MOLECULAR MECHANISMS OF ZEBRAFISH PHOTORECEPTOR REGENERATION

by

Zhao Qin

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Molecular, Cellular, and Developmental Biology) in The University of Michigan 2010

Doctoral Committee:

Professor Pamela A. Raymond, Chair
Professor Peter F. Hitchcock
Professor Richard I. Hume
Assistant Professor Haoxing Xu
To my parents, Youhong Qin and Yanmin Wu
for their love and support
ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my advisor, Prof. Pamela Raymond, for giving me the opportunity to work in her lab and for her continuous support during my study and research. This dissertation would not have been possible without her inspiration, motivation, and personal guidance.

I would like to thank the rest of my dissertation committee: Prof. Richard Hume, Prof. Peter Hitchcock, and Prof. Haoxing Xu, for their insightful comments, helpful suggestions, and encouragement.

I am grateful to the present and former members of the Raymond lab who helped me in many ways over the past years and who made my experience in the Raymond lab pleasant and memorable. Especially, I would like to thank Linda Barthel, Rebecca Bernardos, Jason Meyers, Dilip Pawar, and Chen Kuang for their technical assistance and many interesting non-science-related conversations.

Last but not the least, I would like to thank my parents Youhong Qin and Yanmin Wu for their understanding, encouragement, and support, and my husband Yiming Liu for his love and support.

Chapter 4 of this dissertation describes part of the results of a project in collaboration with Prof. Kenneth Poss’s laboratory at Duke University and Prof. David Hyde’s laboratory at University of Notre Dame. I did all the experiments presented in this chapter.
# TABLE OF CONTENTS

**DEDICATION** ................................................................................................................... ii  
**ACKNOWLEDGEMENTS** ............................................................................................... iii  
**PREFACE** ........................................................................................................................ iv  
**LIST OF FIGURES** ........................................................................................................ vii  
**LIST OF TABLES** .......................................................................................................... ix  

**CHAPTER**  

1. **INTRODUCTION** .......................................................................................................... 1  
   Regeneration: an old bottle with new wine ................................................................. 1  
   Strategies for generating the cellular substrate for regeneration ............................. 3  
   Genetic insights from zebrafish regeneration research ............................................. 7  
   The zebrafish retina ................................................................................................. 12  
   Müller glia function as retinal stem cells during zebrafish retinal regeneration 14  
   Molecular mechanisms of zebrafish retinal regeneration ....................................... 16  
   References ............................................................................................................. 24  

2. **GENETIC EVIDENCE FOR SHARED MECHANISMS OF EPIMORPHIC REGENERATION IN ZEBRAFISH** ........................................................................... 31  
   Introduction ............................................................................................................. 31  
   Methods .................................................................................................................. 33  
   Results .................................................................................................................... 36  
   Discussion .............................................................................................................. 45  
   References ............................................................................................................. 62  

3. **SIX3-RELATED GENES IN ZEBRAFISH PHOTORECEPTOR REGENERATION** ........................................................................................................... 65  
   Introduction ............................................................................................................. 65  
   Methods .................................................................................................................. 67  
   Results .................................................................................................................... 70
4. FGF SIGNALING IN ZEBRAFISH PHOTORECEPTOR REGENERATION AND HOMEOSTASIS .................................................. 89
   Introduction ........................................................................................................ 89
   Methods .............................................................................................................. 92
   Results ................................................................................................................ 94
   Discussion .......................................................................................................... 96
   References ........................................................................................................ 103

5. CONCLUSION AND FUTURE DIRECTIONS .............................................. 106
   Summary of results ........................................................................................... 106
   Common molecular program of tissue regeneration in zebrafish ....................... 109
   HPE genes and tissue regeneration ..................................................................... 111
   Mammalian Müller glia and retinal regeneration ................................................. 112
   References ........................................................................................................ 115
LIST OF FIGURES

Figure
1.1. Regeneration models ........................................................................................................... 19
1.2. Model for zebrafish fin regeneration ................................................................................. 21
1.3. The zebrafish retina ............................................................................................................. 22
1.4. Model for photoreceptor production by Müller glia............................................................ 23
2.1. Cone photoreceptor regeneration in adult zebrafish .............................................................. 49
2.2. Isolation of GFP\(^+\) Müller glia ...................................................................................... 50
2.3. Gene expression profiling of isolated Müller glia from intact and regenerating zebrafish retinas ....................................................................................................................... 51
2.4. Gene ontology grouping of genes within each cluster .......................................................... 52
2.5. *hspd1* and *mps1* are up-regulated in injury-activated Müller glia during zebrafish photoreceptor regeneration ............................................................................................... 53
2.6. qRT-PCR validation of expression patterns of selected genes .............................................. 54
2.7. Cones fail to regenerate in *nbl* and *ncp* mutants at the restrictive temperature ............... 55
2.8. Cone regeneration defect in *nbl* and *ncp* mutants at the restrictive temperature ............. 56
2.9. Retinal regeneration defects of *nbl* and *ncp* ................................................................. 57
2.10. Transmission electron micrographs of mitochondria in injury-activated Müller glia in WT siblings and *nbl* mutants after acute exposure to 33°C ................................................. 58
2.11. Model for Müller glia-based photoreceptor regeneration in adult zebrafish retina ............................................................................................................................... 61
3.1. qRT-PCR validation of expression patterns of *six3a* and *six3b* during early stages of zebrafish photoreceptor regeneration ................................................................. 77
3.2. Fluorescent *in situ* hybridization of *six3a* on retinal sections of *Tg(gfap:GFP)m2002* zebrafish ......................................................................................................................... 78
3.3. Expression of *six3a* in the normal adult zebrafish retina .................................................. 79
3.4. Fluorescent *in situ* hybridization of *six3b* on retinal sections of *Tg(gfap:GFP)m2002* zebrafish ......................................................................................................................... 80
3.5. Expression of six3b in the normal adult zebrafish retina................................. 81
3.6. Expression of six3b at 48 hpl........................................................................ 82
3.7. Fluorescent in situ hybridization of six7 on retinal sections of
Tg(gfap:GFP)mi2002 zebrafish.............................................................................. 83
3.8. Photoreceptor regeneration is not affected in the six3b<sup>vu87/vu87</sup> mutants .......... 84
4.1. Experimental design ........................................................................................ 98
4.2. Retinal section from adult Tg(hsp70:dn-fgfr1) fish........................................ 99
4.3. Inhibition of Fgf signaling reduces rod but not cone regeneration............... 100
4.4. Quantification of photoreceptor regeneration at 14 dpl............................... 101
4.5. Expression of fgfr1 in the intact, normal adult zebrafish retina ................. 102
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Four distinct strategies for generating the regeneration substrate</td>
<td>20</td>
</tr>
<tr>
<td>2.1.</td>
<td>Transcriptionally-regulated genes common to regenerating retina, fin</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>and/or heart</td>
<td></td>
</tr>
<tr>
<td>3.1.</td>
<td>The number of PCNA$^+$ cells per 100 μm on retinal sections of wild</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>type (wt), heterozygous (het), and $\textit{six3}^{b\text{vu87/vu87}}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mutant (mut) fish at 2 dpl</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Regeneration: an old bottle with new wine

Regeneration, *i.e.*, the extraordinary phenomenon of regrowing and repairing missing or damaged tissues and organs in a grown organism, is a classic topic in biology research. The study of regeneration can be backdated to the beginning of experimental biology over two centuries ago when it was believed that only plants and certain microscopic animals could regenerate. To test if a polyp he had found in fresh pond water was a plant or an animal, Abraham Trembley conducted an experiment in 1740 in which he sectioned the organism into two pieces and asked if regeneration could occur in this species (Birnbaum and Sanchez Alvarado, 2008).

“I speculated anew that perhaps these organisms were plants, and fortunately I did not reject this idea. I say fortunately because, although it was the less natural idea, it made me think of cutting up the polyps. I conjectured that if a polyp were cut in two and if each of the severed parts lived and became a complete polyp, it would be clear that these organisms were plants… On November 25, 1740 I sectioned a polyp for the first time…the first polyps I cut were green in color. The two parts extended the same day that I separated them. They were quite easy to distinguish from one another because the first had its anterior end bedecked with those fine threads which serve as the polyp's arms and legs, whereas the second had none at all… I assumed that the second part was only a kind of tail without the organs vital to the life of the animal… Who would have imagined that it would grow back a head! I was observing this second half to find out how long it would retain the remnants of life; I had not the least expectation of being a spectator to this
It is now known that the replacement of amputated body parts in the polyp (*Hydra*; Fig. 1.1A) is just one example of regeneration in the metazoans. The ability to regenerate lost and damaged tissues and organs is widely represented among the various phyla of the animal kingdom and different levels of regenerative capacities are present (Sanchez Alvarado and Tsonis, 2006). The invertebrate *Hydra* and planarians (Fig. 1.1B), which are free-living, freshwater flatworms, have the most robust regenerative responses studied so far. These animals can regenerate essentially all tissues and organs lost to injuries (Wolpert et al., 1971; Reddien and Sanchez Alvarado, 2004) as well as normal physiological turnover (Holstein et al., 1991; Newmark and Sanchez Alvarado, 2000), therefore they are considered immortal (Martinez, 1998). Among vertebrates, the champions of regeneration are the urodele amphibians, newt (Fig. 1.1D) and salamander, as they can successfully regenerate many tissues and organs such as limbs, tail, central nervous system (both spinal cord and brain), and small sections of heart (Tsonis, 2000). Although there is a continuous cell replacement during mammalian tissue homeostasis (*e.g.*, hematopoiesis, epithelial renewal of the gut and skin), regeneration of missing body parts in mammals including humans largely fails, with few exceptions such as the liver (Vessey and de la Hall, 2001; Michalopoulos and DeFrances, 2005) and digit tips (Han et al., 2003).

