrine cells, Müller glia, and cone photoreceptors at 60 and 180dpi (data shown for 180dpi, Fig 2.11). Ganglion cells lose expression of zn5 shortly after their axons reach their target in the tectum, preventing their identification at these later time points, although these cells were detected at early time points (Fig 2.10). We also detected BrdU$^+$ cells in the correct laminar position for horizontal cells (data not shown) although a marker specific for this cell type is not available for the zebrafish. These data indicate that cells labeled with BrdU at 4dpi are able to produce new neurons and glia. Similar results were obtained when BrdU was administered at 2dpi and cell fate was assayed at 180dpi (data not shown). The fact that new neurons were generated is not surprising, as the capacity to regenerate following injury has been well documented; however, our data strongly suggest that these new neurons were derived from $\alpha$1T-expressing Müller glia.

While nearly all of the BrdU$^+$ cells at 4dpi are Müller glia (see above), we wondered whether it was plausible that the few BrdU$^+/\text{GFP}^-$ cells in the INL that were labeled at 4dpi could give rise to all of the BrdU$^+$ cells we observe at 180dpi. We performed a pulse-chase experiment by labeling with BrdU at 4dpi and examining serial sections from retinas at 4, 7, 11, and 180dpi. We counted the number of BrdU$^+$ cells in each nuclear layer and calculated the total number of BrdU$^+$ cells/lesion by multiplying the average number of BrdU$^+$ cells/lesion by the number of slides collected (we examined 4 lesions for 4 and 11dpi; and 3 lesions for 7 and 180dpi; we collected 5 slides of serial sections for 4, 7, and 11dpi; and 6 slides for 180dpi) (Fig 2.12). Consistent with our observation that the peak of proliferation occurs at 4dpi, we did not detect an increased
number of BrdU$^+$ cells at 7, 11, or 180dpi, indicating that the cells labeled with BrdU at 4dpi did not undergo any significant cell division. Therefore, it is unlikely the few BrdU$^+$/GFP$^-$ cells, that are present at 4dpi, could be responsible for producing all of the BrdU$^+$ cells at 180dpi.

We expected to see a change in the distribution of BrdU labeled cells over time based on the 2dpi pulse-chase experiments (Fig. 2.7). As expected, there is an increase in the number of BrdU$^+$ cells in the GCL at 7dpi, and a concomitant decrease in the number of BrdU$^+$ cells in the INL (Fig 2.12), suggesting cells had migrated. We observed a gradual decrease in the number of BrdU$^+$ cells over time, suggesting that some of these cells did not persist within the retina. This decrease is most striking in the GCL, where there are an estimated 167 BrdU$^+$ cells/lesion at 7dpi, which declines to 57 BrdU$^+$ cells/lesion at 180dpi. This decrease in cells in the GCL suggests that retinal cell death that occurs during development (Cole and Ross, 2001) may also occur as the regenerating retina matures.

Discussion

Müller glia generate new neurons and glia after retinal injury

Identification of the cells responsible for regenerating the injured fish retina has eluded investigators for decades. Although some studies implicate putative INL resident stem cells (Braisted, Essman and Raymond, 1994, Otteson, D'Costa and Hitchcock, 2001, Wu et al., 2001), Müller glia remained as potential injury-induced progenitors. Further support of this idea comes from recent studies demonstrating Müller glia are able to generate neurons in the injured bird and mammalian retina (Fischer and Reh, 2001, Ooto
et al., 2004). Studies designed to directly investigate whether Müller glia give rise to new neurons following retinal injury in zebrafish were not possible until now.

We provide several lines of evidence that are consistent with the hypothesis that Müller glia are a source of retina regeneration in the zebrafish. First, nearly all cells that divide in response to injury are Müller glia, which is consistent with other studies indicating Müller glia are the major population of dividing cells during the first week post-injury (Wu et al., 2001, Yurco and Cameron, 2005). We arrived at this conclusion based on evidence that most BrdU labeled cells (94% at 4dpi) express the -1016\(\alpha\)1T:GFP transgene, and that nearly all GFP\(^{+}\) cells (98%) express Müller glial markers. Second, \(\alpha\)1T-expressing Müller glia give rise to neurogenic clusters (Figs 2.6 and 2.8) that are known to be responsible for generating new neurons in response to injury (Raymond, Reifler and Rivlin, 1988). Third, \(\alpha\)1T-expressing Müller glia induce genes considered to be markers of retinal stem cells such as Pax6 and \(\alpha\)1T (Fig.2.6). Fourth, recently dividing GFP\(^{+}\) cells derived from Müller glia migrate to other nuclear layers (Figs 2.7 and 2.12), demonstrating the capacity to replenish neurons in all layers of the retina. Fifth, GFP\(^{+}\) cells derived from Müller glia begin expressing differentiated cell markers such as zn5, a marker for newborn retinal ganglion cells, and HuC/D (Fig 2.10). In addition, cells that are labeled with BrdU at 4dpi ultimately generate photoreceptors, bipolar, amacrine and Müller cells (Fig 2.11), demonstrating the multipotency of the BrdU\(^{+}\) cells. Lastly, 76% of the cells labeled with BrdU at 2dpi are located within the INL, suggesting the BrdU\(^{+}\) cells found at 180dpi were derived from cells in the INL, where Müller cell bodies are located. When taken together, these data strongly suggest that Müller glia are a major source of retina regeneration in the zebrafish.
Although we did not observe a significant population of BrdU\(^+\) cells other than Müller glia, we do not exclude the possibility that, in addition to Müller glia, putative stem cells in the INL and rod progenitors in the ONL also contribute to regeneration. However, by performing a few simple calculations based on the average number of BrdU\(^+\) cells/lesion present at 4dpi, we conclude that it is unlikely that all of the BrdU\(^+\) cells observed at 180dpi were derived from the putative BrdU\(^+\)/GFP\(^-\) stem cells in the INL. We estimate that there are 14 BrdU\(^+\)/GFP\(^-\) cells/lesion in the INL at 4dpi by multiplying the average number of BrdU\(^+\)/GFP\(^-\) cells observed in the INL at 4dpi by the percentage of GFP\(^-\) cells at the same time point (841 BrdU\(^+\) cells/lesion x 1.6% of cells that are GFP\(^-\) = 14). If these 14 cells were responsible for producing the estimated 550 BrdU\(^+\) cells/lesion we observe at 180dpi, they must undergo at least 5 rounds of cell division between 4 and 180dpi. While there is more than ample time for 5 cell divisions between 4 and 180dpi, we conclude it is unlikely for two reasons. First, we do not detect an increase in the number of BrdU labeled cells over time (Fig 2.12), and second, 5 rounds of cell division would likely dilute the BrdU to undetectable levels. These data suggest that cells labeled with BrdU at 4dpi do not undergo any significant cell division at later time points. An alternative explanation is that the generation of BrdU\(^+\) cells is countered by cell death. However, since apoptosis is not observed to occur in Müller glia following injury (Yurco and Cameron, 2005), and the number of proliferating cells is declining rapidly between 4-7dpi, it appears that BrdU-labeled cells exhibit very little cell division after 4dpi.
**Interkinetic nuclear migration as a mechanism to distribute newly born cells**

In the developing mammalian cortex, radial glia produce new neurons that inherit a cellular process that connects the newly born cell to the pial surface of the brain (Noctor et al., 2001). The newly born cell nucleus translocates from its origin through the cell process to its destination. This pattern of neurogenesis produces radial columns of clonal cells that form a functional unit in the adult cortex. During fish retina embryogenesis, a similar process occurs in which each daughter cell of a division maintains a portion of a basal process that spans the developing retina (Das et al., 2003). The basal process provides a conduit for the newly born nucleus to move through, allowing the cell body to travel without extending new connections to the basal retina. Three observations suggest a similar mechanism may be operating in the regenerating zebrafish retina. First, nuclei from proliferating α1T-expressing Müller glia are observed to migrate from the INL to other nuclear layers in response to injury (Figs 2.7 and Fig 2.9) (Braisted, Essman and Raymond, 1994, Wu et al., 2001, Yurco and Cameron, 2005). Second, GFP+/zn5+ cell bodies derived from Müller glia appear to be migrating to the GCL to become new retinal ganglion cells (Fig 2.10). Third, newly born cells appear to migrate along Müller processes to reach their destination (Vihtelic and Hyde, 2000). In light of our observation that newly born cells are derived from α1T-expressing Müller glia, we can propose a model wherein during cell division, newly born cells retain a portion of the parental Müller glial process that spans the retina. The movement of these newly born nuclei would therefore be similar to the interkinetic nuclear movement observed during mammalian cortex and zebrafish retina development, namely that the new nucleus simply
migrates through its cytoplasmic connection to the appropriate nuclear layer, and the cellular process is retracted or used in the formation of an axon. Migrating through the cytoplasm of a cell derived from a dividing Müller glia is likely a relatively easy path to navigate when compared to the numerous synapses a cell would encounter migrating through the plexiform layers of a mature retina. It would be interesting to investigate whether newborn cells derived from a single Müller glia form a new functional retinal unit, or whether the newborn cells integrate into existing retinal units to replace lost cells.

-1016α1TGFP expression as a reporter for an injury-induced signal

We noticed that there appeared to be a higher percentage of Müller glia that expressed GFP near the injury site when compared to GFP-expressing Müller glia distal to the lesion. The percentage of GFP-expressing Müller glia goes from upwards of 90% near the lesion to 0% at sites distal to the lesion (data not shown). This observation is consistent with previous work demonstrating a direct correlation between distance from the injury site and the number of proliferating cells (Yurco and Cameron, 2005), and that there is more α1T transgene expression in the portion of the retina that receives the most damage (Vihtelic et al., 2006). These data suggest that Müller glia may respond to an injury-induced signal emanating from the injury site, and that this signal may be involved in the activation of α1T gene expression. Although the signals initiating retinal regeneration are not known, it they may emanate from photoreceptors since they must be damaged to initiate a regenerative response (Braisted and Raymond, 1992, Braisted, Essman and Raymond, 1994). In the mouse, there is evidence that photoreceptors signal damage to Müller glia through endothelin receptors (Rattner and Nathans, 2005). It will
be interesting to investigate whether endothelin signaling is involved in regulating the response of Müller glia to retinal injury. In addition, transgenic fish harboring wild-type and mutant versions of the α1 tubulin promoter driving GFP expression provide a convenient system for investigating promoter regulatory elements that mediate gene regulation in response to retinal injury. Characterization of these elements will likely lead to the identification of transcription factors required for successful retina regeneration.

**Implications for regeneration in mammals**

It is interesting to note that Müller glia from a variety of organisms begin to proliferate in response to injury and induce expression of filamentous proteins such as GFAP (Grosche, Hartig and Reichenbach, 1995, McGillem and Dacheux, 1999, Sarthy and Egal, 1995), α1T (Senut, Gulati-Leekha and Goldman, 2004, Vihtelic et al., 2006), Neurofilament (Fischer and Reh, 2001), and Vimentin (Lewis and Fisher, 2003). The induction of a group of architectural proteins across species suggests that an evolutionarily conserved mechanism may be acting to induce Müller glia to proliferate in all these species. In humans, Müller glial proliferation is known as reactive gliosis, which could be an attempt to limit retinal damage and is often associated with pathology (Bringmann and Reichenbach, 2001). Gliosis may occur in an attempt to clear neuronal debris, (Pearson, Payne and Cunningham, 1993), but in fish, Müller glia do not phagocytose in vivo (Wagner and Raymond, 1991), suggesting these cells may play a different role in teleosts. Our data suggest that Müller glia in zebrafish play a role in regeneration of the damaged retina, and although retina regeneration in birds and
mammals is limited compared to fish, the common use of Müller glia as a retinal progenitor suggests that a similar mechanism may be acting to produce new neurons in all these species. Zebrafish represent an ideal system to unravel the mechanisms of retina regeneration because of their robust regenerative response and amenability to molecular studies. In contrast, regeneration in the chick retina is meager and restricted to the postnatal period. Regeneration in the mammalian retina is even poorer. Nonetheless, it is interesting that the limited amount of regeneration observed in the mammalian retina can be attributed to Müller glia (Ooto et al., 2004). Thus it is likely that studies of fish retina regeneration will reveal mechanisms that are also relevant to repair the damaged mammalian retina.

Experimental Procedures

Animals

The animals used in this study were treated in accordance with the guidelines of the University Committee on Use and Care of Animals at the University of Michigan. 31 Adult zebrafish (Danio rerio) were used in this study. They were obtained from our breeding colony and raised with a 14:10 light/dark cycle at a temperature of 28°C.