To understand how regeneration proceeds in those highly regenerative animals, therefore, is not only intellectually intriguing, but also could revolutionize our way to treat human degenerative diseases and to repair injured or dysfunctional organs if the
human body could be somehow manipulated and become regeneration-competent. Despite being extremely important, the study of the mechanisms that underpin regeneration has been an overwhelming challenge to researchers for many years because of the inability to conduct genetic analyses in those traditional regeneration models. It was not until recently that several breakthroughs were made in this field (Sanchez Alvarado and Tsonis, 2006). Two types of advances led to recent progress in regeneration research. First, a diverse array of genetic tools, such as transgenesis, gene knockdown techniques, and functional genomics, were introduced into classic regeneration models like the Hydra, planarians, newt, and salamander. Second, extensive examination of regenerative capabilities of several available genetic models provided additional experimental paradigms for studying the cellular and molecular mechanisms of regeneration. It was shown that the amphibian Xenopus tadpole can regenerate many tissues and organs during its pre-metamorphic stages (Yokoyama et al., 2000; Beck et al., 2003). Another vertebrate model organism, the zebrafish (Fig. 1.1C) was found to be capable of regenerating many structures as an adult, including fins (Johnson and Weston, 1995), heart muscle (Poss et al., 2002a), spinal cord (Becker et al., 1997), optic nerve (Bernhardt et al., 1996), retinal neurons (Vihtelic and Hyde, 2000), and hair cells in the inner ear and lateral line (Harris et al., 2003), thus providing an exceptional animal model to study the regeneration of adult organs. Several animal regeneration models are shown and their regenerative capabilities are summarized in Figure 1.1.

**Strategies for generating the cellular substrate for regeneration**
The ability to perform genetic analyses in diverse regeneration models has greatly advanced our understanding of the underlying mechanisms of regeneration, and the enigma of regeneration has finally begun to unravel. One of the most fundamental questions in regeneration research is how do animals acquire the cellular substrate for restoring lost tissues and organs?

Interestingly, but not surprisingly, distinct cellular strategies are deployed in the various regeneration paradigms. Although in most cases, the cellular substrate of regenerated tissues is provided by proliferation of remaining cells; alternatively, regeneration of missing body parts sometimes is achieved by repatterning of pre-existing structures without detectable cell proliferation. For example, after removal of the “head” (the oral end that contains a ring of tentacles and a primitive mouth; see Fig. 1.1A) in Hydra, a tissue remodeling event called “morphallaxis” is soon triggered in the remaining body column so that positional values are reassigned along the apical-basal axis and cells at the oral end are respecified to form a new “head” (Wolpert et al., 1971). No cell proliferation occurs during this initial reorganization process. As a result, a smaller but functional organism is formed (Holstein et al., 1991). Another noteworthy phenomenon during Hydra regeneration is that tissue polarity is maintained after regeneration—when a Hydra is cut at both ends, the new “head” is always formed at the original “head” end (Meinhardt, 2002).

More commonly, regeneration requires generation of new cells by mitotic divisions. Based on the identity of the proliferating cells, three different mechanisms can be distinguished in regeneration scenarios where cell proliferation is required to generate the regeneration substrate. First, regeneration of lost cells and functional recovery of an
injured organ can be accomplished by proliferation of remaining differentiated cells in the same lineage. After surgical removal of parts of the mammalian liver, all five types of differentiated liver cells in the remaining lobes re-enter the cell cycle, without obvious dedifferentiation or transdifferentiation. Each cell type proliferates to produce additional cells, while retaining cellular identity and function, for example, the ability to synthesize the many liver-specific enzymes necessary for normal hepatic function (Michalopoulos and DeFrances, 1997; Michalopoulos and DeFrances, 2005). Such cellular replenishment through proliferation of mature cells in the same lineage is not unique to the mammalian liver. When up to 20% of the ventricle is amputated in the adult zebrafish heart, cardiomyocytes at the injury site proliferate and complete regeneration of the missing myocardium is achieved within two months (Poss et al., 2002a; Jopling et al., 2010; Kikuchi et al., 2010).

A second mechanism involves proliferation of a resident adult somatic stem cell population that gives rise to an undifferentiated cell mass, which subsequently becomes patterned and differentiates to replace lost tissues. This mechanism is used by the regeneration-competent planarians. These organisms maintain a population of undifferentiated cells known as neoblasts throughout their body plans. In response to tissue injuries, these pre-existing somatic stem cells are triggered to proliferate and migrate, and a specialized structure called the regeneration blastema, is assembled at the injury site. This structure comprises a mesodermally-derived, undifferentiated inner cell mass covered by an outer epithelial layer, manifesting a canonical epithelial-mesenchymal tissue relationship that is reiterated many times during animal morphogenesis in embryonic development (Sanchez Alvarado and Tsonis, 2006;
Birnbaum and Sanchez Alvarado, 2008). In addition to their importance in regeneration, neoblasts are also responsible for homeostatic replacement of cells lost through normal cell turnover. They are pluripotent and capable of replacing the about forty different types of cells found in planarians (Sanchez Alvarado, 2007).

The third major way is to acquire the undifferentiated cellular substrate for tissue regeneration through dedifferentiation followed by proliferation of mature, differentiated cells. This strategy is well-demonstrated by appendage regeneration both in the amphibians and in zebrafish (limb and tail regeneration in salamanders, fin regeneration in zebrafish). A common theme in these regeneration paradigms is the proliferation of dedifferentiated cells to form a regeneration blastema similar to that found during planarian regeneration. Evidence for this mechanism first came from Thornton’s analysis of salamander limb regeneration, in which he found dedifferentiated muscles and connective tissues contributed to a blastema that gave rise to the cartilage of the regenerating limb skeleton (Thornton, 1938). This observation was confirmed by Hay twenty years later by electron microscopic examination (Hay, 1959). More recent studies of salamander muscle fibers showed that dedifferentiation of these cells could occur both \textit{in vivo} (Echeverri et al., 2001) and \textit{in vitro} (Brockes and Kumar, 2002), and the resulting dedifferentiated cells could then contribute to multiple lineages (Brockes and Kumar, 2002). Lineage switching was also seen in salamander tail regeneration, where ectodermally-derived spinal cord cells produced tissues of mesoderm origin, \textit{e.g.}, muscle and cartilage (Echeverri and Tanaka, 2002). Similarly, during zebrafish appendage (fin) regeneration, a regeneration blastema, derived from proliferation of dedifferentiated
mesodermal cells, gives rise to cells in the multiple lineages that compose the new fin structure. This process will be discussed in more detail in the next section.

The strategies for generating the cellular substrate for tissue regeneration are summarized in Table 1.1. It is likely that these mechanisms are not mutually exclusive and in some cases, successful regeneration may involve more than one strategy, especially in tissues where a population of resident adult somatic stem cells exists (Susick et al., 2001; Vessey and de la Hall, 2001). In the next section, the molecular mechanisms of regeneration will be reviewed, with an emphasis on genetic insights that have been gained so far from regeneration studies of several body parts in zebrafish.

**Genetic insights from zebrafish regeneration research**

Zebrafish (*Danio rerio*) provide an excellent model for studying regeneration in vertebrates (Sanchez Alvarado and Tsonis, 2006) because they have remarkable capabilities to regenerate many tissues and organs following injury (Johnson and Weston, 1995; Bernhardt et al., 1996; Becker et al., 1997; Vihtelic and Hyde, 2000; Poss et al., 2002a; Harris et al., 2003). As a genetic model organism, zebrafish also possess many experimental advantages including: (1) ease to raise and maintain in large quantities in the laboratory; (2) relatively short generation time (about three months); (3) nearly complete genome sequencing; (4) availability of numerous genetic tools—transgenesis, forward mutagenesis screens, gene knockdown by morpholinos, and microarray analyses (Nechiporuk and Keating, 2002; Poss et al., 2003). For these reasons, the zebrafish has become an emerging system to molecularly dissect the mechanisms of adult regeneration.
Genetic studies of zebrafish regeneration were initiated in the caudal fin system as it is readily accessible, thus easy to perform surgeries and to observe phenotypes (Poss et al., 2002b). The zebrafish caudal fin is composed of multiple segmented, bony fin rays separated by mesenchymal compartments containing nerves, blood vessels, and connective tissue (Nechiporuk and Keating, 2002; Poss et al., 2002b). Regeneration of amputated caudal fins is completed within two weeks under normal laboratory rearing conditions (25-28.5°C). This process can be broken down into four steps: (1) Injury signal. Unidentified signals from the amputated fin trigger regenerative response. (2) Wound healing. During the first 12 hours after amputation, nearby epithelial cells migrate to the amputation site to cover the wound. (3) Blastema formation. In the next 36 hours (12-48 hours post amputation), mesenchymal cells immediately underneath the wound epidermis become disorganized and dedifferentiated, and they begin to proliferate to form the initial blastema. These blastemal cells express the homeodomain transcriptional repressor, msxb (Akimenko et al., 1995). (4) Regenerative outgrowth. From 48 hours to 14 days post amputation, the early blastema becomes compartmentalized, forming a slow-cycling, msxb+ distal blastema (stem cells) and an intensely proliferative, msxb- proximal blastema (transient-amplifying progenitors), which ultimately drives regenerative outgrowth (Fig. 1.2) (Poss et al., 2000; Nechiporuk and Keating, 2002; Makino et al., 2005).