Generation of transgenic zebrafish

-1016α1TIpEGFP expression vector contains -1016 bp of 5’ flanking α1-tubulin DNA, exon 1, and the first intron fused in frame to the GFP sequence. This promoter fragment is similar to the full-length 1696α1TIpEGFP expression vector from previous
work (Goldman and Ding, 2000, Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004) except that it is lacking 680 bp from the 5’ end. -1016α1TIpEGFP DNA was resuspended in injection buffer, and single-cell zebrafish embryos were injected as previously described (Goldman and Ding, 2000). Injected fish were bred and screened for reporter gene expression (Goldman and Ding, 2000, Goldman et al., 2001).

**Optic nerve lesions**

Fish were anesthetized in 0.02% tricaine methane sulfonate (Sigma, St. Louis, MO) before surgery. Optic nerve crushes were performed as described previously (Hieber et al., 1998). Under microscopic visualization, the right eye was gently pulled from its socket, and the exposed optic nerve was crushed behind the eyeball using watchmaker’s forceps. Care was taken not to lesion the ophthalmic artery running along the optic nerve. The left optic nerve was kept intact, its retina serving as an unoperated control. After surgery, fish were returned to their tanks for 4 days.

**Eye lesions**

Eye lesions were performed as described previously (Senut, Gulati-Leekha and Goldman, 2004). Briefly, fish were anesthetized and under microscopic visualization, the right eye was gently pulled from its socket and stabbed four times (once in each quadrant) through the sclera with a 30 gauge needle. The needle was inserted to the length of the bevel (~5 mm) to achieve similar lesions from case to case. The left eye served as an unoperated control. After surgery, fish were returned to their tanks to recover.
**Bromodeoxyuridine injections**

To identify dividing cells, bromodeoxyuridine (BrdU) injections were performed as described (Byrd and Brunjes, 2001) with minor modifications. Briefly, after anesthesia in 0.02% tricaine methane sulfonate, fish in which the retina had been punctured earlier received a single intraperitoneal injection of 20µl of a 25 mg/ml BrdU (Sigma, St. Louis, MO) stock solution. Fish were sacrificed at various times after BrdU injection.

**Tissue preparation**

Fish were given an overdose of tricaine methane sulfonate and eyes from adult fish were dissected, enucleated, and fixed by immersion in fresh 1% (to preserve GFP fluorescence) or 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 16 hr. After fixation, samples were cryoprotected in phosphate-buffered 20% sucrose before embedding with O.C.T. mounting medium (Sakura Finetek USA, Inc., Torrance, CA). Embedded samples were kept at -70°C until sectioning. Six to eight micrometer serial sections were obtained on a cryostat (CM3050S; Leica, Nussloch, Germany), collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), dried overnight at room temperature, and stored at -70°C.

**Immunohistochemistry**

Immunohistochemistry was performed as described (Senut, Gulati-Leekha and Goldman, 2004) using the following primary antibodies: rat anti-BrdU (dividing cell
marker; 1:250; Harlan; Sera-Lab); mouse zpr1 (double-cone photoreceptor marker; (Larison and Bremiller, 1990)) (1:250; Zebrafish International Resource Center (ZIRC), Eugene, OR); rabbit anti-GFP (1:1000; Molecular Probes, Eugene, OR); mouse anti-HuC/D (1/500; Molecular Probes); rabbit anti-protein kinase C (PKC; ON bipolar cell marker; (Yazulla and Studholme, 2001)) (1:250; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-tyrosine hydroxylase (TH; interplexiform cell marker;) (1:500; Chemicon,); mouse anti-zrf1 (glial marker) (1:250; ZIRC); mouse anti-zn5 (retinal ganglion cell marker) (1:500; ZIRC); and mouse anti-glutamine synthetase (GS; glial marker) (1:500; Chemicon). For BrdU immunostaining, sections were pretreated with 2 N HCl for 30 min at 37°C, two 5 min rinses in 0.1 M Sodium Borate buffer, and three 5 min rinses in PBS, pH 7.4. Primary antibodies were diluted in PBS containing 1% donkey serum and 0.3% Triton X-100 [normal donkey serum Triton (NDST) 1%]. Cryostat sections were first rehydrated 10 min in PBS, preincubated in NDST 3% for 30 min at room temperature, and then incubated in the primary antibodies overnight at 4°C. Sections were rinsed three times in NDST 1% and incubated for 2-3 hr at room temperature with secondary anti-mouse, anti-rabbit, or anti-rat antibodies conjugated to Alexa 488 (1:1000; Molecular Probes,) or cyanin 3 (1:250; The Jackson Laboratory, West Grove, PA). Sections were then washed twice in PBS, once in PB and then with water containing 10 ng/ml 4, 6-diamidino-2-phenylindole (DAPI; Sigma) for nuclear staining. Slides were washed twice with water and allowed to dry in the dark. Slides were then coverslipped using MOWIOL and subsequently stored in the dark at 4°C.
**In situ hybridization**

In situ hybridizations were performed with digoxigenin labeled cRNA probes as described (Barthel and Raymond, 2000). Pax6 cRNA was prepared from a full length Pax6a cDNA clone (Open Biosystems, Huntsville, AL) linearized by EcoRV digestion, followed by transcription with T7 RNA polymerase. A sense control probes was made and produced no signal.

**Imaging**

Slides were examined using a Zeiss (Oberkochen, Germany) Axiophot or LSM510 confocal microscope. Images were captured using a digital camera adapted onto the Axiophot microscope or with a Zeiss LSM510 Confocal microscope. Images were processed and annotated with Adobe Photoshop CS.

**Transmission Electron Microscopy**

Enucleated eyes from 4-5dpi -1016 α1T transgenic fish were fixed in 2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M Sorenson’s buffer, pH 7.4, for four hours. After several buffer rinses, eyes were post-fixed in 1% osmium tetroxide in the same buffer, rinsed in ddH2O to remove phosphate and then en bloc stained with aqueous 3% uranyl acetate for one hour. Eyes were then dehydrated in ascending concentrations of ethanol, treated with propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections were obtained and stained with toluidine blue to identify lesions. 70nm ultra-thin sections were then taken for selected regions of interest and stained with uranyl acetate and lead citrate and examined using a Philips CM100 electron microscope at 60
kV. Digital images were recorded with a Hamamatsu ORCA-HR digital camera system operated with AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).
Figure 2.1  -1016α1T:GFP transgenic fish induce GFP expression in Müller glia following retinal injury. Three independent lines of transgenic fish (-1016 L1, L2, L3) were tested for induction of GFP in the retina 4 days following retinal injury. The panels labeled GFP show transgene GFP expression in Müller-like cells. The middle panels show staining for GFAP, a Müller glial marker. The merged images (right panels) show GFP expressing cells also express GFAP (arrows). Retinal ganglion cells do not express GFP in response to injury (arrowheads). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
Figure 2.2  Retinal ganglion cells from -1016α1T:GFP transgenic fish do not express GFP after optic nerve crush. Three lines of -1016α1T:GFP transgenic zebrafish (-1016 L1, L2, L3) were analyzed for transgene expression in axotomized ganglion cells 4 days post crush. The transgene is not expressed in damaged axons (GFP panels) although GAP43 induction indicates ganglion cells were successfully axotomized (GAP 43 panels). The DAPI panels show merged images from each transgenic line.
Figure 2.3. -1016α1T:GFP transgene expression in the circumferential germinal zone (CGZ). In the uninjured retina, -1016α1T:GFP transgene expression is limited to cells at the CGZ (arrowhead) and newly born cells that remain GFP⁺ near the CGZ (arrow).
Figure 2.4 Injury-induced cell proliferation and GFP expression. (a) Graph showing the average number of BrdU⁺ cells per lesion at 24hr intervals after injury. Four lesions (1-5dpi) or two lesions (6-7dpi) were counted to obtain averages. The increase in proliferation in the INL at 2dpi precedes the increase in proliferation in the ONL at 3dpi. At 4dpi, there are approximately 850 cells/lesion in the INL that are labeled by BrdU uptake. By 7dpi, cell proliferation returns to near baseline levels. (b) Graph showing the percentage of BrdU⁺ cells quantified in (a) that are also GFP⁺ at each day post injury. (c-k) Images of BrdU⁺/GFP⁺ cells from 2-5dpi. (c-e) At 1dpi, few cells were BrdU⁺, although rare cells considered to be rod progenitors (c) and microglia (d) based on their position within the retina could be identified. Rarely, a GFP⁺ proliferating cell in the INL could be found (e-g). (h) At 2dpi, a robust induction of GFP in Müller-like cells was observed, which correlates with the rise in the number of BrdU⁺ cells in the INL at 2dpi. BrdU⁺ putative microglia were also present (grey arrow). (i) At 3dpi, more BrdU⁺/GFP⁺ cells were observed in the INL. (j) At 4dpi, putative rod progenitors in the ONL can be identified as BrdU⁺/GFP⁺ cells (grey arrow) and BrdU⁺/GFP⁺ cells are abundant (white arrow). In addition, we identified some nuclei that were GFP⁺/BrdU⁻ (arrowhead). (k) At 5dpi, the number of BrdU⁺ cells in the INL is decreasing, but GFP⁺ proliferative cells can be identified in all three nuclear layers (arrows). ◇ ONL, outer nuclear layer; ■ INL inner nuclear layer; ▲ GCL, ganglion cell layer.

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Figure 2.5 -1016α1T:GFP expression is specific to Müller glia. Sections from -1016 transgenic 4 day post injured retinas were examined by confocal microscopy to determine whether GFP expression was induced specifically in Müller glia. Cells were assayed for colocalization of GFP (green panels) with the Müller glial markers glutamine synthetase (GS) (red in a) and GFAP (red in b), and a neuronal marker, HuC/D (red in c). (a) GFP is expressed in Müller cells which are labeled by GS immunostaining. There are three GFP+ nuclei that appear as GS- holes in this micrograph (arrows). Note the coexpression of GFP and GS in the cytoplasm (arrowheads). Of 205 GFP+ cells assayed for GS expression, 200 (98%) were double-labeled. (b) GFP positive cells also express GFAP (arrows). Of 107 GFP+ cells assayed for GFAP expression, 105 (98%) were double-labeled. (c) Amacrine cells labeled with the neuronal marker HuC/D do not express GFP after injury (wide arrows). Cells double labeled with HuC/D and GFP were never observed at 4dpi.
Figure 2.6 Neurogenic clusters are derived from Müller glia. Sections from 4dpi retinas were processed for GFP expression (green) and then *in situ* hybridization for Pax6 mRNA (blue) was performed. (a) Pax6 is induced in columns of elongated cells known as ‘neurogenic clusters’ in response to injury (arrowheads). Amacrine cells normally express Pax6 (arrow, see also d). (b) GFP is expressed by Müller glia in response to injury (arrowheads). (c) GFP+ cells derived from Müller glia correspond to Pax6 expressing neurogenic clusters (arrowheads). (d) Pax6 is expressed in the uninjured retina in amacrine cells (arrow). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
Figure 2.7. Proliferating cells migrate to other nuclear layers. Fish injured on day 0 were given a single dose of BrdU at 2dpi and sacrificed at 3, 4, 5, or 7dpi to follow the progress of the BrdU\(^+\) cell population. Labeled cells can be found in all nuclear layers at all time points, suggesting the cells had migrated soon after BrdU labeling had occurred. Some nuclei have elongated or stretched morphology, also suggesting migration (arrows). At 7dpi, numerous BrdU\(^+\) cells can be seen forming what appears to be a neurogenic cluster of proliferating cells (arrowheads), suggesting these neurogenic clusters are derived from cells in the INL that were labeled with BrdU at 2dpi. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
Figure 2.8  Neurogenic clusters are tightly apposed cells with Müller glial characteristics. (a) Sections from 4dpi retina were processed for BrdU (red) and GFP (green). A stack of confocal images of were obtained and used to create images in the z-plane (the top panel and right panel represent a slice taken at the green and red lines, respectively). Note the multiple BrdU\(^+\) nuclei within the area that corresponds to GFP\(^+\) Müller glia (arrows). The inset shows a higher magnification of the BrdU\(^+\) (red) nucleus marked with an asterisk in grayscale for greater clarity. Individual nuclei are outlined. (b) A representative image from a stack of confocal images taken from 4dpi retina sections processed for GFP (green) and GS (glutamine synthetase) (red) demonstrating multiple nuclei within the area of a single Müller glia (the top panel and right panel represent a slice taken at the red and green lines, respectively). Nuclei that are either surrounded by GFP\(^+\) Müller cell processes, or are within Müller glia, express GFP (white arrows) while a nucleus that is outside the GFP\(^+\) Müller glia does not (the arrowhead in each panel identifies the same nucleus). The typical glial morphology can be clearly seen in the z-plane (right panel, slice taken from the green line) as the cell extends from the ONL to the GCL and contacts the vitreous. A nucleus that appears to be within the Müller cell when viewed in the xy-plane (grey arrow – main panel) is clearly not within the Müller cell when viewed in the z-plane (grey arrow – right panel) and does not express GFP. The inset shows a higher magnification of the nucleus marked with an asterisk. (c-f) Transmission electron micrographs of a neurogenic cluster at 4dpi. (c) Two nuclei with elongated morphology typical of proliferating neurogenic clusters (asterisks) near the inner plexiform layer. The boxes represent the areas shown at higher magnification in (d) and (e). The scale bar represents 3\(\mu\)m. (d) Composite image from two high magnification micrographs of the cells shown in (c) demonstrating these cells are separated by plasma membrane (arrowheads). The scale bar is equivalent to 500nm. (e) Higher magnification of the area outlined in (c). The cytoplasm of the Müller glia (black arrows, see (f)) is very similar to the cytoplasm of the cells with elongated nuclei (arrowhead), while the cytoplasm of other cells is very different (red arrows). The box represents the area shown in (f). The scale bar represents 500nm. (f) High magnification of the area shown in (e) demonstrating a junction between Müller glia (arrowheads), indicating the elongated cells shown in (c) are surrounded by Müller processes. The scale bar represents 500nm. IPL, inner plexiform layer.
Figure 2.9  Electron micrograph of a migrating cell. Cells in the inner plexiform layer (IPL) at 5dpi are presumed to be migrating to the ganglion cell layer (GCL). (a) An example of a putative migrating cell. The inner nuclear layer (INL) is just outside the field of view to the top left, and the GCL is further to the bottom right. The identified cell appears to be a Müller cell based on the presence of lateral processes (arrows) and cytoplasm density similar to that of Müller glia (see Fig 2.8f). The inset shows a higher magnification of the boxed area. A nuclear pore (white arrowhead) is visible in the nuclear envelope (black arrowheads). Note the lack of plasma membrane separating the two nuclear envelopes. (b) Pseudocolored electron micrograph of (a). The cytoplasm (green) was identified by tracing plasma membrane at high magnification. Nuclear envelopes were identified by the presence of nuclear pores (white arrowhead in (a) and were traced to define nuclei (purple). The presence of two apparent nuclei is most likely due to sectioning through a single spiral-shaped nucleus, although it may also represent two nuclei within the same cell. The scale bar represents 2\( \mu m \).
Figure 2.10  Cells derived from GFP$^+$ Müller glia express markers of differentiating amacrine and retinal ganglion cells. -1016 transgenic fish were injured at day 0 and allowed to recover until 7 or 11dpi (HuC/D shown at 7dpi and zn5 at 11dpi). Sections were stained for HuC/D or zn5 (red), markers for differentiating amacrine and retinal ganglion cells respectively, and GFP (green) to detect whether GFP$^+$ cells derived from Müller glia begin to differentiate. DAPI is shown in purple to indicate the laminar position of the labeled cells. (a-d) Some GFP$^+$ cells begin to express HuC/D at 7dpi (arrows). (e-h) Zn5 labeled cells in the IPL and GCL express GFP (arrows). (i) A GFP$^+$ Müller-like cell with a thin axon-like projection (arrowheads). (j) A zn5 labeled cell extending an axon (arrowheads) into the inner plexiform layer (IPL). (k) The zn5$^+$ cell also expresses GFP (arrow). (l) The newly born ganglion cell sits at the edge of the INL. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 2.11 Cells labeled with BrdU at 4dpi become new neurons and glia. Fish injured at day 0 received a single injection of BrdU at 4dpi and were allowed to recover for 180 days. Sections from these retinas were processed for cell specific markers to identify retinal cell types (red) that were derived from cells labeled with BrdU (green) at 4dpi. (a) BrdU+ cells become cone photoreceptors following injury as indicated by a BrdU labeled nucleus within a zpr1+ cell (arrow). (b) An example of a BrdU+/GS+ Müller glia at 180dpi (arrow). (c) A HuC/D+ amacrine cell derived from a cell labeled with BrdU at 4dpi (arrow). (d) A PKC+ bipolar cell derived from a cell labeled at 4dpi (arrow). Marker, cell specific marker; GS, glutamine synthetase; PKC, protein kinase C.
Figure 2.12  Cells labeled with BrdU at 4dpi exhibit little proliferation at later times. Fish injured at day 0 were given a single dose of BrdU at 4dpi and sacrificed at 4, 7, 11, or 180dpi. Serial sections were collected and processed for BrdU labeling to follow the cells labeled at 4dpi. The average number of BrdU labeled cells per lesion is shown for each time point examined. The number of BrdU\(^+\) cells in the ONL and INL declines over time, while the number of labeled cells in the GCL increases at 7dpi, suggesting cells had migrated there. The lack of an increase in the overall number of BrdU labeled cells over time suggests that cells labeled with BrdU at 4dpi generally do not continue to progress through the cell cycle. In fact, there is a decline in the total number of cells, suggesting some of the labeled cells had undergone apoptosis.
References


Chapter III

The proneural bHLH gene ascl1a is required for retina regeneration

Summary

Unlike mammals, fish can regenerate their central nervous system following injury. Although successful regeneration has been observed for decades, little is known of the molecular events that govern it. We previously identified Müller glia as a source of injury-induced retinal progenitors in zebrafish. Following injury, Müller glia re-enter the cell cycle, dramatically increase α1tubulin (α1T) promoter activity and generate new neurons and glia to facilitate retinal repair. Here we report the identification of an E-box in the α1T promoter that is necessary for its activity in proliferating Müller glia. In a search for E-box binding proteins that may mediate α1T induction during retina regeneration we found that the basic helix-loop-helix transcription factor ascl1a is induced in Müller glia within four hours following injury and regulates α1T promoter activity via the E-box in vitro. Knockdown of ascl1a expression in the regenerating retina confirmed that ascl1a regulates α1T transgene expression and is necessary for the generation of retinal progenitors and their differentiating progeny in vivo. These data suggest ascl1a is a key regulator of retina regeneration in zebrafish.
Introduction

The fish retina has a remarkable capacity to regenerate following injury (reviewed in Hitchcock et al., 2004). Several injury models have been used to elicit a regenerative response in the zebrafish retina (Cameron, 2000, Vihtelic and Hyde, 2000, Chapter II, Fausett and Goldman, 2006, Raymond et al., 2006, Fimbel et al., 2007). In all these regeneration paradigms, Müller glia respond to injury by proliferating to produce multipotent cells which become neurons and glia (Cameron, 2000, Vihtelic and Hyde, 2000, Chapter II, Fausett and Goldman, 2006, Raymond et al., 2006, Fimbel et al., 2007). Interestingly, Müller glia function as injury-induced progenitors in rats and chicks also, but regeneration is limited and not all cell types are produced (Fischer and Reh, 2001, Ooto et al., 2004). In humans, retinal disease causes Müller glia to proliferate, often with pathologic consequences (Bringmann and Reichenbach, 2001). The fact that Müller glia re-enter the cell cycle in response to injury makes them an attractive candidate for cell replacement therapies.

Numerous genes that are either expressed in retinal progenitors or are thought to be involved in maintenance of stem cells are induced in response to injury including: Pax6 (Chapter II, Fausett and Goldman, 2006, Hitchcock et al., 1996), notch and cadherin (Raymond et al., 2006, Wu et al., 2001), delta, vsx and rx (Raymond et al., 2006), olig2 (Fimbel et al., 2007), and stat3 (Kassen et al., 2007). In addition, microarray analysis has identified genes that are regulated in response to physical or light-induced injury in zebrafish (Cameron et al., 2005, Kassen et al., 2007), and mice (Rattner and Nathans, 2005). Despite recent advances in our understanding of the genes implicated in retina regeneration, surprisingly little is known about what role these genes play during
regeneration and whether they are necessary for successful regeneration. The \textit{wnt}
signaling pathway seems to be important for initiating cell cycle re-entry in rats (Osakada
et al., 2007), and FGF and insulin injection into uninjured chick retinas causes Müller
glia to proliferate (Fischer et al., 2002). Retinal disease prompts photoreceptors to
initiate signals that impinge on Müller glia (Rattner and Nathans, 2005), but it is not
known whether any of these signaling pathways transform Müller glia into retinal
progenitors.

Our lab developed a transgenic zebrafish model to study central nervous system
(CNS) regeneration (Goldman et al., 2001), and used this transgenic model to show
Müller glia are a source of multipotent progenitors which contribute newborn cells
toward regeneration in zebrafish (Chapter II, Fausett and Goldman, 2006). In order to
identify genes that regulate this process, we focused on DNA elements that mediate \textit{\alpha}1T
transgene expression in proliferating Müller glia. Here we report the identification of an
E-box that is required for \textit{\alpha}1T transgene expression \textit{in vivo} and provide evidence that the
basic helix-loop-helix transcription factor, ascl1a, regulates the \textit{\alpha}1T promoter via this E-
box \textit{in vitro}. To test whether ascl1a regulates \textit{\alpha}1T transgene induction \textit{in vivo}, we
developed a technique to inhibit gene expression in the injured retina using antisense
morpholino oligonucleotides. Ascl1a knockdown prevents Müller glia from re-entering
the cell cycle in response to injury, thereby preventing successful retina regeneration. To
our knowledge, this is the first identified gene that is required for successful retina
regeneration.
Results

An E-box is required for α1T promoter activation in proliferating Müller glia following retinal injury

We previously reported that lesion of the zebrafish retina causes Müller glia to proliferate and almost simultaneously activate the α1T promoter (Chapter II, Fausett and Goldman, 2006). In addition, we showed that these α1T expressing Müller glia can generate most major retinal neurons and glia that are responsible for repairing the damaged retina. To begin to identify the mechanism by which these Müller glia respond to injury-induced signals and apparently dedifferentiate into a dividing population of retinal stem cells, we wanted to identify α1T promoter elements mediating its induction following retinal injury. We hypothesized that identifying these putative elements would lead us to transcription factors which regulate retina regeneration.

We first tested whether a 200 base pair (bp) DNA fragment that is necessary for α1T transgene expression in the developing CNS (Goldman and Ding, 2000) is also required for transgene expression in proliferating Müller glia after injury (Fig 3.1a, Δ-1046-846). Administration of BrdU to transgenic fish harboring the -1696 (wild-type) or -1016 α1T:GFP transgene labels dividing cells, the vast majority of which are α1T transgene expressing Müller glia (Fig 3.1b -1016 panel, Chapter II, Fausett and Goldman, 2006). BrdU labeled cells in transgenic fish harboring a 200bp internal α1T promoter deletion do not express GFP (Fig 3.1b, arrowheads in Δ-1046-846 panels), suggesting there is an element within the deleted 200bp region that is required for transgene expression in proliferating Müller glia. Fish harboring the -907α1T:GFP transgene (Fig 3.1a, -907) also do not express GFP in proliferating Müller glia (Fig 3.1b, panels -907L1,
L2 and L3). These results suggest that a DNA element located between nucleotides -1016 and -907 of the α1T promoter is required for transgene expression in proliferating Müller glia.