The first studies attempting to elucidate the molecular mechanisms of zebrafish fin regeneration were conducted by the Keating group. Assuming that many of the genes required for regeneration would be necessary for normal development, they performed a forward mutagenesis screen for temperature-sensitive mutants of zebrafish fin
regeneration (Nechiporuk et al., 1999). So far, four mutants identified from this screen have been reported—*nightcap* (*ncp*) (Poss et al., 2002b), *emmental* (*emm*) (Nechiporuk et al., 2003), *no blastema* (*nbl*) (Makino et al., 2005), and *devoid of blastema* (*dob*) (Whitehead et al., 2005). Positional cloning identified the affected genes in these mutants as *mps1*, *sly1*, *hspd1*, and *fgf20a*, respectively. Detailed analyses of regeneration defects in these mutants revealed that these genes function at different stages during fin regeneration (Fig. 1.2). *mps1*, also called *ttk*, encodes a protein kinase involved in the mitotic checkpoint regulation (Poss et al., 2004). It is induced and required for cell cycle progression only in those rapidly proliferating cells of the proximal blastema during regenerative outgrowth (Poss et al., 2002b). *sly1*, a gene important for intracellular protein and vesicular trafficking, is necessary for both blastemal cell proliferation and organization during the two steps of blastema formation and regenerative outgrowth (Nechiporuk et al., 2003). *hspd1*, which encodes the heat shock protein 60, is up-regulated in blastemal cells during early blastema formation and in distal blastemal cells later during regenerative outgrowth. Loss of *hspd1* function specifically targets *msxb*-expressing mesenchymal stem cells. It causes mitochondrial defects and apoptosis of these cells (Makino et al., 2005). One of the fibroblast growth factor (Fgf) ligands, *fgf20a*, is expressed at the epithelial-mesenchymal boundary as early as 1 hour post amputation and its expression is maintained in the blastemal cells during blastema formation and regenerative outgrowth. Dysfunction of *fgf20a* results in early defects in regeneration initiation, including formation of an abnormal wound epidermis and failure of blastema formation (Whitehead et al., 2005).
Meanwhile, using a candidate approach, the role of several developmental signaling pathways, e.g., Fgf, Wnt, and BMP, has been investigated during zebrafish fin regeneration. In addition to *fgf20a*, components of Fgf signaling, *fgf24* (previously called *wfgf*) and *fgfr1*, are expressed in the regenerating zebrafish caudal fin (Poss et al., 2000). Functional disruption of the fibroblast growth factor receptor (Fgfr1) by treating fish with a specific pharmacological inhibitor (SU5402) leads to defective blastemal cell proliferation and *msxb* expression during blastema formation and blocks regenerative outgrowth (Poss et al., 2000). Injection and *in vivo* electroporation of a morpholino against *fgfr1* into zebrafish fin regenerates could phenocopy the outgrowth defect observed with the inhibitor (Thummel et al., 2006). Moreover, when Fgf signaling is blocked by expression of a dominant-negative *fgfr1* under the control of a heat shock promoter in a stable transgenic line, *Tg(hsp70:dn-fgfr1)*, regeneration of the amputated caudal fin fails. Further analysis using this transgenic line revealed another function of Fgf signaling in defining position-dependent blastemal properties and regenerative growth rates during zebrafish appendage regeneration (Lee et al., 2005). Genetic manipulations of members of the Wnt signaling pathway suggested opposing roles for distinct Wnt pathways in zebrafish fin regeneration: the canonical Wnt/β-catenin signaling enhances regeneration (Kawakami et al., 2006; Stoick-Cooper et al., 2007), whereas the β-catenin-independent signaling acts in a negative feedback loop to suppress regeneration (Stoick-Cooper et al., 2007). Gain- and loss-of-function of BMP signaling showed two distinct functions of BMP signaling during regenerative outgrowth: it is important for *msxb* expression and proliferation of the blastemal cells, and also for patterning the newly generated fin structure (Quint et al., 2002; Smith et al., 2006).
Recently, unbiased, genome-wide microarray analyses revealed dynamic regulation of gene and microRNA expression during zebrafish caudal fin regeneration (Schebesta et al., 2006; Yin et al., 2008). These studies identified many genes and microRNAs whose expressions are altered during regeneration, and the functions of some of these genes and microRNAs have begun to be elucidated. For example, activin-βA, a gene encoding a TGFβ-related ligand, is induced early during wound healing and later in the blastema. Inhibition of Activin-βA signaling affects cell migration during wound healing and blastema formation, thereby causes an early and complete block of regeneration (Jazwinska et al., 2007). In contrast, expression of the highly conserved microRNA-133 is down-regulated during zebrafish fin regeneration, and depletion of microRNA-133 was found to be downstream of Fgf signaling to promote blastemal proliferation and regeneration progression (Yin et al., 2008).

As described previously, zebrafish can regenerate part of their heart muscle through proliferation of remaining cardiomyocytes in the ventricle (Poss et al., 2002a). Interestingly, when kept at the restrictive temperature (33°C), two of the temperature-sensitive fin regeneration mutants, nbl and ncp, fail to regenerate amputated myocardium, suggesting hspdl and mpsl are also required for zebrafish heart regeneration (Poss et al., 2002a; Makino et al., 2005). Genetic attenuation of Fgf signaling by heat-shock induction in the Tg(hsp70:dn-fgfr1) fish demonstrated that functional Fgf signaling in the epicardial tissue is necessary for neovascularization in the regenerated myocardium and completion of cardiac regeneration (Lepilina et al., 2006). Identified in a gene expression profiling analysis of regenerating zebrafish heart, PDGF signaling has been shown to be required for DNA synthesis of cardiomyocytes both in culture and during regeneration.
Comparison of differentially expressed genes during fin and heart regeneration indicates that although fin and heart regeneration use many tissue-specific molecules, they may share a common set of core factors, mostly involved in tissue remodeling and cell migration (Lien et al., 2006).

This dissertation focuses on analyzing regeneration of retinal neurons in the adult zebrafish. In the next several sections, recent progresses in zebrafish retinal regeneration research will be discussed.

The zebrafish retina

The structure and function of the neural retina are highly conserved among all vertebrates. Similar to its mammalian counterpart, the zebrafish retina contains six major classes of neurons (rod and cone photoreceptors, horizontal, bipolar, amacrine, and ganglion cells) and one type of radial glial cell (Müller glia). The cell bodies of zebrafish retinal cells are also organized into three cellular layers (outer nuclear layer, onl; inner nuclear layer, inl; and ganglion cell layer, gcl), separated by two synaptic layers (outer plexiform layer, opl; and inner plexiform layer, ipl): rod and cone photoreceptors are in the onl; interneurons (horizontal, bipolar, and amacrine cells) and Müller glia in the inl; and projection neurons (ganglion cells) in the gcl (Fig. 1.3) (Goldsmith and Harris, 2003). At the molecular level, many of the genetic pathways that control retinal development are conserved in vertebrates, e.g., the zebrafish retinal field is defined at the end of gastrulation (approximately 8 hours post fertilization, hpf) in a region of the anterior neural plate that coincidently expresses several homeobox transcription factors necessary for vertebrate eye development: orthodenticle homolog 2 (otx2), paired box gene 6
(pax6), retinal homeobox (rx), and sine oculis homeobox homolog 3 (six3) (Chow and Lang, 2001; Livesey and Cepko, 2001).

Although the zebrafish retina begins to function at as early as 72 hpf (Hu and Easter, 1999; Malicki, 1999), neurogenesis persists in the adult zebrafish retina as part of continued body growth of the fish. In fact, the majority of the zebrafish retinal tissue is generated postembryonically through proliferation of retinal stem cells located at the boundary between the neural retina and the ciliary epithelium—the ciliary marginal zone (CMZ) (Johns and Easter, 1977; Moshiri et al., 2004; Raymond et al., 2006). This is different from what happens in the mammalian retina, where the entire retinal tissue is generated during embryonic or early postembryonic development (Moshiri et al., 2004). The molecular profile of these CMZ retinal stem cells have been characterized: they co-express homeobox-containing genes pax6a, rx1, and visual system homeobox 2 (vsx2), and they have diffuse distribution of N-cadherin on their plasma membranes and activated Notch-Delta signaling (Raymond et al., 2006).

The CMZ retinal stem cells can give rise to all retinal cell types except rod photoreceptors (Raymond, 1986; Hitchcock and Raymond, 2004). Rod photoreceptors in the central differentiated regions of the growing zebrafish retina are generated by proliferation and subsequent differentiation of a separate population of progenitors in the inl that is exclusive for the rod lineage (Raymond and Rivlin, 1987; Julian et al., 1998; Otteson and Hitchcock, 2003; Hitchcock and Raymond, 2004). Through a lineage tracing experiment, Bernardos et al. (Bernardos et al., 2007) showed that these rod-specific progenitors are derived from Müller glia. Müller cells in the intact, growing zebrafish retina proliferate at a low frequency and express low levels of the retinal progenitor
marker, Pax6, which has also been implicated in neurogenesis by radial glia and astrocytes in the developing and adult mammalian cortex (Gotz and Barde, 2005). The slow-cycling zebrafish Müller glial cells produce photoreceptor progenitors that express another homeobox transcription factor, *cone-rod homeobox* (*crx*), and migrate along the radial processes of Müller glia to the onl where they differentiate into rod photoreceptors (Fig. 1.4A) (Bernardos et al., 2007).

**Müller glia function as retinal stem cells during zebrafish retinal regeneration**

In addition to the persistent neurogenesis at the CMZ and in the rod lineage, the adult zebrafish retina possesses a robust capacity to replace lost neurons following injury. Several lesion paradigms have been used to study zebrafish retinal regeneration, including light lesions, to specifically destroy photoreceptors (Vihtelic and Hyde, 2000; Bernardos et al., 2007); intravitreal injection of the neurotoxin ouabain, to destroy ganglion cells and inl neurons (Fimbel et al., 2007); and physical lesions, to cause a local damage in the retina (Fausett and Goldman, 2006). In all cases, the missing retinal neurons are regenerated, and the retinal laminar architecture and visual function restored (Mensinger and Powers, 1999, 2007; Sherpa et al., 2008).

In response to retinal injuries, Müller glia are activated locally within the region of the lesion. They become dedifferentiated, re-enter the mitotic cycle, and begin to express molecular markers of the CMZ retinal stem cells (co-expression of the homeobox transcription factors *pax6a*, *rx1*, and *vsx2*, diffuse distribution of N-cadherin on plasma membranes, and activated Notch-Delta signaling) (Wu et al., 2001; Yurco and Cameron, 2005; Fausett and Goldman, 2006; Raymond et al., 2006; Fimbel et al., 2007; Yurco and
Cameron, 2007). These results suggested that Müller glia might be the source of the regeneration substrate that replenishes damaged retinal neurons. However, this remained uncertain until a lineage tracing experiment was done by using a glial specific marker to follow Müller glia lineages in response to retinal injury.

Using a transgenic zebrafish line, in which the green fluorescence protein (GFP) is driven by the zebrafish promoter sequence of a glial specific gene, *glial fibrillary acidic protein* (*gfap*) (Bernardos and Raymond, 2006), Bernardos et al. (2007) analyzed Müller glia responses following light-induced photoreceptor cell death. Adult zebrafish were briefly treated with ultra-high-intensity light from a spot source (~120,000 lux, approximately the light intensity when looking directly at the sun) and retinal sections were examined at 1, 2, 3, 4, 5, and 6 days post lesion (dpl). In the intact retina, GFP is exclusively expressed in Müller cells. Intense light exposure causes photoreceptor cell death in the central, differentiated regions of the retina. In response to photoreceptor loss, Müller glia within the lesioned area are activated—their nuclei migrate apically within a few hours after the light treatment and they re-enter the cell cycle without retracting their radial processes within the first 48 hours post lesion (hpl). By 3 days, Müller glia-derived, groups of proliferating, multipotent retinal progenitors, called neurogenic clusters are formed in the inl within the lesioned region. Cells in these neurogenic clusters express low levels of the retinal progenitor marker Pax6. These neurogenic clusters are characteristic of zebrafish retinal regeneration in all kinds of lesion paradigms (Vihtelic and Hyde, 2000; Faillace et al., 2002; Yurco and Cameron, 2005). In the case of photoreceptor regeneration, retinal progenitors in the neurogenic clusters become committed to the photoreceptor lineage (down-regulating Pax6 and up-regulating Crx).
while migrating along the Müller glial processes to the onl. Although the glial specific expression of the transgene would be turned off in these neuronal progenitors, perdurance of the GFP protein allowed the authors to observe some GFP\(^+\) cells that were also positive for a marker for differentiated cone photoreceptors, zpr-1, suggesting these Müller glia progeny had differentiated into photoreceptors. These results demonstrated that although Müller glia produce only rod photoreceptors in the uninjured adult zebrafish retina, they can switch their lineage to function as retinal stem cells to regenerate other retinal cell types, in this case, the cone photoreceptors (Fig. 1.4B) (Bernardos et al., 2007).

**Molecular mechanisms of zebrafish retinal regeneration**

Several microarray-based gene expression profiling analyses have been conducted in order to uncover the molecular mechanisms of retinal regeneration in adult zebrafish (Cameron et al., 2005; Kassen et al., 2007; Craig et al., 2008). By using whole-retina RNA samples, genes that are differentially expressed during zebrafish retinal regeneration have been identified in two lesion paradigms—surgical removal of a small piece of retina (Cameron et al., 2005) and exposure of fish to constant light for several days (Kassen et al., 2007; Craig et al., 2008). Another study used RNA samples harvested from laser-captured onl tissue in attempt to identify injury signals from the damaged/dying photoreceptors in light-treated zebrafish retinas (Craig et al., 2008).

These expression profiling analyses provided many candidate genes whose function during retinal regeneration needs further interrogation. Loss-of-function experiments of several regeneration-responsive genes were performed by using an injection and *in vivo* electroporation technique that delivers morpholino antisense
oligonucleotides into adult zebrafish retinas (Fausett et al., 2008; Thummel et al., 2008; Craig et al., 2010; Thummel et al., 2010). The proneural basic helix-loop-helix (bHLH) transcription factor *achaete-scute complex-like 1a* (*ascl1a*) is up-regulated in injured-activated Müller cells (Yurco and Cameron, 2007; Fausett et al., 2008). A recent analysis of mechanically injured zebrafish retinas in which *ascl1a* expression was knocked-down with morpholinos suggested that it is required for Müller glial proliferation and *pax6* induction in retinal progenitors (Fausett et al., 2008). Morpholino injection and electroporation have also been used to study the function of several genes during photoreceptor regeneration in adult zebrafish (Thummel et al., 2008; Craig et al., 2010; Thummel et al., 2010). Knockdown of *proliferating cell nuclear antigen* (*pcna*) blocks Müller glial cell cycle re-entry, causes Müller glial cell death, and results in failure to regenerate both rod and cone photoreceptors (Thummel et al., 2008). The two copies of the zebrafish *pax6* gene, *pax6a* and *pax6b*, were shown to be required at different points of neuronal progenitor proliferation necessary for zebrafish cone photoreceptor regeneration. Loss of *pax6b* expression affects the first cell division of neuronal progenitors, whereas loss of *pax6a* expression prevents later cell divisions (Thummel et al., 2010). A secreted factor *galectin 1-like 2* (*Drgal1-L2*) is induced in proliferating Müller glia and their progeny by photoreceptor cell loss. Knockdown of *Drgal1-L2* function with a specific morpholino results in defective regeneration of rod photoreceptors. *Drgal1-L2* is the first secreted molecule shown to be important for regenerative neurogenesis in the adult zebrafish retina (Craig et al., 2010).

This dissertation focuses on studying the molecular mechanisms of zebrafish photoreceptor regeneration. In order to discover the molecular triggers that mediate the
transition of Müller glia to function as retinal stem cells, a transcriptional profiling analysis of isolated Müller cells from light-lesioned/regenerating zebrafish retinas is described in chapter 2. This cell-specific expression profiling identified many genes regulated in injury-activated Müller glia during the early stages of zebrafish photoreceptor regeneration. Functional analyses have been focused on two genes shown to be essential for zebrafish fin and heart regeneration (chapter 2) and a highly conserved transcription factor, *six3* (chapter 3). Using a candidate approach, the role of a conserved developmental signaling pathway, Fgf signaling, during photoreceptor regeneration is evaluated in chapter 4.
Figure 1.1. Regeneration models. (A) Hydra and (B) planarian Schmidtea mediterranea can regenerate all tissues and organs lost to injuries. (C) Zebrafish (Danio rerio) can regenerate fins, heart muscle, spinal cord, optic nerve, retinal neurons and hair cells in the inner ear and lateral line. (D) Newt (Notophthalmus iridescens) can regenerate limbs, tail, heart, spinal cord, retina, lens, and inner ear hair cells. Scale bars, 2mm. Adapted from Sanchez Alvarado and Tsonis, 2006.
### Table 1.1. Four distinct strategies for generating the regeneration substrate.

<table>
<thead>
<tr>
<th>Strategy for generating the regeneration substrate</th>
<th>Require cell proliferation?</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repatterning of pre-existing structures</td>
<td>No</td>
<td><em>Hydra</em> regeneration</td>
</tr>
<tr>
<td>Proliferation of remaining differentiated cells in the same lineage</td>
<td>Yes</td>
<td>Mammalian liver regeneration; zebrafish heart regeneration</td>
</tr>
<tr>
<td>Proliferation of resident adult somatic stem cells</td>
<td>Yes</td>
<td>Planarian regeneration</td>
</tr>
<tr>
<td>Dedifferentiation and subsequent proliferation of mature, differentiated cells</td>
<td>Yes</td>
<td>Appendage regeneration in amphibians and zebrafish</td>
</tr>
</tbody>
</table>
Figure 1.2. Model for zebrafish fin regeneration. This process can be broken down into four steps. (1) Injury signal. Signals from the amputated fin trigger regenerative response. (2) Wound healing. During the first 12 hours after amputation, nearby epithelial cells migrate to the amputation site to cover the wound. (3) Blastema formation. In the next 36 hours (12-48 hours post amputation), mesenchymal cells immediately underneath the wound epidermis become disorganized and dedifferentiated, and they begin to proliferate to form the initial blastema. These blastemal cells express the homeodomain transcriptional repressor, msxb. (4) Regenerative outgrowth (48 hours-14 days post amputation). At this stage, the early blastema becomes compartmentalized, forming a slow-cycling, msxb+ distal blastema (stem cells) and an intensely proliferative, msxb- proximal blastema (transient-amplifying progenitors), which ultimately drives regenerative outgrowth. So far, four genes (fgf20a, hspd1, mps1, and sly1) have been identified in a forward genetic screen for temperature-sensitive mutants of zebrafish fin regeneration. Their affected regeneration stages are indicated in this figure. Adapted from Poss et al., 2002b.
Figure 1.3. The zebrafish retina. Similar to its mammalian counterpart, the zebrafish retina contains six major classes of neurons (rod and cone photoreceptors, horizontal, bipolar, amacrine, and ganglion cells) and one type of radial glial cell (Müller glia). The cell bodies of zebrafish retinal cells are also organized into three cellular layers (outer nuclear layer, onl; inner nuclear layer, inl; and ganglion cell layer, gcl), separated by two synaptic layers (outer plexiform layer, opl; and inner plexiform layer, ipl): rod and cone photoreceptors are in the onl; interneurons (horizontal, bipolar, and amacrine cells) and Müller glia in the inl; and projection neurons (ganglion cells) in the gcl. Adapted from Goldsmith and Harris, 2003.
Figure 1.4. Model for photoreceptor production by Müller glia. (A) In the intact, growing zebrafish retina, Müller glia express low levels of the retinal progenitor marker, Pax6. They proliferate at a low frequency to produce Crx\(^+\) photoreceptor progenitors, which migrate along the radial processes of Müller glia to the outer nuclear layer (onl) and differentiate into Rho4D2\(^+\) rod photoreceptors. (B) In response to photoreceptor cell loss, Müller glia within the lesioned area are activated. Their nuclei migrate apically and they re-enter the cell cycle without retracting their radial processes within the first 48 hours. By 3 days, Müller glia-derived, groups of proliferating, multipotent retinal progenitors, called neurogenic clusters are formed in the inner nuclear layer (inl) within the lesioned region. Cells in these neurogenic clusters express low levels of Pax6. Later, retinal progenitors in the neurogenic clusters become committed to the photoreceptor lineage (down-regulating Pax6 and up-regulating Crx) while migrating to the onl. Newly generated cone photoreceptors (zpr-1\(^+\)) first appear at 5 dpl. Blue, cones; magenta, rods; green, Müller glia and their progeny; red ovals, blood vessels (BV). ilm, inner limiting membrane; olm, outer limiting membrane. Adapted from Bernardos et al., 2007.
References


Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.