We next searched for potential transcription factor binding sites within this 109bp region by performing Electrophoretic Mobility Shift Assays (EMSA). Radiolabeled oligonucleotide probes spanning this region and nuclear extracts prepared from zebrafish or rat brain revealed specific binding to one probe (Fig 3.2a, arrow) which contains a single E-box sequence CATGTG (Fig 3.2b probe 4). To test whether the E-box was required for protein binding, we used probes with 2 bp mutations (Fig 3.2b) to either the E-box (probes 4-3 and 4-4) or surrounding nucleotides (probes 4-1, 4-2, and 4-5). E-box mutations disrupted protein binding (Fig 3.2c, lanes 3 and 4), but mutations to other nucleotides did not (Fig. 3.2c, lanes 1, 2 and 5). In addition E-box mutations prevented unlabeled probes from competing for binding with wild-type radiolabelled probes even at 50-fold molar excess (Fig 3.2c, lanes 6-10). These experiments indicate the E-box is required for binding nuclear proteins prepared from brain extracts. Nuclear extracts isolated from zebrafish retina also bound specifically to the E-box (Fig 3.2d), although nuclear extracts isolated from rat liver, fish gill, fish muscle, and fish kidney do not (data not shown). A probe containing an E-box that is not required for transgene expression following retinal injury (Senut, Gulati-Leekha and Goldman, 2004) does not bind nuclear extracts from zebrafish brain (Fig. 3.2e, lanes 4-6). These results suggest that the binding we observe is specific to the E-box at position -954, and is due to putative transcription factors which are present in the zebrafish brain and retina.
Because the E-box resides within the 109bp region required for transgene expression in proliferating Müller glia following retinal injury, we hypothesized that this E-box directs transgene expression in these cells. To test this hypothesis, we placed the 2bp mutation from probe 4-4 in the full length α1T promoter to create TG-954CAα1TpEGFP transgenic zebrafish (Fig 3.3a). Three independent lines of these transgenic fish were identified and characterized for transgene expression during development. The TG-954CA promoter directs transgene expression to the brain, spinal cord and retina during development, which is similar to the wild-type promoter (Fig 3.4). Mature transgenic fish harboring the wild-type or -1016 α1T promoters express GFP in stem cells located at the circumferential germinal zone (CGZ) (Chapter II, Fausett and Goldman, 2006, Goldman et al., 2001). However, TG-954CA transgenic fish do not express the transgene in stem cells at the CGZ, suggesting the E-box is required for transgene expression in retinal stem cells (data not shown). We next tested whether the E-box was required for transgene expression in injury-induced retinal stem cells by lesioning retinas from TG-954CA transgenic fish. Like the Δ-1046-846 and -907 transgenic fish, BrdU\(^+\) cells in TG-954CA transgenic fish do not express GFP (Fig 3.3b), suggesting the E-box is required for transgene expression in proliferating Müller glia. Occasionally we noticed GFP\(^+\) cells in the ganglion cell layer (Fig 3.3, arrows). These appeared to be axotomized retinal ganglion cells (RGCs) because in wild-type transgenic fish, axotomized RGCs robustly induce GFP expression (Goldman and Ding, 2000, Senut, Gulati-Leekha and Goldman, 2004). We confirmed that axotomized RGCs in TG-954CA transgenic fish express GFP by performing optic nerve crush injuries on these fish (Fig 3.5). These results demonstrate the E-box is required specifically for transgene
expression in stem cells of the adult retina residing either in the retinal periphery (CGZ), or in the central retina following injury (proliferating Müller glia).

**Ascl1a is induced in proliferating Müller glia**

Identifying the E-box as a regulator of α1T transgene expression in proliferating Müller glia focused our attention on basic helix-loop-helix (bHLH) transcription factors which are expressed in retinal progenitors (Brown et al., 2001, Marquardt et al., 2001, Perron et al., 1999, Tomita et al., 1996, Tomita et al., 2000, Wang et al., 2001) and are known to bind E-boxes (Massari and Murre, 2000). These transcription factors play a role in retinal cell fate specification (Brown et al., 1998, Inoue et al., 2002, Kay et al., 2001, Marquardt et al., 2001, Morrow et al., 1999) and may also play a critical role during retina regeneration.

One particular bHLH protein, *ascl1a*, attracted our attention because: 1) the chick homolog of *ascl1a* is expressed in the injured chick retina (Fischer and Reh, 2001), and other genes that are induced in the injured chick retina are also expressed in the injured fish retina (Chapter II, Fausett and Goldman, 2006, Hitchcock et al., 1996, Raymond et al., 2006); 2) *ascl1a* is reported to be induced during regeneration of the zebrafish retina, although its expression was not characterized (Cameron et al., 2005, Kassen et al., 2007); 3) the mouse homolog of *ascl1a* is expressed in retinal (Jasoni and Reh, 1996) and neural progenitors (Torii et al., 1999, Yun et al., 2002), which is what Müller glia appear to become as they dedifferentiate in response to retinal injury. To determine whether *ascl1a* was induced in proliferating Müller glia, we examined injured retinas from -1016 transgenic fish for *ascl1a* expression. We observed that *ascl1a* expressing cells
correspond precisely to GFP+ Müller glia at 4dpi (Fig. 3.6c), indicating \textit{ascl1a} is expressed in proliferating Müller glia.

If \textit{ascl1a} regulates \(\alpha 1T\) transgene expression, it should be induced prior to transgene expression in Müller glia. The earliest \(\alpha 1T\) transgene expression is detectable at 24 hours post injury (hpi) (Chapter II, Fausett and Goldman, 2006). We therefore examined retinas at 6, 12, 18 and 24hpi for \textit{ascl1a} expression by in situ hybridization (ISH). We detected a low level of expression at 6, 12 and 18hpi, and stronger expression at 24hpi (Fig 3.6d-g), indicating \textit{ascl1a} is induced prior to transgene induction. To confirm that \textit{ascl1a} induction was occurring in Müller glia, we performed ISH for \textit{ascl1a}, followed by \textit{glutamine synthetase} antibody immunostaining at 6 and 24hpi. Indeed, \textit{ascl1a}/\textit{GS} cells were found at both time points (Fig 3.6h-m). These results indicate that \textit{ascl1a} is induced in Müller glia at least 18 hours before \(\alpha 1T\) transgene expression is detectable.

Other genes are upregulated in response to injury, but exactly how early these genes are expressed has not been described. We assayed for expression of endogenous \(\alpha 1T\), \textit{pax6}, \textit{delta}, \textit{notch}, and \textit{olig2} expression at various times after injury to find out whether \textit{ascl1a} induction occurred before, concomitantly, or after induction of these other genes. The results of these ISHs are summarized in Fig 3.7. \textit{Ascl1a} was induced as early as 4hpi, although the level of expression was very low. \textit{Ascl1a} expression was higher at 24hpi and was very strong by 48hpi. The increased \textit{ascl1a} expression precedes endogenous \(\alpha 1T\) induction, consistent with the idea that \textit{ascl1a} regulates \(\alpha 1T\). Because \textit{pax6} is expressed by amacrine cells it was difficult to detect induction until ectopic expression appeared in the inner and outer nuclear layers at 4dpi. This result is consistent
with the observation that not all GFP⁺ Müller glia express pax6, (Chapter II, Fausett and Goldman, 2006), suggesting pax6 induction in Müller glia occurs after GFP expression. However, to further test whether pax6 is induced earlier, we examined glutamine synthetase⁺ Müller glia for pax6 expression at 6 and 24hpi. We did not detect any pax6⁺/GS⁺ cells (data not shown), suggesting pax6 is not induced at these early time points. We observed delta, notch and olig2 induction at 96hpi, but not at earlier times, suggesting these genes are not induced until Müller glia have already adopted a stem cell fate and begun proliferating.

*Ascl1a regulates α1T expression in vitro and in vivo*

If ascl1a regulates α1T expression in proliferating Müller glia, it should be able to transactivate the α1T promoter via the E-box. To test this hypothesis, we created luciferase reporter vectors carrying three copies of the wild-type (CATGTG) or mutant (CATGCA) E-box upstream of a minimal B-globin promoter driving luciferase expression. In addition, we created luciferase vectors harboring full-length wild-type or TG-954CA α1T promoters. *Ascl1a* overexpression in HEK293T cells transactivated the E-box promoter, indicating *ascl1a* acts as a transcriptional activator (Fig. 3.8a). This *ascl1a* mediated transactivation requires an intact E-box, indicating *ascl1a* acts through the E-box. Similar results were obtained with the full-length promoter vectors, although the transactivation was less dramatic (Fig. 3.8b), possibly due to high basal expression because of endogenous transcriptional activators which act at sites other than the E-box.

We performed EMSA with *in vitro* synthesized ascl1a in an attempt to determine whether *ascl1a* could bind the E-box *in vitro*, but were unable to detect specific binding
Despite the lack of evidence that \( asc11a \) can bind the E-box \textit{in vitro}, the expression profile of \( asc11a \) and its ability to regulate \( \alpha 1T \) through the E-box \textit{in vitro} suggest it may regulate \( \alpha 1T \) transcription through the E-box \textit{in vivo}. In order to test this hypothesis, we needed to block \( asc11a \) expression in Müller glia following retinal injury.

Morpholino oligonucleotides (MOs) have been widely used to block gene expression during development (Nasevicius and Ekker, 2000) and recently have been used to block gene expression in regenerating tail fins (Thummel et al., 2006). Electroporation was required to introduce MOs into tail fins and we adapted this protocol to introduce \( asc11a \) targeted antisense MOs into the injured retina. We used lissamine labeled MOs in order to visualize MO treated cells. We compared the effects of control or \( asc11a \) MOs (Cau and Wilson, 2003) on transgene expression in our -1016\( \alpha 1T:GFP \) transgenic fish. We designed these experiments to be blind in order to eliminate potential bias (see methods). Two independent observers scored the number of lissamine-labeled MO treated Müller glia for GFP expression. Normally, GFP\(^+\) Müller glia are first visible at 2dpi, and by 4dpi, there are numerous GFP\(^+\) Müller glia (Fig 3.1). It was readily apparent that \( asc11a \) knockdown suppressed transgene expression in Müller glia following retinal injury (Fig 3.9). Specificity was confirmed using a second MO (Cau and Wilson, 2003) which gave similar results. We counted the number of lissamine\(^+\)/GFP\(^+\) and lissamine\(^+\)/GFP\(^-\) cells in the INL by analyzing confocal images. 11.8\% of the control morpholino treated cells expressed GFP at 2dpi while only 3.9\% the ascl1a MO treated cells expressed GFP, (three fish were counted for each group). At 4dpi, 10.2\% of the control MO treated cells expressed GFP while only 1.3\% and 2.4\% of the ascl1a and ascl1a\(^{5'UTR}\) MO expressed GFP respectively (two fish were counted for control and ascl1a MOs, and three fish for
ascl1a^{5'UTR}). Although electroporation does not deliver MOs specifically to Müller glia, we observe a significant decrease in the percentage of MO^{+} cells that express GFP, suggesting ascl1a is required for GFP transgene expression in vivo.

Another interpretation of these results is that the ascl1a MOs directly block GFP expression. Although it is unlikely that two different MOs both designed to target ascl1a would non-specifically inhibit GFP expression, we wanted to formally exclude this possibility. Our lab developed a strategy to deliver MOs to axotomized retinal ganglion cells via the optic nerve (Veldman and Goldman, in prep). As mentioned above, axotomy causes robust GFP expression in wild-type transgenics (Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004). We treated axotomized RGCs with the control and ascl1a MOs to test whether they would inhibit GFP expression. We predicted that since the E-box is not required for transgene expression in axotomized retinal ganglion cells (Fig 3.5), and ascl1a is not induced in retinal ganglion cells following optic nerve crush (Veldman and Goldman, in prep), that the MOs would not have an impact on transgene expression. Indeed, ascl1a MO treatment did not block transgene expression (Fig 3.10). We also injected ascl1a MOs into wild-type transgenic fish and did not detect a decrease in GFP expression (data not shown). These results indicate the ascl1a MOs do not inhibit GFP expression.

To determine whether ascl1a regulates endogenous α1T expression during retina regeneration, we analyzed α1T expression in injured retinas treated with control or ascl1a MOs by in situ hybridization (Fig 3.11). While the control MO had no effect on endogenous α1T expression, ascl1a knockdown prevented α1T induction, suggesting ascl1a regulates α1T expression in vivo. Because pax6 induction is seen as an indication
that multipotent progenitors have formed in response to injury, and α1T expression precedes *pax6* induction we examined whether *ascl1a* knockdown also prevented *pax6* induction. Indeed, ascl1a knockdown prevents *pax6* induction in response to injury (Fig 3.11).

**Ascl1a is required for regeneration**

The above results establish that *ascl1a* is required for transgene induction in proliferating Müller glia and suggest that *ascl1a* directly regulates α1T in these cells. The mammalian homolog of *ascl1a* is expressed in a population of retinal progenitors during development (Jasoni and Reh, 1996), in neural progenitors prior to terminal differentiation (Torii et al., 1999, Yun et al., 2002), and is required for formation of neural progenitors in the brain (Casarosa, Fode and Guillemot, 1999). In *Drosophila*, *acheate-scute* genes regulate neurogenesis (Campos-Ortega and Jan, 1991). Since *ascl1a* is expressed as early as 4hpi, and ascl1a knockdown inhibits α1T and *pax6* expression, we wondered whether *ascl1a* induction in Müller glia directed these cells to become injury induced retinal progenitors. We hypothesized that if *ascl1a* is involved in activating Müller glia, that *ascl1a* knockdown would prevent Müller glia from re-entering the cell cycle. Since most BrdU-labeled cells in the injured retina are Müller glia (Chapter II, Fausett and Goldman, 2006), MO+/BrdU+ cells represent MO+ Müller glia that proliferated. In control MO treated retinas, many MO+/BrdU+ cells could be identified (Fig 3.12). However, we did not detect MO+/BrdU+ cells in *ascl1a* MO treated retinas, suggesting *ascl1a* knockdown prevents Müller glia from proliferating.
Discussion

Ascl1a regulates α1T expression in proliferating Müller glia

We are interested in identifying transcription factors which regulate α1T expression in proliferating Müller glia following retinal injury because we suspect these transcription factors may direct retina regeneration. Müller glia appear to dedifferentiate in response to retinal injury and produce new neurons for retinal repair (Fischer and Reh, 2001, Ooto et al., 2004, Chapter II, Fausett and Goldman, 2006, Raymond et al., 2006, Fimbel et al., 2007, Kassen et al., 2007). We used promoter analysis in transgenic fish to identify an E-box that is required for α1T transgene expression in proliferating Müller glia (Fig 3.3). We then searched for transcription factors which could regulate α1T expression through this E-box. We imagined that the putative transcription factor would be expressed in Müller glia prior to the onset of transgene expression, positively regulate α1T promoter activity through the E-box, and interact with the E-box in vitro. The bHLH transcription factor ascl1a met all but the last criteria (Figs 3.6, 3.7 and 3.8). Although we were unable to demonstrate a direct interaction between ascl1a and the E-box by EMSA, other positive results suggested that ascl1a was a good candidate for regulating α1T expression in vivo. We blocked ascl1a expression in the regenerating retina with antisense MOs and found that while control MO had no effect, two different ascl1a MOs inhibit transgene expression after injury (Fig 3.9). The ascl1a MOs do not affect GFP expression directly (Fig 3.10), therefore they must prevent transgene expression indirectly, presumably by inhibiting ascl1a expression. Furthermore, ascl1a knockdown prevents endogenous α1T induction (Fig 3.11). Although these results do not prove a direct interaction between ascl1a and the α1T E-box, when taken together
they convincingly demonstrate that *ascl1a* regulates α1T expression in proliferating Müller glia.

**Ascl1a activates quiescent Müller glia**

In response to injury, Müller glia are transformed from a quiescent state into a proliferative state to generate multipotent retinal progenitors. α1T transgene expression, cell cycle re-entry and expression of other injury-induced genes (Fig 3.7) are all indicators of this process. How are Müller glia transformed? We detected *ascl1a* induction in the retina at 4hpi and in Müller glia as early as 6hpi, a full 18 hours before any other indicator of dedifferentiation appears. Such an early response to injury suggests *ascl1a* plays a role in activating Müller glia. Proneural genes such as *ascl1a* are often involved in cell fate specification. In *Drosophila*, achaete-scute genes promote neuroblast formation (Campos-Ortega and Jan, 1991). In mice, *Ascl1* specifies neural precursors in the brain and olfactory epithelium (Casarosa, Fode and Guillemot, 1999, Cau et al., 1997) and seems to play a role in causing stem cells to differentiate (Torii et al., 1999). We suspect *ascl1a* expression in Müller glia instructs them to become retinal progenitors in the regenerating zebrafish retina. When *ascl1a* expression is blocked, Müller glia do not divide (Fig 3.11). These observations are consistent with *ascl1a* activating Müller glia to become retinal progenitors. *Ascl1a* may commit Müller glia to become injury-induced progenitors by initiating expression of target genes such as α1T.

There are undoubtedly other genes which are regulated by *ascl1a* which probably include cell cycle regulators. When *ascl1a* expression is blocked, Müller glia do not divide,
retinal progenitors are not produced, newborn cells are not generated, and regeneration does not occur (Fig 3.12).

Ascl1a induction at 4hpi is the earliest indication of change in Müller glia after retinal injury. How is ascl1a induction regulated? Because of the rapid onset of expression, we suspect it may be regulated by transcription factors already present in Müller glia. Signal transduction cascades can occur very rapidly and initiate transcription of target genes without waiting for transcription factors to be synthesized. Ascl1a may act downstream of FGF in developing zebrafish pituitary gland (Herzog et al., 2004, Pogoda et al., 2006) and FGF injection can cause Müller glia to re-enter the cell cycle in the absence of injury (Fischer et al., 2002), suggesting FGF may initiate ascl1a expression in proliferating Müller glia. Another potential regulator is the wnt signaling pathway. In rats, adding wnt3a to retinal explant cultures increases the number of Müller glia that respond to injury by dedifferentiating (Osakada et al., 2007). It would be interesting to know whether the wnt3a mediated effect is due to Ascl1 induction, as Ascl1 induction has not been reported in mammalian Müller glia in response to injury. In response to intense light exposure, stat3 becomes localized to Müller glia within 16 hours, and although it is impossible to determine the precise time when injury occurs in this model, stat3 is present before Müller glia re-enter the cell cycle and begin to express injury induced genes such as ascl1a, α1T, delta, notch, and olig2 (Kassen et al., 2007).

**Does retina regeneration recapitulate development?**

Because many genes that are expressed during retinal development are also induced during regeneration, it seems logical that regeneration would reinitiate a
developmental genetic program. However, these two processes are vastly different. During development, an entire retina is formed from a neuroepithelial layer of heterogeneous retinal progenitors. This amorphous mass is transformed into the highly arrayed structure of a mature retina over a period of weeks in mammals or days in fish. Millions of cells proliferate and are specified to become different retinal cell types. In contrast, the structure of the retina is already established in the regenerating retina. Müller glia exist in a very different environment than developmental progenitors. Müller glia have already differentiated into functioning cells. In response to injury, they undergo a few rounds of proliferation to replace neurons that were lost. Müller glia may need to be converted into retinal progenitors to allow them to proliferate, which is what \textit{ascl1a} appears to do. This process likely involves a different genetic program than is required to initiate eye formation. Indeed, there seem to be different genetic programs for embryonic and CGZ based retinogenesis (Wehman et al., 2005).

Development and regeneration may converge once progenitors are formed and new cells are born. Indeed, the environment of a regenerating retina is very similar to the developing retina once production of newborn cells begins (Raymond et al., 2006). The same genes that are required for retinal cell type specification in the developing retina may dictate cell type specification during regeneration. This may explain why \textit{Pax6}, \textit{delta}, \textit{notch}, and \textit{olig2} are expressed once proliferation has reached its peak (Chapter II, Fausett and Goldman, 2006) and (Fig 3.7), to specify newborn cells. Expression of these genes at four days post injury suggests they regulate cell type specification, since differentiated neurons are present by seven days after injury (Chapter II, Fausett and Goldman, 2006, Raymond et al., 2006). \textit{Delta-notch} signaling has long been known to
regulate cell fate decisions in *Drosophila* (reviewed in (Bray,1998)), and in zebrafish, *notch* signaling regulates neuronal cell type in the spinal cord (Shin et al., 2007). The presence of *delta-notch* signaling components at a time when cells are likely being specified suggests that newborn cells are communicating via *delta-notch* signaling.

**Implications for retina regeneration in mammals**

*Ascl1a* expression four hours after retinal injury suggests it initiates the transformation of Müller glia into multipotent progenitors. The observation that this gene is a key regulator of successful retina regeneration in zebrafish makes it a potential target for therapeutic applications in mammals. There are no reports of *Ascl1* expression in mammalian retinas following injury. Could regeneration be limited in mammalian retinas due to a lack of *Ascl1* expression? Although some Müller glia do proliferate in response to injury in rats (Ooto et al., 2004), they do not spontaneously produce retinal neurons, suggesting they may not be true progenitors. It would be very interesting if these proliferative Müller glia did not express *Ascl1*, but were activated some other way. If this is the case, *Ascl1* expression may be sufficient to transform Müller glia into retinal progenitors. Transgene expression can be targeted to the mammalian retina by electroporation (Matsuda and Cepko, 2004), and specifically to Müller glia by using glial specific promoters in lentiviral vectors (Greenberg et al., 2007). Targeted expression of *Ascl1* or *ascl1a* using these methods will be a useful approach to determine whether mammalian Müller glia can be transformed into progenitors to facilitate retina regeneration.
Until now, there was no way to test the function of genes that are expressed during retina regeneration in vivo. By using lissamine labeled morpholinos and transgenic fish that express fluorescent reporters in regenerating cells, we can now perform loss of function studies on many genes by blocking their expression in vivo. This will be an extremely valuable tool to identify the role genes play during successful regeneration and may guide strategies to initiate a regenerative response in human retinal disease.

**Experimental Procedures**

**Animals**

The animals used in this study were treated in accordance with the guidelines of the University Committee on Use and Care of Animals at the University of Michigan. Fish were obtained from our breeding colony and raised with a 14:10 light/dark cycle at a temperature of 28°C.

**Transgenic zebrafish**

1696α1TlpEGFP, del1046-846α1TlpEGFP, and 1016α1TlpEGFP transgenic fish have been described previously (Chapter II, Fausett and Goldman, 2006, Goldman and Ding, 2000, Goldman et al., 2001). -907α1TlpEGFP and TG-954CAα1TlpEGFP constructs were resuspended in injection buffer, and single-cell zebrafish embryos were injected, raised to adulthood and screened for transgenic progeny as previously described (Goldman and Ding, 2000, Goldman et al., 2001)
**Optic nerve lesions**

Fish were anesthetized in 0.02% tricaine methane sulfonate (Sigma, St. Louis, MO) before surgery. Optic nerve crushes were performed as described previously (Chapter II, Fausett and Goldman, 2006, Hieber et al., 1998, Senut, Gulati-Leekha and Goldman, 2004). To treat axotomized ganglion cells with morpholinos oligonucleotides, the optic nerve was severed with a pair of scissors and a small piece of gelfoam which had been soaked in 1mM MO was applied to the stump of the optic nerve where it remained for 24 hours. The gelfoam was removed 24 after application and the fish recovered for 5 more days until they were killed to harvest the eye. The eye was then fixed for 4 hours in 1% paraformaldehyde and the retina was dissected out and flat mounted onto a glass slide with the ganglion cell layer up. A coverslip was placed onto the retina for confocal imaging.

**Eye lesions and Morpholino mediated gene knockdown**

Eye lesions were performed as described previously (Chapter II, Fausett and Goldman, 2006, Senut, Gulati-Leekha and Goldman, 2004). To deliver morpholinos to the injured retina, a 30-gauge needle was attached to a Hamilton syringe (Hamilton, Reno, NV) containing 1mM morpholino (Gene Tools, Philomath, OR). Approximately 0.5 µl was injected into the vitreous after inserting the needle at each injury site. We used the following lissamine labeled morpholinos: Control MO 5’CCTCTTACCTCAGTTACAATTATA-3’; \textit{ascl1a} MO, 5’ATCTTGGCGGTGATGTCCATTTCGC-3’; \textit{ascl1a}^{5'UTR} MO, 5’AAGGAGTGAGTCAAAGCACTAAAGT-3’ [The latter two MOs have been
described previously as *ash1a* MOs (Cau and Wilson, 2003)]. Custom electrodes were then placed across the head of the fish with the cathode on the left eye and the anode on the right eye. An ECM 830 Electro Square Porator (BTX, San Diego, CA) was used to deliver 5 consecutive 50ms pulses at 70V with a 950ms interval between pulses. The uninjected eye served as a negative control. One observer assigned letters to control and *ash1a* MOs. A second observer then electroporated these MOs into fish and assigned the fish with numbers. This way, both observers could score MO treated cells for GFP expression without any bias.

**Bromodeoxyuridine labeling**

To identify dividing cells, fish were either given a single injection of BrdU as described (Chapter II, Fausett and Goldman, 2006) or housed in 10mM BrdU for 24 hours (from 24-48 or 36-60 hpi). Fish were transferred to tanks with fresh water and killed at various times after BrdU administration to harvest the retinas.

**Tissue preparation**

Fish were given an overdose of tricaine methane sulfonate and eyes from adult fish were dissected, enucleated, and fixed as described (Chapter II, Fausett and Goldman, 2006, Senut, Gulati-Leekha and Goldman, 2004) by immersion in fresh 1% (to preserve GFP fluorescence) or 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 16 hr. After fixation, samples were cryoprotected in phosphate-buffered 20% sucrose before embedding with O.C.T. mounting medium (Sakura Finetek USA, Inc., Torrance, CA). Embedded samples were kept at -70°C until sectioning. Six to eight micrometer
serial sections were obtained on a cryostat (CM3050S; Leica, Nussloch, Germany), collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), dried overnight at room temperature, and stored at -70°C.