CHAPTER 2

GENETIC EVIDENCE FOR SHARED MECHANISMS OF EPIMORPHIC REGENERATION IN ZEBRAFISH

Introduction

The study of regeneration has long fascinated biologists and has lately experienced a renaissance associated with growing interest in regenerative medicine and the therapeutic potential of stem cells. Zebrafish (Danio rerio) are an ideal genetic model for studying regeneration in vertebrates (Sanchez Alvarado and Tsonis, 2006) because they have remarkable capabilities to regenerate fins (Johnson and Weston, 1995), heart muscle (Poss et al., 2002a), and nervous tissues (Bernhardt et al., 1996) following injury. A forward mutagenesis screen for temperature-sensitive mutations that interfere with regeneration of amputated caudal fin identified several genes whose functions are critical for specific steps in fin regeneration, including mps1 (also called ttk, a kinase required for mitotic checkpoint regulation), hspd1 (heat shock protein 60, a mitochondrial chaperone), and fgf20 (fibroblast growth factor 20) (Poss et al., 2002b; Makino et al., 2005; Whitehead et al., 2005). In addition, gene profiling analysis of regenerating tissues has provided lists of candidate genes associated with regeneration in fin
The regeneration of retinal neurons in adult zebrafish is an especially powerful model for studying regeneration of neuronal tissues: laminar retinal architecture and visual function are restored following damage inflicted by surgical lesions, neurotoxins, laser or photic lesions of retina (Hitchcock and Raymond, 2004). The neural stem cells in the retina arise from differentiated Müller glia, which respond to local retinal injuries by dedifferentiation, proliferation and production of multipotent neuronal progenitors (retinal stem cells) that can regenerate all types of retinal neurons (Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007). To discover genes expressed in injury-activated, neurogenic Müller glial cells that activate stem cell properties and trigger a neurogenic program, we generated transcriptional profiles of isolated, fluorescent-tagged Müller glial cells from light-lesioned adult transgenic zebrafish retinas during the early stages of photoreceptor regeneration. We found two genes required for fin regeneration, \( hspd1 \) and \( mps1 \), are also up-regulated in the injury-activated Müller glia. Functional analyses of \( hspd1 \) and \( mps1 \) mutants revealed that both genes are required for regeneration of cone photoreceptors. Moreover, consistent with the temporal sequence of mutant phenotypes in regenerating fins (Poss et al., 2002b; Makino et al., 2005), we found that \( hspd1 \) is required for an early step in retinal regeneration (formation of retinal stem cells from dedifferentiated, proliferating Müller glia), whereas defects in \( mps1 \) function block regeneration at a later step (proliferation of specialized photoreceptor progenitors).
Methods

Zebrafish

Zebrafish lines *Tg(gfap:GFP)mi2002* (Bernardos and Raymond, 2006), *nbl* (kindly provided by M. Keating) (Makino et al., 2005) and *ncp* (kindly provided by K. Poss) (Poss et al., 2002b) were maintained according to standard methods. The Committee on Use and Care of Animals in Research at the University of Michigan approved all procedures using animals. Adult fish (3-month to 1-year old) were used for all experiments. Light lesions were as described previously (Bernardos et al., 2007).

Retinal dissociation and isolation of Müller glia

Retinas were dissected from dark-adapted *Tg(gfap:GFP)mi2002* zebrafish at 8, 16, 24 and 36 hpl and non-light-treated controls (0 hpl). Tissues were minced with a razor blade and dissociated by enzymatic digestion with 16 U/ml papain (Worthington), 0.2 U/ml dispase (Worthington) (Nelson et al., 2003) in phosphate buffered saline (PBS) at pH 6.5 for 30 minutes at 28°C and triturated. Cells were pelleted at 6000 rpm for 3 minutes, resuspended in 1 mg/ml papain inhibitor (Worthington), 100 µg/ml DNase I (Sigma-Aldrich) with 2 mM MgCl₂ in PBS at pH 7.4 for 10 minutes at room temperature and then put on ice. GFP⁺ cells were isolated on a Vantage SE cell sorter (BD Biosciences). Gating was based on cell size and fluorescence intensity, with parameters set by reference to a control sample of dissociated retinal cells from wildtype zebrafish.

Microarray analysis
At each sample time, retinas from three or four fish were pooled for cell
dissociation and cell sorting. Total RNA was extracted and purified from 1-2 x 10^5
freshly sorted GFP^+ cells using the RNAqueous-4PCR kit (Ambion). The interval
between retinal isolation and cell lysis was ~2.5 hours. The quality and quantity of RNA
were assessed with a 2100 BioAnalyzer (Agilent Technologies). For microarray gene
profiling, 20 ng of total RNA was used for linear amplification with Ovation Biotin
Labeling System (NuGEN) and 2.75 μg of biotin-labeled, fragmented cDNA was
hybridized to a GeneChip Zebrafish Genome Array (Affymetrix) with 15,617 probe sets.
Independent hybridizations of three biological replicates were performed for each time
interval.

For data analysis, the “AFFY” package was used to filter probe sets based on
absent-present call; the Robust Multichip Average method and a two-stage filtering
procedure based on false discovery rate confidence interval (FDRCI) was used as
described (Akimoto et al., 2006). Genes differentially expressed at one or more time
intervals compared to the untreated control were identified by a fold change ≥ 2 and an
FDRCI P-value ≤ 0.15. Hierarchical clustering was performed as described (Weber et al.,
2005). Gene ontology analysis used the Affymetrix NetAFFX web interface and the
DAVID annotation tool (Dennis et al., 2003). Statistically over-represented (P ≤ 0.1)
gene ontological groups were identified as described (Raffatellu et al., 2008).

qRT-PCR

Total RNA was reverse transcribed and linearly amplified with the Ovation Biotin
Labeling System (NuGEN). All real-time PCR reactions were carried out in duplicate
with iQ SYBR Green Supermix (BioRad) on a iCycler iQ real-time PCR detection system (BioRad). The standard curve method was used to determine levels of expression of the genes of interest relative to *gpi*a (*glucose phosphate isomerase a*) and relative fold changes in gene expression after lesion. Sequences used for qRT-PCR (F, forward primer; R, reverse primer) are: *ascl1a* (*achaete-scute complex-like 1a*): F 5’- CAACTGGTTTTGAGCGTTCG-3’, R 5’-GACATCCTCCCAAGCGAGTG-3’; *dlg7* (*discs, large homolog 7*): F 5’-AGGCAGTCTCCTGTGGATG-3’, R 5’-TCCCTCTGTTCTGGGTGAA-3’; *gpi*a: F 5’-TCCAAGGAACACGCAAG-3’, R 5’-TTCCACATCACACCTGCA-3’; *hspd1* (*heat shock 60kD protein 1*): F 5’- AGGCTCTCTGGTGTTGGA-3’, R 5’-GCATCTAGCAGTGCCGT-3’; *id3* (*inhibitor of DNA binding 3*): F 5’-TGCCATTAGGATGGATG-3’, R 5’-CGCAGATTGCTTTCCACAC-3’; *mps1* (*monopolar spindle 1*): F 5’- ACTCGCAGGTGCGAACTC-3’, R 5’-CCACACGTCCTTTCAG-3’; *pcna* (*proliferating cell nuclear antigen*): F 5’-CATGATCTCCTTGCAAGG-3’, R 5’-TGAGCTGACTGGCTATT-3’; *pdgfa* (*platelet-derived growth factor a*): F 5’- TTCCCGAGAGCTTAGTGG-3’, R 5’-TGCTCCTATGGTGCC-3’; *six3b* (*sine oculis homeobox homolog 3b*): F 5’-CCATCCGAGCAAGAAAG-3’, R 5’- CAGACTGCTTTGGCCAGTC-3’.

**Tissue processing**

Immunohistochemistry was performed as described (Bernardos et al., 2007). For *in situ* hybridization digoxigenin (DIG)-labeled cRNA probes for *hspd1* (IMAGE clone ID: 3819432) and *mps1* (IMAGE clone ID: 6797095) were prepared and hybridized at 5
μg/ml as described (Raymond et al., 2006). Light microscopy was with AxioImager epifluorescent compound microscope; images were processed with Adobe PhotoShop (Adobe Systems) as described previously (Bernardos et al., 2007). All adjustments were applied to the entire image. Cells expressing the nuclear cell proliferation marker PCNA were counted in cryosections through the dorsoventral axis in the plane of the optic disc and expressed as number of cells per 100 μm linear length as described (Bernardos et al., 2007). The selection of regions for counting was done 'blind' (without viewing PCNA immunofluorescence). PCNA⁺ cells were counted in ten retinal sections from each of three fish for both mutants and wildtype siblings. Unpaired Student’s t-test was used for statistical analysis. Transmission electron microscopy was performed as described (Rivlin and Raymond, 1987) and ultrathin sections were viewed with a Phillips CM-100 equipped with an AMT digital camera.

Results

Photoreceptor regeneration after ultra-intense light treatment

The injury model we used is a light-lesion paradigm. Freely-swimming adult zebrafish were briefly exposed (20-30 minutes) to a spot source of ultra-intense light that selectively destroys cone and rod photoreceptors while leaving the inner retina intact (Bernardos et al., 2007). Postembryonic generation of rod photoreceptors continues in the differentiated retina of adult teleost fish, so here we specifically examined regeneration of cone photoreceptors, which are not produced in central, differentiated regions in the intact retina (Hitchcock and Raymond, 2004). To visualize the entire retinal lesion and
subsequent regeneration of cones, we examined isolated, flat-mounted retinas immunolabeled with zpr-1, a specific marker for red-green double cones in zebrafish (Fig. 2.1B). The lesion is confined to a central region approximately 1/4 to 1/3 of the total retinal area in a horizontal band along the nasal-temporal axis (Fig. 2.1E).

Cones completely regenerate by 14 days (Fig. 2.1H). In the intact zebrafish retina, cones form a highly regular, square mosaic pattern (Stenkamp and Cameron, 2002), with red-green double cones arranged in rows (Fig. 2.1C). Previous studies have shown that the regular cone mosaic pattern is not restored during regeneration (Stenkamp and Cameron, 2002) although the photoreceptors are functional and vision is restored (Mensinger and Powers, 2007). The disruption in the arrangement of red-green cones within the lesioned/regenerated area of the retina (Fig. 2.1I) was used in subsequent experiments to identify the regenerated region within the lesioned retina.

**Gene expression profiling of isolated Müller glia from intact and regenerating zebrafish retinas**

Injury-activated Müller glia dedifferentiate, proliferate and give rise to radial clusters of neuronal progenitors that migrate into the layer of damaged/dying photoreceptors (outer nuclear layer) where they differentiate to replace the missing cone and rod photoreceptors (Yurco and Cameron, 2005; Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Kassen et al., 2007). By using the transgenic zebrafish reporter line, \textit{Tg(gfap:GFP)mi2002}, in which expression of GFP is controlled by the \textit{cis}-regulatory sequences of a glial-specific gene, \textit{gfap} (Bernardos and Raymond, 2006), we previously showed that after destruction of photoreceptors the
progeny of dividing Müller glia differentiate into cone photoreceptors (Bernardos et al., 2007). To discover the cell-intrinsic, regeneration-responsive factors in the neurogenic Müller glia, we compared gene expression profiles of GFP+ cells isolated from intact and light-lesioned Tg(gfap:GFP)mi2002 zebrafish retinas at 8, 16, 24 and 36 hours following light treatment. These intervals are within the window during which Müller glia are activated but prior to the ‘birth’ (terminal mitotic division) of the first regenerated cone photoreceptors at 2 days post-lesion (dpl) (Raymond et al., 2006). We thereby limited our dataset to genes regulated at the early stages of regeneration in order to discover the molecular triggers that mediate the transformation of Müller glia into retinal stem cells. Harvested retinas were dissociated enzymatically and GFP+ cells were isolated from the resulting cell suspension by fluorescence-activated cell sorting (Fig. 2.2).

With microarray gene profiling we identified a total of 953 transcripts differentially expressed in at least one of the four sample times compared with the untreated control. The complete microarray dataset is available in the Gene Expression Omnibus database GEO (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE14495. Hierarchical clustering grouped these genes into three distinct groups based on their temporal expression patterns (Fig. 2.3). Expression of genes in clusters I and II (n=745) were up-regulated but with different time courses: cluster I, immediate up-regulation after the lesion (n=644); cluster II, delayed up-regulation (n=101). Cluster III includes all genes that were down-regulated after the lesion (n=208). For a broad overview of the major biological functions associated with each cluster, we grouped genes according to gene ontology terms: biological process, cellular component and molecular function (Fig. 2.4). In cluster I, the translation/protein biosynthesis group of
genes predominates (Fig. 2.4A); accordingly, genes classified as cellular components and molecular functions of ribosome are highly represented (data not shown). This suggests that an early step in the injury-induced activation of Müller glia is stimulating protein synthesis and metabolism. The enrichment of DNA replication/cell cycle genes in the genes up-regulated with a delayed onset (cluster II, Fig. 2.4B) is consistent with the observation that most or all Müller glia within the lesioned area re-enter the cell cycle by 48 hours post-lesion (hpl) (Bernardos et al., 2007). The down-regulation of genes involved in chromatin assembly and ion homeostasis (cluster III, Fig. 2.4C) is consistent with the dedifferentiation of injury-activated Müller glia described previously (Yurco and Cameron, 2005; Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Kassen et al., 2007).

As an initial validation of the microarray data, eight genes with different temporal expression patterns from distinct gene ontological groups were selected for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis; the two methods showed excellent agreement (Fig. 2.5B, E, also see Fig. 2.6). Among the genes we investigated, ascl1a (formerly zash1a, Fig. 2.6A) and six3b (Fig. 2.6C) are transcription factors expressed in retinal progenitors in the ciliary marginal zone (CMZ) of postembryonic zebrafish retina (Raymond et al., 2006). The proneural basic helix-loop-helix (bHLH) gene ascl1a is induced in activated Müller glia and their neurogenic progeny following retinal lesions (Fausett et al., 2008). The homeobox transcription factor six3 plays a crucial role in early eye development and interacts with bHLH proteins (Marquardt and Gruss, 2002). Another up-regulated gene, sox4a, belongs to the C-group Sox family of HMG-box transcription factors, which are expressed in committed
neuronal progenitors and operate downstream of proneural bHLH genes to establish neuronal properties (Bergsland et al., 2006). Consistent with the increased expression of ascl1a, six3b, and sox4a, a negative regulator of the bHLH genes, id3, was down-regulated (Fig. 2.6F). Interactions among these genes may form a transcription regulatory network to initiate a neurogenic program in the injury-activated Müller glia.

As expected, many cell cycle genes and growth factors were up-regulated during regeneration, such as pcna (Fig. 2.6E) and pdgfa (Fig. 2.6B), respectively. A recent gene expression profiling analysis of regenerating zebrafish heart muscle found that pdgfa is also up-regulated during heart regeneration, and PDGF signaling is necessary for cardiomyocyte proliferation (Lien et al., 2006).

**hspd1 and mps1 are up-regulated in injury-activated Müller glia with different time courses**

We were intrigued to find that two genes required for fin and heart regeneration in zebrafish are up-regulated in injury-activated Müller glia. One is hspd1, which encodes heat shock protein 60 (Hsp60), an ancient, highly conserved protein that functions in the cellular stress response as a chaperone for protein folding and assembly (Deocaris et al., 2006). In the regenerating zebrafish caudal fin, hspd1 is required for the formation and maintenance of the early/distal blastemal stem cells derived from mesenchyme (Makino et al., 2005). The other regeneration gene is mps1 (monopolar spindle 1, also called ttk), a protein kinase involved in mitotic checkpoint regulation (Abrieu et al., 2001). In the regenerating fin, mps1 is not required for the activation of mesenchymal stem cells or initial establishment of the blastema, but is required later in rapidly proliferating
progenitor cells at the outgrowth stage (Poss et al., 2002b). We observed a similar
temporal sequence of gene regulation in the light-lesioned retina, in that \textit{hspd1} was up-
regulated immediately after the light treatment at 8 hpl, while \textit{mps1} was not induced until
36 hpl in both our microarray and qRT-PCR analyses (Fig. 2.5B, E).

To define the spatial expression patterns of \textit{hspd1} and \textit{mps1} during retinal
regeneration, we performed fluorescent \textit{in situ} hybridization for both genes on retinal
sections of light-lesioned Tg(\text{gfap}:\text{GFP})\text{mi}2002 zebrafish. These results confirmed the
timing of gene expression following injury: \textit{hspd1} was up-regulated locally within the
region of the lesion at both 24 and 48 hpl (Fig. 2.5A); \textit{mps1} was undetectable in
unlesioned retina and at 24 hpl, but was induced within the lesioned area at 48 hpl (Fig.
2.5D). Both genes were up-regulated specifically in the inner nuclear layer of the retina
where the cell somas of Müller glia reside. In addition, \textit{hspd1} was also expressed in the
damaged/dying photoreceptors within the lesioned region at 24 hpl (Fig. 2.5A). Co-
labeling with the GFP transgenic reporter and PCNA at 48 hpl confirmed that both genes
were expressed in the injury-activated Müller glia and their progeny (Fig. 2.5C, F).

\textit{hspd1} and \textit{mps1} are required for zebrafish cone photoreceptor regeneration

We next asked whether \textit{hspd1} and \textit{mps1} are necessary for retinal regeneration.
The zebrafish mutant \textit{nbl} (\textit{no blastema}) is a temperature-sensitive null allele of \textit{hspd1} for
the chaperone activity (Makino et al., 2005); \textit{ncp} (\textit{nightcap}) has a missense substitution in
the conserved kinase domain of \textit{mps1} and also exhibits a temperature-sensitive phenotype
(Poss et al., 2002b). Homozygous \textit{nbl} or \textit{ncp} mutants and their homozygous wildtype
siblings (WT) were light-lesioned and allowed to recover at the restrictive temperature
(33°C) after the injury. We found that cone photoreceptor regeneration takes place much faster at 33°C than at the standard temperature of 28°C: by 7 dpl cones were fully regenerated in WT (nbl+/+, n=4; ncp+/+, n=5; Fig. 2.7A, C), whereas ~14 days were required to achieve a comparable stage of recovery at 28°C (data not shown). In contrast, both mutants did not regenerate cones, or did so only sporadically, at the restrictive temperature of 33°C (nbl−/−, n=6; ncp−/−, n=3; Fig. 2.7B, D, also see Fig. 2.8).

To characterize the cellular nature of the retinal regeneration defects, we collected eyes from light-lesioned nbl or ncp mutants and wildtype siblings held at 33°C for 1, 2 or 3 days after the lesion. Retinal regeneration in zebrafish requires mitotic activation of Müller glial cells (Thummel et al., 2008), and thus we first quantified the proliferative response of Müller glia at 1 dpl by counting PCNA+ cells in the inner nuclear layer (inl) of the lesioned region, nearly all of which appear to be injury-activated Müller glia. We found 6.8 ± 0.8 PCNA+ cells per 100 µm linear length retina in nbl mutants and 6.2 ± 1.1 PCNA+ cells per 100 µm in ncp mutants; neither are significantly different from WT: 6.1 ± 1.0 PCNA+ cells per 100 µm (P = 0.31) and 6.8 ± 0.9 PCNA+ cells per 100 µm (P = 0.36), respectively. At 2 dpl, clusters of proliferating, Müller glia-derived, multipotent retinal progenitors weakly immunoreactive for the retinal progenitor marker Pax6 are seen in the inl within the lesioned area in WT. These regularly spaced, radially oriented groups of PCNA+/Pax6+ cells associated with Müller glia, called 'neurogenic clusters', are characteristic of retinal regeneration in teleost fish (Hitchcock and Raymond, 2004). The number of proliferating progenitors in the neurogenic clusters of nbl was reduced to ~50% of WT: 11.3 ± 0.5 PCNA+/Pax6+ cells per 100 µm in nbl compared with 21.7 ± 3.2 PCNA+/Pax6+ cells per 100 µm in WT; P < 0.05 (Fig. 2.9A, C). The ncp mutants showed
a slight but not statistically significant reduction in formation of neurogenic clusters: 12.2 ± 0.2 PCNA+/Pax6+ cells per 100 µm in ncp compared with 14.6 ± 1.9 PCNA+/Pax6+ cells per 100 µm in WT; $P = 0.17$ (Fig. 2.9C).

Proliferation of retinal progenitors in ncp mutants was reduced at 3 dpl, when the neuronal progenitors have migrated into the outer nuclear layer (onl) and become committed to the photoreceptor lineage, as evidenced by expression of a photoreceptor-specific homeobox gene, Crx (Bernardos et al., 2007). At 3 dpl ncp mutants had fewer than half as many photoreceptor progenitors (PCNA+ cells in the onl) relative to WT: 16.8 ± 3.2 PCNA+ cells per 100 µm in ncp compared with 34.9 ± 2.1 PCNA+ cells per 100 µm in WT; $P < 0.05$ (Fig. 2.9B, C). Consistent with the reduction in multipotent retinal progenitors at 2 dpl, the nbl mutants showed a substantial decrease (~90% reduction) in the number of photoreceptor progenitors at 3 dpl: 4.1 ± 1.6 PCNA+ cells per 100 µm in nbl and 43.5 ± 1.2 PCNA+ cells per 100 µm in WT; $P < 0.0001$ (Fig. 2.9C). Note that the number of PCNA+ cells in the WT retinas varies between the two mutant lines and across experiments; this variability in the absolute rate of cell proliferation in teleost fish retinas is typical (Julian et al., 1998), and likely reflects environmental modulation of endogenous growth rates. Taken together these data suggest that nbl blocks cone photoreceptor regeneration at an earlier step compared with ncp, and are consistent with the differential time course of hspd1 and mps1 expression during retinal regeneration.