**Immunohistochemistry**

Immunohistochemistry was performed as described (Chapter II, Fausett and Goldman, 2006, Senut, Gulati-Leekha and Goldman, 2004) using the following primary antibodies: rat anti-BrdU (dividing cell marker; 1:250; Harlan; Sera-Lab); rabbit anti-GFP (1:1000; Molecular Probes, Eugene, OR); and mouse anti-glutamine synthetase (GS; glial marker;) (1:500; Chemicon). For BrdU immunostaining, sections were pretreated with 2 N HCl for 30 min at 37°C, two 5 min rinses in 0.1 M Sodium Borate buffer, and three 5 min rinses in PBS, pH 7.4. Primary antibodies were diluted in PBS containing 1% donkey serum and 0.3% Triton X-100 [normal donkey serum Triton (NDST) 1%]. Cryostat sections were first rehydrated 10 min in PBS, preincubated in NDST 3% for 30 min at room temperature, and then incubated in the primary antibodies overnight at 4°C. Sections were rinsed three times in NDST 1% and incubated for 2-3 hr at room temperature with secondary anti-mouse, anti-rabbit, or anti-rat antibodies conjugated to Alexa 488 (1:1000; Molecular Probes,) or cyanin 3 (1:250; The Jackson Laboratory, West Grove, PA). Sections were then washed twice in PBS, once in PB and then with water containing 10 ng/ml 4, 6-diamidino-2-phenylindole (DAPI; Sigma) for nuclear staining. Slides were washed twice with water and allowed to dry in the dark. Slides were then coverslipped using PVA-Dabco and subsequently stored in the dark at 4°C.
In situ hybridization

In situ hybridizations were performed with digoxigenin labeled cRNA probes as described (Barthel and Raymond, 2000). Ascl1a probe was a gift from Eric Weinberg, University of Pennsylvania. Pax6 cRNA was prepared from a full length Pax6a cDNA clone (Open Biosystems, Huntsville, AL). α1T probe was described previously (Hieber et al., 1998); notch1b and notch3 were a gift from Michael Lardelli, University of Adelaide; deltaA, deltaD and olig2 were a gift from Bruce Appel, Vanderbilt University; and deltaB was a gift from Julian Lewis, University College London. For timeline expression analysis (Table 1), notch3 hybridization was done individually and in combination with notch1b; similar results were obtained in each case. Delta in situ hybridization was done with deltaA individually and in combination with deltaB and deltaD; similar results were obtained in each case. Negative results were repeated twice for the 24 and 48hpi time points except for olig2.

Imaging

Slides were examined using a Zeiss (Oberkochen, Germany) Axiophot or Olympus Fluoview FV1000 laser scanning confocal microscope. Images were captured using a digital camera adapted onto the Axiophot microscope or Olympus confocal microscope. Images were processed and annotated with Adobe Photoshop CS.

Vectors for creating transgenic zebrafish

-907α1TIpEGFP expression vector contains 907 bp of 5’ flanking α1-tubulin DNA, exon 1, and the first intron fused in frame to the GFP sequence. This promoter
fragment is similar to the full-length 1696α1TIpEGFP expression vector from previous work (Goldman and Ding, 2000, Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004) except that it is lacking 789 bp from the 5’ end. A 2 bp mutation (TG-CA) was introduced into the full length α1T promoter by amplifying a PCR product containing the 2bp E-box mutation and cloning it into the α1TIpEGFP vector. The TG-954CAα1TIpEGFP construct is identical to the -1696 expression vector except for the TG:CA mutation at position -954.

**Vectors for in vitro assays**

Luciferase reporter vectors are based on the pXP2 construct (Nordeen, 1988). The α1TpXP2 reporter (wild-type) contains 1696 bp of 5’ flanking α1T sequence and the first exon of GFP cloned in frame and upstream of the luciferase coding sequence by BglII/SacI digestion and ligation into pXP2 (Veldman and Goldman, in prep). The TG-954CAα1T-pXP2 vector is identical to the wild-type vector except for a 2bp substitution at position 954 (TG-CA). This vector was made using BglII/SacI restriction sites. The E-box-pXP2 vector contains 3 copies of the α1T E-box at position 954 5’CATGTG 3’ upstream of a minimal β-globin promoter in pXP2 (Veldman and Goldman, in prep). The mE-box-pXP2 vector is identical to the E-box-pXP2 vector except each E-box has a 2 bp mutation (CATGTG to CATGCA). The coding sequence of ascl1a was cloned into pCS2+ by restriction digest of ascl1a (a gift from Eric Weinberg, University of Pennsylvania) with BamHI and XhoI, followed by ligation to create pCS2+ascl1a. A myc-tagged construct was made by amplifying a PCR fragment of ascl1a (Forward primer - 5’AGAGAGAATTCATGGACATCACCACCGCCAAGATGG-3’ Reverse primer
- 5’ ATCTCTTCTAGATCAAAACCAGTTGAGTGAAGTCTCTCTTCTAGATCAAAACCAGTTGAGTGAAGTC-3’), digesting with EcoRI and XbaI, followed by ligation into pCS2+MT to create pCS2+MT:ascl1a. pCS2+B-globin was a gift from Audrey Seasholtz, (University of Michigan, Ann Arbor, Michigan). ascl1a:MT was a gift from Matthias Hammerschmidt, (Freiburg, Germany). Vectors were confirmed by sequence analysis.

**Transactivation Assays**

HEK293T or rMC-1 (Sarthy et al., 1998) cells were plated in 24 well plates 24 hr before transfection. Cells were transfected via CaCl precipitation. Luciferase assays were performed in duplicate for each sample and values were normalized to B-globin.

**Nuclear Extracts**

Nuclear extracts were isolated from zebrafish brain, retina, and HEK293T transfected cells by rinsing in PBS three times before centrifugation. Cells were then resuspended in hypotonic buffer and allowed to swell on ice for 10 minutes. Next, cells were homogenized with 10 strokes of a dounce homogenizer. Cell lysis was verified by trypan blue staining. Nuclei were then pelleted and resuspended in low salt buffer, followed by drop-wise addition of high salt buffer. Nuclei were incubated for 30 minutes to allow extraction of nuclear proteins. Nuclei were pelleted and the supernatant was collected and dialyzed for >1hr in Slide-dialyzer tubes (Pierce). Aliquots were frozen on dry ice and stored at -80C. Total protein concentration was calculated using the BCA Assay (Pierce).
Electrophoretic Mobility Shift Assays

Oligonucleotide probes labeled with $^{32}$PdCTP (ICN, San Diego, CA) were incubated with 5-10µg of nuclear extracts prepared from: zebrafish brain or retina, rat brain, or with in vitro synthesized proteins using TNT Coupled Transcription/Translation (Promega, Madison, WI) for ten minutes at room temperature in binding buffer. Non-radiolabeled oligonucleotides were incubated at >50 fold excess to test binding specificity. The protein:DNA mix was then ran on a non-denaturing polyacrylamide gel at 250V for 3.5-4hrs and bands were visualized by exposing the gel to Kodak imaging film.
Figure 3.1 A 109bp region of the α1T promoter is required for transgene expression in dedifferentiating Müller glia. (a) Schematic representation of α1T promoter constructs. The bars represent promoter sequence and the numbers indicate relative position from the start codon. -1696 is the wild-type promoter described previously (Goldman et al., 2001, Senut, Gulati-Leekha and Goldman, 2004). Δ-1046-846 has been described (Goldman and Ding, 2000). The -1016 promoter directs transgene expression in Müller glia (Fausett and Goldman, 2006). The -907 promoter lacks 789bp of upstream sequence. (b) GFP transgene expression (or lack thereof) in transgenic fish shown in (a). Transgenic fish were injured on day 0 and given a single injection of BrdU at 4 days post injury (dpi) and killed four hours later. Fish carrying the required DNA element express GFP in BrdU labeled Müller glia (-1016 panel), while transgenic fish lacking the element do not (Δ-1046-846 and -907 panels). Two independent lines of Δ-1046-846 and three independent lines of -907 transgenic fish all display a lack of GFP expression in BrdU labeled cells. The images for -1016 and Δ-1046-846 are from the same sections. Because the -907 transgenic fish display very weak GFP expression in general (data not shown), we used serial sections to obtain the -907 images. ONL – outer nuclear layer, INL inner nuclear layer, GCL, ganglion cell layer.
Figure 3.2 An E-box within the 109bp region that is required for transgene expression in Müller glia binds nuclear extracts from zebrafish brain and retina. (a) EMSA using a probe from the 109bp region binds nuclear extracts from zebrafish brain and rat brain. The arrow indicates specific binding. Cold indicates where 50-fold molar excess unlabeled probe was added as competition. Extract indicates whether zebrafish (zf) or rat brain extracts were added. (b) Nucleotide sequence of the probes used for EMSA. The E-box is outlined in probe 4 with a box. Mutations are indicated by italicized and underlined text. (c) Mutations to the E-box (lanes 3 & 4) render the probe unable to bind nuclear extracts from zebrafish brain, while mutations to non-E-box nucleotides do not affect binding (lanes 1, 2 and 5). Probes correspond to those shown in (b). Unlabeled mutant probes compete with wild-type probe binding when the E-box is intact (lanes 6, 7 and 10), but not when the E-box is mutated (lanes 8 and 9), even at 50-fold molar excess. (d) Nuclear extracts from zebrafish retina bind specifically to the E-box. (e) An E-box probe from a different region of the promoter (Eb) is unable to compete with the E-box from probe 4 (lane 3), and does not bind to zebrafish brain nuclear extracts (lane 4).
Figure 3.3  The E-box is required for transgene expression in vivo.  (a) Schematic diagram showing the -1696 (wild-type) and TG-954CA α1T promoters.  (b) Transgenic fish received retinal injuries on day 0 and were given a single injection of BrdU on day 4 and were killed 4 hours later.  BrdU labeled cells which normally correspond to proliferating Müller glia do not express GFP in TG-954CA transgenic fish.  Arrowheads indicate BrdU labeled cells that normally correspond to transgene expressing Müller glia.  There are some cells which express GFP in the ganglion cell layer which are most likely axotomized ganglion cells (arrows).
Figure 3.4 Transgene expression in developing zebrafish. (a) The wild-type promoter directs GFP expression in the eye, brain and spinal cord of two day old zebrafish larvae. (b-d) Transgenic fish harboring a mutation to the E-box also express GFP in the eye, brain and spinal cord, although the expression is not as intense and uniform. (e) Immunostaining indicates GFP is expressed in the developing retina of wild-type transgenic fish at 3 days. (f-h) GFP expression in TG-945CA transgenic retinas is similar to wild-type GFP expression.
Figure 3.5 TG-954CA transgenic fish express GFP in axotomized retinal ganglion cells. GFP is induced in regenerating ganglion cells six days after optic nerve crush in all three lines of transgenic fish harboring the E-box mutation (arrows in GFP panel). DAPI staining is shown in blue. ONL – outer nuclear layer, INL – inner nuclear layer, GCL – ganglion cell layer.
Figure 3.6 Ascl1a is induced in proliferating Müller glia following retinal injury. (a-c) Ascl1a expression is detected by in situ hybridization in GFP+ Müller glia at 4dpi (arrows). (d-g) In situ hybridization for ascl1a from 6-24hpi. Ascl1a is induced in cells of the INL at 6hpi (arrowheads). Ascl1a expression gradually increases and is easily detected at 24 (arrowheads). (h-m) Ascl1a in situ hybridization (red) and glutamine synthetase immunostaining (green) ascl1a is induced in Müller glia at 6 (arrows in j) and 24hpi (arrows in m). DAPI nuclear staining is shown in the merged panels.
Figure 3.7 Timeline of gene expression following retinal injury. Probes corresponding to the genes indicated were used for in situ hybridization on injured retinas at various times after injury. The solid bars indicate when expression was first detected and the dashed bars indicate when expression likely begins. The width of the bars indicates relative signal strength.
Figure 3.8 Ascl1a regulates the α1T promoter through the E-box in vitro. (a) Luciferase reporter vectors with either a minimal B-globin promoter alone (light grey bars), or three copies of the E-box (dark grey bars) or three copies of a mutant E-box (black bars) were transfected in combination with ascl1a into HEK293T cells. Ascl1a transactivates the reporter when a functional E-box is present, but not when the E-box is mutated. (b) Full-length α1T constructs harboring either the wild-type (-1696) or mutant E-box (TG-954CA) promoters were transfected into HEK293T cells with or without ascl1a. Ascl1a induces reporter expression when the E-box is intact, but not when the E-box is mutated. Error bars indicate standard error of the mean for three replicates.
Figure 3.9  Ascl1a is required for transgene expression in vivo.  -1016 transgenic fish retinas were injured and electroporated with either control or ascl1a MOs on day 0. Eyes were harvested 4 days later and prepared for sectioning. Control morpholino treatment does not inhibit transgene expression in Müller glia (white arrows). Some Müller glia do not receive morpholino (green arrows). Morpholinos targeting ascl1a (ascl1a and 5’UTR panels) prevent transgene expression (arrowheads). Although not all of the MO treated cells are Müller glia, the ascl1a targeting morpholinos clearly prevent GFP expression. Müller glia that did not receive morpholino are able to express GFP (green arrows). ONL – outer nuclear layer, INL – inner nuclear layer, GCL – ganglion cell layer.
Figure 3.10 Ascl1a morpholinos do not block GFP expression in axotomized ganglion cells. Axotomized retinal ganglion cells labeled with the indicated lissamine tagged morpholinos (red) were examined for GFP expression (green). The morpholinos do not prevent GFP expression in these cells (arrows).
Figure 3.11 Ascl1a knockdown prevents induction of α1T and pax6. Retinas from -1016 transgenic fish were injured and electroporated with lissamine-labeled morpholinos (MO) on day 0 and harvested on day 4. (a-f) α1T expression detected by in situ hybridization (ISH) is shown in blue, native GFP expression in green and MO in red. The injury site is marked by an asterisk. (a-c) Control MO treatment does not affect α1T induction (arrows). (d-f) Ascl1a knockdown prevents α1T induction. Note the lack of α1T and GFP expression between the asterisk and the arrows, where the retina is treated with MO. Where the retina did not receive MO, α1T and GFP are expressed (arrows). (g-l) Pax6 expression detected by ISH is shown in blue, native GFP expression in green and MO in red. (g-i) Control MO treatment does not affect pax6 induction at 4dpi. Arrows indicate pax6+/MO+/GFP+ cells. (j-l) Ascl1a MO treatment prevents pax6 induction. Arrowheads indicate MO+/pax6− cells. Rare GFP+ cells are sometimes present but were not treated with MO (blue arrowhead)
Figure 3.12  Ascl1a is required for proliferation of Müller glia. Morpholinos were electroporated into injured -1016 retinas on day 0 and fish were housed in BrdU treated water from 36-60 hours post injury to label dividing cells. The fish recovered until day 10 when eyes were harvested. The control morpholino does not prevent treated cells from labeling with BrdU (white arrowheads). Morpholinos targeting ascl1a (ascl1a and ascl1a\(^{5'UTR}\)) prevent cells from labeling with BrdU (white arrows). Some cells are able to proliferate, but did not receive morpholino (grey arrowheads). (b) Ascl1a MO treatment leads to an overall decrease in the number of BrdU labeled cells at 2, 4, and 10dpi. ONL – outer nuclear layer, INL – inner nuclear layer, GCL – ganglion cell layer.
References


Chapter IV

Conclusion

Teleost fish have the ability to regenerate and repair damage to their central nervous system. This capacity is reflected both in the ability to regrow damaged axons and replace lost neurons with newly generated cells. The zebrafish retina is a convenient model to study both axonal and cellular regeneration. Although the capacity to repair an injured retina was discovered years ago, the precise cellular source of multipotent retinal progenitors which generate new neurons and glia had not been identified. Using transgenic zebrafish harboring a -1016α1T:GFP transgene which is expressed in a population of dividing cells after retinal injury (Chapter II, Fausett and Goldman, 2006), we set out to determine whether Müller glia are a source of multipotent progenitors in the injured zebrafish retina. We characterized cells that express GFP in response to injury and found that the proliferating cells had many characteristics of Müller glia which led us to conclude that indeed Müller glia are a source of multipotent progenitors for retina regeneration (Chapter II, Fausett and Goldman, 2006).

To begin to characterize the mechanisms underlying successful retina regeneration, we searched for DNA elements which mediate GFP expression in proliferating Muller glia. We identified an E-box that is required for transgene expression in Muller glia, which prompted us to search for bHLH transcription factors
which could regulate α1T expression (Chapter III). \textit{Ascl1a} seemed like a reasonable candidate, based on its expression in retinal progenitors, and induction following injury. We found that \textit{ascl1a} is expressed in Muller glia four hours after retinal injury, making it the earliest indicator of the initial stages of retina regeneration (Chapter III). We found that \textit{ascl1a} not only regulates the α1T promoter via the Ebox \textit{in vivo}, but is also required for successful retina regeneration in zebrafish, suggesting \textit{ascl1a} may be a good candidate to induce Muller glia to become multipotent retinal progenitors in mammals (Chapter III).

\textbf{Müller glia as retinal sensors?}

Müller glia are intimately associated with retinal neurons and seem to be involved in every aspect of retinal biology. They remove the neurotransmitter glutamate from synapses and convert it to glutamine, scavenge free radicals, and provide neighboring neurons with substrates for the Kreb’s cycle (Bringmann et al., 2000, Newman and Reichenbach, 1996, Tsacopoulos and Magistretti, 1996)). They also seem to participate in the cone visual pigment cycle (Mata et al., 2002, Mata et al., 2005). When Müller glia are not formed in the developing retina, normal lamination is disrupted (Bernardos et al., 2005, Takatsuka et al., 2004), suggesting they help form the laminated structure of the retina. Müller glia also conduct light to photoreceptors by acting as optical fibers (Franze et al., 2007). With their intimate connections to neurons, Müller glia are perfectly situated to provide metabolic support and sense synaptic activity (Barres, 1991). Does this close contact with all of the retinal cell types provide a way for Müller glia to observe retinal function? As Müller glia remove ions and neurotransmitters from
synapses, they may be monitoring synaptic transmission and sensing the health of the surrounding cells.

Circumstantial evidence that Müller glia act as sensors comes from rod photoreceptor production in the mature fish retina, where rod photoreceptors are produced as the eye expands. Rods are derived from rod precursors which are produced by stem cells in the inner nuclear layer (Otteson, D'Costa and Hitchcock, 2001). Since Müller glia are responsible for producing new cells after injury, it is likely they are the stem cells in the inner nuclear layer which are responsible for generating rod precursors. How is rod production initiated? Müller glia may be intrinsically programmed to produce rods at specified time intervals, which could be controlled by regulating the length of the cell cycle (Otteson, D'Costa and Hitchcock, 2001, Conlon and Raff, 1999). However, if photoreceptor density is the factor that dictates when rods are produced, then there must be a way to detect it. Rod production could be regulated by detecting when more rods need to be produced. A change in the physical density of rods could be measured physically, or by detecting a change in metabolic or synaptic activity in a given area. These signals could prompt Müller glia to initiate rod production. It seems that producing rods only when more rods are needed would be simpler than regulating cell cycle length in all Müller glia. This would also provide a mechanism for specifically producing rods where they are needed, in areas of decreased rod density.

How do Müller glia detect when regeneration is necessary? It was initially thought that photoreceptor loss was required to initiate retina regeneration. Physical injury, constant light exposure, heat lesion, and laser ablation all cause photoreceptor loss and trigger Müller glia to re-enter the cell cycle and generate new neurons. The death of
photoreceptors could be a signal to Müller glia that they should re-enter the cell cycle. Indeed, photoreceptors can signal to Müller glia during disease or injury (Rattner and Nathans, 2005). However, injection of ouabain into the fish eye kills amacrine and ganglion cells without damaging photoreceptors (Fimbel et al., 2007), yet Müller glia still proliferate and produce new neurons. If signals from photoreceptors are not required to initiate retina regeneration, how are Müller glia activated in response to cell death? Although photoreceptors may signal to Müller glia, there must be another mechanism to activate these cells in the absence of photoreceptor damage. Could a decrease in synaptic activity be detected by Müller glia and cause them to initiate neurogenesis? Death of surrounding cells could be detected by Muller glia which may remove cellular debris. If Müller glia are monitoring amacrine and ganglion cells, they could sense when these cells are damaged. Indeed, when amacrine and ganglion cells are destroyed due to ouabain injection, they are replaced, and although not explicitly demonstrated, they are most likely derived from Müller glia (Fimbel et al., 2007). It is not clear however, if other cell types are also produced; it would be very interesting if only cells that need to be replaced are generated in response to injury. When photoreceptors are destroyed by laser ablation or heat lesioning, they are the predominant cells that are regenerated (Raymond et al., 2006, Wu et al., 2001), suggesting Müller glia may be able to sense which cells need to be replaced.

In the developing cortex, cells derived from a particular radial glia function as a unit (Noctor et al., 2001). In other words, cells that are clonally related are connected to each other through synapses and work together to produce signals in the brain. Organization of these functional units could be a consequence of how development
occurs. Radial glia produce newborn cells that migrate along the radial process of their parent cell to travel within the cortex. If all daughter cells of a radial glia remain closely associated with it, these cells could be connected to form a functional unit. Stereotypical connections between cells in the retina suggest there may also be functional units in the retina. Müller glia provide structure and support for neurons in their putative retinal units. Clusters of proliferating cells are always closely associated with Müller glia as they migrate to their final location within the regenerating retina. Close association may allow these cells to be easily integrated into existing retinal units. It seems plausible that all of these intimate contacts with retinal neurons may provide a way for Müller glia to monitor the condition of the retinal unit which they anchor. When part of a unit is defective, Müller glia could revert to a stem cell state and produce cells to replace the ones that were defective or lost. When specific cells are destroyed, are they integrated into the existing retinal circuitry or is the entire retina re-wired? If each Müller glia produced new cells for their respective retinal units, the newborn cells could easily be integrated into the existing wiring. After a retinal puncture injury, most Müller glia near the injury respond by proliferating, while only some Müller glia at more distal locations respond. This may indicate that more retinal units are damaged near the injury site; the closer cells are to the injury, the more likely the retinal units are to be disrupted, therefore more cells respond near the injury site.

Müller glia respond to a variety of injuries in the same way, by dedifferentiating and initiating production of new neurons. Maybe they respond so quickly to retinal injury by sensing disruption of signaling within a retinal unit. Then, when any part of the pathway is disrupted, they initiate regeneration to replace the part of the unit that is
defective. Müller glia could anchor the newborn cells by maintaining very close contact, allowing new cells to be integrated into existing retinal units. This way, regeneration would not disrupt existing retinal units, and complete re-wiring of retinal circuitry would not be necessary.

The idea that Müller glia monitor the condition of retinal units is an interesting intellectual exercise, and there is some data consistent with this idea. Lesion models that destroy specific cell types suggest that cells that are destroyed are selectively replaced. Lineage tracing strategies using selective expression of Cre recombinase in combination with fluorescent reporter vectors would be very useful in tracking what types of cells are regenerated after specific cells are destroyed. Lineage tracing may also be able to provide information about whether newborn cells are integrated into existing retinal units and whether they remain associated with their Müller glia precursor.

Other more traditional signaling pathways have also been implicated in activating Müller glia. FGF is known to influence dedifferentiation of Müller glia in chicks (Fischer et al., 2002). In fish, addition of exogenous FGF in an organ culture model did not increase the number of proliferating cells at the injury site (Boucher and Hitchcock, 1998), although proliferation may already be saturated in the injured fish retina by endogenous signaling molecules. FGF is thought to act upstream of ascl1a in the development of the zebrafish pituitary (Herzog et al., 2004a, Pogoda et al., 2006); therefore it is possible that FGF is released after retinal injury to initiate progenitor formation. Photoreceptors can send signals that impinge on Müller glia via endothelin receptors during disease and injury (Rattner and Nathans, 2005), therefore endothelin could initiate dedifferentiation of Müller glia in response to injury. Wnt signaling has
been shown to be important for activating Müller glia in mammals (Das et al., 2006, Osakada et al., 2007), and may stimulate Müller glia to divide in fish. Stat3 is upregulated in response to constant light exposure in fish before the onset of cell division (Kassen et al., 2007), suggesting it too could drive dedifferentiation. Adding these signaling factors to the uninjured retina and inhibiting them in the regenerating retina could provide direct evidence that these signaling methods participate in directing Müller glia to proliferate after injury.

What is the role of ascl1a in Müller glia?