In amputated fins, nbl causes structural defects in mitochondria specifically in the putative blastemal stem cells (Makino et al., 2005). To determine whether Müller glia-derived retinal stem cells are similarly differentially affected by the nbl mutation, we
used a temperature shift paradigm and examined retinas with transmission electron microscopy. Regeneration was allowed to proceed normally at 28°C for 2 or 3 days, before fish were shifted to 33°C for 4 or 8 hours. Müller glia were identified by the position (in the inner half of the inner nuclear layer) and morphological features of their nuclei (polygonal, often lobulated with clumped heterochromatin), and the presence of cytoplasmic glycogen granules. In nbl (but not WT) at 2 dpl following 8 hours at 33°C, most of the identified Müller glia within the lesioned area had swollen, distorted mitochondria with empty matrix (Fig. 2.9D, also see Fig. 2.10A-C): of 29 Müller glia we examined, 20 had defective mitochondria, and 3 of the 20 also had one or more mitochondria with normal morphology. This mitochondrial defect was not seen in the neurogenic progeny of Müller glia, i.e., the radial clusters of neuronal progenitors migrating into the outer nuclear layer, which were increased in abundance at 3 dpl (Fig. 2.10D-F). These results suggest that the defect in nbl is confined to injury-induced stem cells derived from differentiated cells in both neural retina and mesenchymal tissues in the caudal fin.

**Additional genes shared in regenerating tissues**

To identify additional candidate genes that might be involved in epimorphic regeneration independent of the body structure damaged, we compared our microarray dataset from isolated injury-induced Müller glia/progenitors with published gene profiling results from two other zebrafish regeneration models: amputated caudal tail fins (11) and surgically lesioned hearts (12). Table 2.1 lists twenty-eight genes whose expression levels changed in the retinal dataset and in one or both of the comparison
datasets. A large subset of these regeneration-associated genes are involved in the innate immune response to tissue injury, several regulate the immune system by suppressing inflammatory cytokine signaling, and others mediate the stress response. In addition, a number of the regeneration genes regulate developmental signaling pathways (e.g., TGFβ, Hedgehog, Notch) or are transcription factors that regulate progenitor cells. Another recently published retinal regeneration microarray dataset designed to identify molecular signatures of injured and dying photoreceptors and microglia was generated from tissue obtained by laser-capture microdissection of the photoreceptor layer from light-damaged zebrafish retinas (15); at least three of the secreted growth factor signals they found—*midkine, progranulin,* and *galectin*—are also up-regulated in regenerating heart (12). This provides further support for a common molecular program of injury-induced regeneration in mesodermal and neural tissues.

**Discussion**

Our study differs from three previously published microarray-based gene expression profile studies of retinal regeneration in adult zebrafish (Cameron et al., 2005; Kassen et al., 2007; Craig et al., 2008) in two fundamental ways: (1) We used brief exposures to ultra-intense light to induce widespread and rapid photoreceptor death, whereas the earlier studies exposed fish to continuous light at lower intensities for several days (Kassen et al., 2007; Craig et al., 2008) or surgically removed a small piece of retina (Cameron et al., 2005). (2) We isolated the injury-activated Müller glia for RNA extraction and gene profiling analysis, whereas the other studies harvested RNA from the
entire retina (Cameron et al., 2005; Kassen et al., 2007) or from laser-captured outer nuclear layer tissue (Craig et al., 2008). Retinal injury induces a series of complex cellular responses in many cell types, including neurodegeneration and apoptosis of the damaged cells, stress responses in other retinal cells, and activation of microglia/macrophages (Vihtelic and Hyde, 2000; Bernardos et al., 2007; Kassen et al., 2007). By purifying the GFP$^+$ Müller glia, we increased the sensitivity of our analysis to identify injury-induced changes in gene expression that activate the retinal stem cell population and initiate a neurogenic program. Although some of the genes whose expression levels changed dramatically in our dataset were also identified in previous studies, the magnitude of the changes they observed was necessarily diluted by the cellular heterogeneity of the samples. For example, the maximum fold change of hspd1 reported previously was 2.0 (Cameron et al., 2005) or 1.7 (Kassen et al., 2007) compared with 3.6 in our study, and changes in mps1 were not reported in (Kassen et al., 2007). A recent analysis of mechanically injured zebrafish retina in which ascl1a function was knocked-down with morpholino antisense oligonucleotides verified that it is required for the regenerative response (Fausett et al., 2008), which validates the utility of our dataset as a tool for discovering genes that induce a neurogenic program in differentiated glial cells. Consistent with the increased expression of hspd1 we observed in the outer nuclear layer by in situ hybridization, the microarray data from laser-captured outer nuclear layer tissue also showed an up-regulation of hspd1 (Craig et al., 2008). In contrast, neither mps1 nor ascl1a were up-regulated in that analysis, again consistent with our observation that these genes are specifically induced in injury-activated Müller glia during the initial stages of regeneration (Fig. 2.5D, F) (Raymond et al., 2006).
A model of Müller glia-based photoreceptor regeneration in adult zebrafish (Bernardos et al., 2007) is shown in Fig. 2.11. In response to the light lesion (step 1) Müller glia are activated locally in the region where photoreceptors were damaged by the intense light treatment (step 2); Müller glia activation is evidenced by apical nuclear migration and up-regulation of GFAP intermediate filaments. This is followed by dedifferentiation of Müller glia and their entry into the mitotic cycle (step 3). Asymmetric division of Müller glia generates neurogenic clusters of multipotent progenitors that proliferate, migrate into the outer nuclear layer, and differentiate into photoreceptors, and results in the self-renewal of the Müller ‘stem cell’ (step 4). In this study we found that hspd1 is essential for the formation of neurogenic clusters (step 3) whereas mps1 is required for a later step during photoreceptor progenitor proliferation (step 4).