Ascl1a induction at 4hpi is the earliest indication that Müller glia are changing in response to injury. What role does ascl1a play in these cells? Its expression at such an early stage suggests it is involved in specifying Müller glia to become retinal progenitors. In drosophila, proneural genes of the achaete-scute complex commit some ectodermal cells to become neuroblasts, which later proliferate to produce neurons and glia. One of the first achaete-scute homologs in mammals was identified in neural precursors (Lo et al., 1991). Like drosophila achaete-scute genes, Ascl1 is not expressed in differentiated cells, suggesting that achaete-scute gene function in mammals is conserved. Additional studies have subsequently investigated Ascl1 function by examining mice lacking Ascl1 (Guillemot et al., 1993). These mice die at birth from apparent feeding and breathing problems and have many defects in the nervous system. Progenitors from the brain, olfactory epithelium and spinal cord are absent in Ascl1 null mice (Battiste et al., 2007, Casarosa, Fode and Guillemot, 1999, Guillemot et al., 1993, Parras et al., 2004, Parras et al., 2007), suggesting achaete-scute genes have proneural function in vertebrates.
Indeed, cells that express Ascl1 are mitotically active in vivo (Mizuguchi et al., 2006, Parras et al., 2004, Parras et al., 2007, Torii et al., 1999, Yun et al., 2002). Ascl1 can also act as a cell determination factor, causing cells to assume certain fates (Cau et al., 1997, Gordon et al., 1995, Parras et al., 2007, Tomita et al., 1996, Torii et al., 1999, Wildner et al., 2006). There is also evidence that Ascl1 expression can cause neural progenitors to exit the cell cycle in vitro (Farah et al., 2000) although this effect is not immediate. Cells overexpressing Ascl1 undergo several rounds of proliferation before exiting the cell cycle (Lo et al., 2002). Achaete-scute genes can exert their effects by influencing both early and late cell fate determination; early determination in lower organisms such as drosophila, and later determination in vertebrates.

What is the role of ascl1a in fish? Ascl1a is required for pituitary development (Herzog et al., 2004b, Pogoda et al., 2006) and epiphysial neurogenesis (Cau and Wilson, 2003), suggesting it has a proneural function in zebrafish also. There appear to be no major defects in other parts of the nervous system. This is somewhat surprising, because ascl1a is expressed in proliferating regions of the brain and retina. Perhaps there are other transcription factors that can compensate for ascl1a in zebrafish. We investigated whether ascl1a knockdown affects α1T transgene expression in developing zebrafish by injecting morpholinos targeting ascl1a into single cell -1696α1T:GFP transgenic embryos. There was no apparent effect on transgene expression, eye development or brain development, consistent with previous observations. When we examined pools of embryos from ascl1a mutant carriers, we did not observe a difference in eye size or PCNA labeling at the CGZ, suggesting ascl1a is not required for formation of the embryonic retina, and does not affect proliferation at the CGZ. We also injected ascl1a
RNA into single-cell -1696α1T:GFP embryos to investigate its function during development. There seemed to be less neural tissue in these embryos, and many lacked eyes and other neural structures, suggesting *ascl1a* overexpression caused premature cell fate determination in neural cells. If *ascl1a* is not required for neural development, and overexpression causes premature cell determination, how might *ascl1a* expression in Müller glia cause them to dedifferentiate?

As discussed above, *achaete-scute* genes act as cell determination factors. During development, *ascl1a* expression commits cells to become certain types, including pituitary and epiphysial cells (Cau and Wilson, 2003, Herzog et al., 2004b, Pogoda et al., 2006), probably by committing neural precursors to become pituitary and epiphysial progenitors. In the injured retina, *ascl1a* expression four hours after injury is the first indication that Müller glia are changing in response to injury. *Ascl1a* expression precedes cell cycle re-entry and α1T expression, suggesting it plays a role in initiating these downstream events. Indeed, when *ascl1a* expression is inhibited, these downstream events do not occur. Achaete-scute genes specify cells to assume a certain fate and it appears that *ascl1a* expression in Müller glia, which are quiescent stem cells, directs them to become retinal progenitors in response to injury. *Ascl1a* then appears to have a proneural function in the regenerating zebrafish retina. Müller glia that express *ascl1a* are committed to proliferate, whereas Müller glia that do not express *ascl1a* remain quiescent and uncommitted.

The process by which Müller glia re-enter the cell cycle and acquire characteristics of retinal stem cells has been called dedifferentiation. However, since we do not fully understand what actually occurs when Müller glia begin to divide,
dedifferentiation may not accurately describe this process. Dedifferentiation implies that Müller glia are transformed from differentiated cells into multipotent progenitors. But, the observation that Müller glia can become retinal progenitors may suggest that they are already multipotent and that \textit{ascl1a} expression initiates differentiation into progenitors. Therefore retinal stem cell may be a better description of Müller glia. Whether Müller glia divide symmetrically or asymmetrically after retinal injury will significantly influence how we think of these cells. If a Müller glia divides to produce a multipotent progenitor and remains a Müller glia, they truly are stem cells and the term dedifferentiate is not applicable. Since Müller glia appear to give rise to rod precursors and remain as Müller glia, the term retinal stem cell seems appropriate. If, on the other hand Müller glia divide symmetrically to produce two multipotent progenitors, the term dedifferentiate would be appropriate. The observation that \textit{ascl1a} is expressed in Müller glia before the onset of cell division could suggest that these cells may be reverting to a more primitive state, and become multipotent cells with a phenotype completely different than Müller glia. However, \textit{ascl1a} expression may also indicate that Müller glia are stem cells that become committed to generating neurons once \textit{ascl1a} is expressed. Lineage tracing strategies using \textit{ascl1a} driven Cre recombinase may provide an answer to the question of what actually happens when Müller glia divide after injury.

\textbf{Ascl1a regulates \textalpha{}1T expression}

\textit{Ascl1a} is a transcriptional activator that probably exerts its effects in Müller glia by regulating expression of target genes. \textit{Ascl1} seems to regulate \textit{Math4C/neurogenin1} expression which then initiates \textit{NeuroD} expression to drive olfactory neuron production
in mice (Cau et al., 1997). In the regenerating zebrafish retina, \( \alpha 1T \) expression and cell cycle re-entry occur simultaneously, suggesting they may be regulated by the same mechanism. Indeed, \( ascl1a \) is required for both \( \alpha 1T \) expression and cell division. Why then does it take nearly twenty hours for \( ascl1a \) to have an effect on \( \alpha 1T \) expression and cell-cycle re-entry? It is possible that \( ascl1a \) does not directly regulate \( \alpha 1T \) expression, but because \( ascl1a \) is required for \( \alpha 1T \) transgene expression in vivo, it appears to nonetheless regulate the pathway leading to \( \alpha 1T \) transgene expression. \( Ascl1a \) expression is relatively low until 24hpi, which is when GFP expression and cell division can be detected. Perhaps there are inhibitory bHLH proteins expressed in Müller glia which prevent \( ascl1a \) from activating transcription of target genes. Indeed, transcription factors which are present in the brain and retina bind to the \( \alpha 1T \) E-box in vitro (Fig 3.2). These transcription factors may bind the E-box to prevent \( \alpha 1T \) expression, since \( \alpha 1T \) is not expressed in the brain or retina (except for a few cells in the ventricles and CGZ). The low level of \( ascl1a \) expression from 4-18hpi may not be enough to overcome inhibition by inhibitory bHLH proteins such as id proteins. \( Ascl1a \) protein may need to accumulate in order to compete with and overcome inhibitory bHLH binding to initiate target gene expression. When \( ascl1a \) transcript levels are higher at 24hpi, there may be enough \( ascl1a \) protein to overcome putative inhibition by id proteins and induce \( \alpha 1T \) and other target genes. Alternatively, other bHLH factors may be required to enable \( ascl1a \) to regulate \( \alpha 1T \) expression via the E-box.

Although the EMSA experiments failed to produce positive results which would indicate \( ascl1a \) can bind directly to the \( \alpha 1T \) E-box, we can use an alternative method to test if a direct interaction occurs. Chromatin immunoprecipitation (ChIP) methods have
been used to demonstrate protein:DNA interactions in many model systems, including zebrafish (Havis, Anselme and Schneider-Maunoury, 2006). Synthetic RNA is injected into single cell embryos which are allowed to develop for 24 hours prior to performing the ChIP assay. We could inject myc-ascl1a RNA into -1696α1T transgenic zebrafish and use a myc antibody to immunoprecipitate myc-tagged ascl1a. This would provide direct evidence that ascl1a can bind the α1T E-box in vivo. We could also determine whether promoters from other genes that are potentially regulated by ascl1a bind ascl1a protein in vivo.

**Future directions**

Genes that are required for successful regeneration are important because they represent prospective therapeutic targets to transform mammalian Müller glia into multipotent retinal progenitors. These proliferative cells could be used to repair damaged retinas by replacing dead or defective cells with new ones. Although ascl1a is required for Müller glia to proliferate and produce new cells, it is not clear whether ascl1a expression alone is sufficient for this to occur. In order to truly determine whether ascl1a is a good therapeutic target to initiate retinal progenitor formation, it will be necessary to determine whether it can transform Müller glia into progenitors. We could create transgenic fish harboring a GFAP promoter driving ascl1a expression in Müller glia to attempt to answer this question. However, this approach may cause premature or incorrect differentiation of cells that normally express GFAP during development. Therefore, it would be preferable if we could express ascl1a in adult Müller glia. DNA can be electroporated into mammalian retinas (Matsuda and Cepko, 2004) and we may be
able to electroporate a GFAP:ascl1a plasmid into the adult fish retina to cause ascl1a expression in Müller glia. Ascl1a expression in Müller glia of -1016α1T:GFP transgenic fish would indicate whether ascl1a expression alone is sufficient to transform Müller glia into progenitors. Another way to approach this question would be to introduce ascl1a or Ascl1 into Müller glia of adult rats using lentiviral vectors (Greenberg et al., 2007). This would demonstrate whether ascl1a expression in mammals can direct Müller glia to become retinal progenitors. A positive result would be very compelling evidence that Ascl1 expression is a good strategy to transform Müller glia into retinal progenitors, especially because there are no reports of Ascl1 expression in the injured mammalian retina. This would be a significant development in our search to identify strategies to repair the mammalian retina.

Since Müller glia respond to retinal injury by inducing GFP expression at the same time they begin dividing, we can selectively purify proliferating progenitors using fluorescence activated cell sorting (FACS). We can also purify quiescent Müller glia by FACS using transgenic zebrafish which express GFP in all Müller glia (Kassen et al., 2007). We could then compare the gene expression profiles of the two purified cell populations by microarray analysis to identify genes whose expression changes in α1T expressing Müller glia. If we analyze gene expression at 2 and 4 days post injury, we should also be able to identify genes whose expression changes between the initial activation of Müller glia and their transition to multipotent progenitors. These experiments would identify many candidate genes which may be involved in retina regeneration. Since we are now able to test gene function in injured retinas, we will be able to test whether candidate genes are relevant in vivo by blocking their expression in
proliferating Müller glia. This information will likely guide strategies to repair the mammalian retina.

It will also be interesting to test the function of α1T in dedifferentiating Müller glia. Since newborn cells begin migrating shortly after they express α1T, it seems likely that α1T may be required for migration, especially in light of recent evidence that tubulin mutations affect cell migration in humans (Keays et al., 2007).

Additional work with ascl1a mutant fish (Herzog et al., 2004) could help characterize the role of ascl1a during eye development. It will be interesting to cross -1696α1T:GFP transgenic fish with ascl1a mutant fish to see whether ascl1a regulates α1T during development, although ascl1a MO injection into single cells embryos does not appear to affect GFP expression in developing embryos. Retinas of ascl1a mutant fish may lack a particular cell type, which would not have been detected in our preliminary studies. It is also possible that ascl1a may not have an effect on neurogenesis in the CGZ until after larval development is complete. The α1T transgene is not expressed in the CGZ of young zebrafish, but it is expressed in the adult CGZ (Goldman et al., 2001). α1T expression appears to reflect a transition in the state of the stem cells at the CGZ. During development, cells at the CGZ do not express GFP until fish are approximately two weeks old, at which time cells in the CGZ begin expressing GFP. Interestingly, E-box mutant transgenic fish do not go through this transition, suggesting ascl1a or another bHLH protein may regulate transgene expression in the adult CGZ. This could represent a change in the state of stem cells in the CGZ, which may be regulated by ascl1a. Neural stem cells in mice appear to undergo a transition also (Imura, Kornblum and Sofroniew, 2003). Since most of the adult retina is derived from
CGZ-based retinogenesis, it would be interesting to investigate whether *ascl1a* plays a role in this putative stem cell transition by conditionally interfering with *ascl1a* in the CGZ. If *ascl1a* expression is required for the putative stem cell transition at the CGZ, it would provide evidence that retina regeneration and CGZ based retinogenesis use similar genetic programs.

We now know that Müller glia are a source of retina regeneration and that *ascl1a* is required to transform these cells into multipotent progenitors, but there is still much to be learned. This work provides some insight into the mechanisms underlying successful retina regeneration and lays the groundwork for additional studies that will identify how Müller glia are able to dedifferentiate and produce new neurons for retinal repair.
References