Comparison of gene expression profiles from regenerating zebrafish caudal fin, heart muscle and neural retina revealed a number of shared genes even though different cellular substrates are required for regeneration of these diverse structures: amputated fins regenerate from a blastema derived from dedifferentiated, mesenchymal stem cells (Poss et al., 2003); hearts regenerate by cardiomyocyte proliferation (Poss et al., 2002a); the neural retina regenerates from progenitors derived from non-neuronal, Müller glial cells. What each of these regenerating tissues have in common, however, is that the stem cells responsible for replacing the missing cells and repairing the damaged tissue arise from differentiated cells that respond to injury by dedifferentiation and proliferation. The fundamental nature of the proteins encoded by the two genes on which we performed functional analysis—Hsp60, a mitochondrial protein chaperone important in the cellular stress response, and Mps1, a kinase with a function in mitotic checkpoint regulation—
hints at a universal mechanism of epimorphic regeneration. These results, together with the comparative analysis of regeneration transcriptomes, suggest that the capacity of diverse cell types to respond to tissue injury by dedifferentiation and acquisition of stem cell properties may require the activation of conserved cellular and molecular mechanisms that regulate choice of cell fate and morphogenetic patterning during embryogenesis.
**Figure 2.1. Cone photoreceptor regeneration in adult zebrafish.** Flat-mounted zebrafish retinas immunolabeled with cone-specific zpr-1 (red). Retinas are oriented dorsal up, ventral down, nasal left, temporal right. (A, B) Intact retina. Asterisk, attached retinal pigment epithelium. (D, E) At 3 days after exposure to intense light, cones are missing in a horizontal band across the retina. (G, H) By 14 days cones have regenerated within the lesioned region (dashed lines). (C, F, I) are magnified images of the boxes in (B, E, H), respectively. Scale bars, 300 µm in (A, B, D, E, G, H); 20 µm in (C, F, I).
Figure 2.2. Isolation of GFP⁺ Müller glia. (A) A dissociated GFP⁺ Müller glial cell (green). Counterstained with DAPI (blue). (B, C) Flow cytometry scatter plots. (B) Dissociated cells from adult Tg(gfap:GFP)mi2002 zebrafish retinas were gated by forward and side scatters and (C) GFP⁺ Müller glia were isolated based on fluorescence in the FITC channel (R5). Our yield of dissociated retinal cells from adult zebrafish (5- to 6-month old) was ~2.5 x 10⁵ cells/retina of which ~9% were GFP⁺ Müller glia. With flow cytometry, we could recover ~2.1 x 10⁴ Müller glia/retina, an efficiency of ~84%. Scale bar, 10 µm.
Figure 2.3. Gene expression profiling of isolated Müller glia from intact and regenerating zebrafish retinas. 'Heat map' fold changes of gene expression at 8, 16, 24 and 36 hpl relative to unlesioned retina on a log2 scale. Hierarchical clustering analysis revealed three major groups: I, II, III.
Figure 2.4. *Gene ontology grouping of genes within each cluster.* Differentially expressed genes in the microarray analysis were subjected to hierarchical clustering, followed by functional and statistical analysis of the genes in each cluster. The number of genes in each biological process (columns) and the corresponding $P$-values (diamonds) are indicated. (A) Cluster I. (B) Cluster II. (C) Cluster III.
Figure 2.5. *hspd1* and *mps1* are up-regulated in injury-activated Müller glia during zebrafish photoreceptor regeneration. Expression patterns of *hspd1* (A-C) and *mps1* (D-F). (A, D) Fluorescent *in situ* hybridization of *hspd1* and *mps1* on retinal sections of *Tg(gfap:GFP)mi2002* zebrafish. Autofluorescence in cones (arrow), rods (solid arrowhead) and red blood cells (empty arrowhead). Asterisks, lesioned area (note the disrupted retinal pigment epithelium). (B, E) Expression fold changes of *hspd1* and *mps1* in isolated GFP+ cells detected by qRT-PCR (grey) and microarray (red). Error bars, standard error of the mean for three independent biological replicates. (C, F) Within the lesioned region at 48 hpl: *in situ* hybridization with *hspd1* and *mps1*, respectively (magenta), produces discrete fluorescent dots associated with GFP+ neurogenic Müller glia (green) and anti-PCNA (red). Arrows indicate triple-labeled cells; onl, outer nuclear layer; inl, inner nuclear layer. These are not microglia, which are confined to the onl in the lesioned region (Raymond et al., 2006). Scale bars, 50 µm in (A, D); 10 µm in (C, F).
Figure 2.6. qRT-PCR validation of expression patterns of selected genes. Expression fold changes of a subset of injury-responsive genes detected by qRT-PCR (grey) and microarray (red). (A-C) Genes from cluster I: ascl1a, pdgfa, six3b. (D, E) Genes from cluster II: dlg7, pcna. (F) Gene from cluster III: id3. Error bars, standard error of the mean for three independent biological replicates.
Figure 2.7. Cones fail to regenerate in nbl and ncp mutants at the restrictive temperature. Flat-mounted retinas at 7 dpl immunolabeled with zpr-1 (red). (A, C) Regenerated cones between dashed lines in WT. (B, D) Few or no cones are seen in the lesioned central area in nbl and ncp, respectively. The occasional zpr-1+ profile in the region of the lesion might represent a spared cone photoreceptor. Scale bars, 300 µm.
Figure 2.8. Cone regeneration defect in \textit{nbl} and \textit{ncp} mutants at the restrictive temperature. (A-E, G, H) Flat-mounted retinas at 7 dpl immunolabeled with zpr-1 (red). (A-E) One retina from each of five \textit{nbl} mutants. (F) Brightfield image of (E). (G, H) One retina from each of two \textit{ncp} mutants. Dashed lines, light-damaged areas have few or no zpr-1 labeled cones; we cannot determine from these preparations whether the rare, scattered cones sometimes observed within the light-damaged areas survived the lesion or have regenerated. Asterisk, attached retinal pigment epithelium. Scale bars, 300 µm.
Figure 2.9. Retinal regeneration defects of nbl and ncp. (A) Neurogenic clusters at 2 dpl in the inner nuclear layer (inl) immunolabeled with anti-PCNA (magenta) and weakly labeled with anti-Pax6 (green) in WT and nbl. Note that Pax6 is also expressed at high levels in amacrine cells at the inner boundary of the inl. (B) PNCA⁺ photoreceptor progenitors at 3 dpl in the outer nuclear layer (onl) of WT and ncp. (C) Number of PNCA⁺ cells in the inl or onl per 100 µm linear length retina at 2 or 3 dpl, respectively. Error bars, standard error of the mean for three individuals. *, P < 0.05; **, P < 0.0001. (D) Transmission electron micrographs of injury-activated Müller glia in WT and nbl. See text for description of temperature shift paradigm. Müller glia (M) are shown by the magenta wash. Mitochondria (arrows) in Müller glia of WT appear normal after 8 hours at 33°C, whereas in nbl mutants Müller glia contain swollen mitochondria. Scale bars, 10 µm in (A, B); 100 µm in (D).
Figure 2.10. Transmission electron micrographs of mitochondria in injury-activated Müller glia in WT siblings and nbl mutants after acute exposure to 33°C. (A-C) High magnification images of mitochondria in injury-activated Müller glia in retinas at 2 dpl after 8 hours of exposure to 33°C. See Fig. 2.9D for lower magnification images of these sections. (A) Glycogen granules (g) and mitochondria (arrows) in Müller glia in WT. (B, C) Swollen mitochondria with empty matrix in Müller glia of nbl. (D) Low magnification view of a neurogenic cluster (within the arrows) in the inner nuclear layer of nbl at 3 dpl after 4 hours of exposure to 33°C. Asterisks, Müller glia; p, progenitor. Note that the mitochondrial defect is present only in injury-activated Müller glia but not in the associated neuronal progenitors. (E, F) High magnification images of mitochondria from the Müller glial cells in (D). Scale bars, 0.5 µm in (A-C) and (E, F); 10 µm in (D).
Table 2.1. Transcriptionally-regulated genes common to regenerating retina, fin and/or heart. The genes listed are in the retinal microarray dataset reported here and are also found in one or both of the two comparison datasets (Lien et al., 2006; Schebesta et al., 2006). The highlighted genes correspond to the temperature-sensitive regeneration mutants. All genes except *nr1d2b* are up-regulated at one or more sample times. *, a closely related gene is found in one of the comparison datasets: *jag1a* in fin; *C4-1* and *C4-2* in heart.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>monopolar spindle 1</td>
<td>mps1(ttk)</td>
<td>cell cycle</td>
</tr>
<tr>
<td>decorin</td>
<td>den</td>
<td>cell signaling</td>
</tr>
<tr>
<td>IGF binding protein 3</td>
<td>igfbp3</td>
<td>cell signaling</td>
</tr>
<tr>
<td>jagged 2</td>
<td>jag2*</td>
<td>cell signaling</td>
</tr>
<tr>
<td>Kallmann syndr. 1b</td>
<td>kal1b</td>
<td>cell signaling</td>
</tr>
<tr>
<td>meteorin</td>
<td>metnrl</td>
<td>cell signaling</td>
</tr>
<tr>
<td>platelet-derived growth factor a</td>
<td>pdgfa</td>
<td>cell signaling</td>
</tr>
<tr>
<td>GLI-Kruppel family member GLI2a</td>
<td>gli2a</td>
<td>cell signaling</td>
</tr>
<tr>
<td>transforming growth factor β-induced</td>
<td>tgfbi</td>
<td>cell signaling</td>
</tr>
<tr>
<td>TGFβ-induced factor homeobox 1</td>
<td>tgif1</td>
<td>cell signaling</td>
</tr>
<tr>
<td>activating transcr. factor 3</td>
<td>atf3</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>clusterin</td>
<td>clu</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>LIM domain only 4</td>
<td>lmo4</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>matrix metalloproteinase 14 beta</td>
<td>mmp14b</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>similar to complement protein C7-1</td>
<td>LOC570832*</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>matrix metalloproteinase 9</td>
<td>mmp9</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>suppressor of cytokine signaling 3b</td>
<td>socs3b</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>tissue inhibitor of metalloproteinase 2</td>
<td>timp2</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>cathepsin C</td>
<td>ctsc</td>
<td>immunoregulation</td>
</tr>
<tr>
<td>cathepsin B, a</td>
<td>ctsba</td>
<td>proteolysis</td>
</tr>
<tr>
<td>karyopherin alpha 2</td>
<td>kpna2</td>
<td>protein import into nucleus</td>
</tr>
<tr>
<td>SRY-box containing gene 11b</td>
<td>sox11b</td>
<td>regulation of transcription</td>
</tr>
<tr>
<td>SRY-box containing gene 4a</td>
<td>sox4a</td>
<td>regulation of transcription</td>
</tr>
<tr>
<td>zic family member 2 (odd-paired-like) b</td>
<td>zic2b</td>
<td>regulation of transcription</td>
</tr>
<tr>
<td>nuclear receptor subfamily 1, group D, member 2b</td>
<td>nr1d2b</td>
<td>regulation of transcription</td>
</tr>
<tr>
<td>calreticulin, like 2</td>
<td>calrl2</td>
<td>stress response</td>
</tr>
<tr>
<td>heat shock 70kDa protein 5</td>
<td>hspa5</td>
<td>stress response</td>
</tr>
<tr>
<td>heat shock 60kD protein 1</td>
<td>hspd1</td>
<td>stress response</td>
</tr>
</tbody>
</table>
Figure 2.11. Model for Müller glia-based photoreceptor regeneration in adult zebrafish retina. Four steps in the regeneration of photoreceptors in the light-damaged retina. In nbl mutants, regeneration is blocked at step 3 and in ncp mutants at step 4. See text for further description.
References


Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.


CHAPTER 3

SIX3-RELATED GENES IN ZEBRAFISH PHOTORECEPTOR REGENERATION

Introduction

In chapter 2, I described a gene expression profiling analysis of isolated Müller glia from regenerating zebrafish retinas (Qin et al., 2009). This study provided a list of candidate genes whose function during photoreceptor regeneration would still need to be addressed. One of the genes that I found up-regulated in this study is *sine oculis homeobox homolog 3b* (*six3b*).

*six3b* is a member of the evolutionarily conserved *Six* gene family, which was identified by homology to the *Drosophila sine oculis (so, without eye)* gene. The *six* genes are transcription factors containing two functional domains, homeodomain and Six domain. The homeodomain specifies DNA binding activity and the Six domain, located just 5’ to the homeodomain, is thought to be involved in both DNA binding and protein-protein interaction (Singh and Tsonis, 2010).

The *Drosophila so* gene is expressed in the rostral end of the embryo during early development. It is required for proper pattern formation in the eye imaginal disc and for development of optic lobes, the brain regions where visual information is processed.
(Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Similar to its fly homolog, the vertebrate six3 gene is expressed exclusively in the most anterior part of the neural ectoderm during early embryonic development and has been shown to be essential for forebrain and eye development in all vertebrates studied. Disruption of six3 function results in truncation of forebrain with loss of eyes (Carl et al., 2002; Lagutin et al., 2003); overexpression of six3 leads to enlargement of forebrain and ectopic formation of eye tissues (Oliver et al., 1996; Kobayashi et al., 1998; Loosli et al., 1999). Recently, mechanisms that mediate six3 function in vertebrate forebrain and eye development have begun to be elucidated: first, six3 has been suggested to promote cell proliferation during anterior neural plate specification by regulating transcription of crucial cell cycle genes—Xenopus six3 regulates the transcription of cyclinD1 and p27Xic1, activating cyclinD1 and inhibiting p27Xic1 (Gestri et al., 2005); second, Six3 directly binds to Geminin, a DNA replication inhibitor, to release the pre-replication complex, demonstrating a non-transcriptional mechanism for six3-dependent cell proliferation during medaka fish eye development (Del Bene et al., 2004); third, Six3 activates expression of Pax6, the “master regulator of eye development”, in mammalian lens formation (Liu et al., 2006).

Three six3-related genes are present in the zebrafish genome: six3a, six3b, and six7. The homeodomain and Six domain of these six3 homologs are highly conserved. These genes exhibit similar expression patterns during early embryogenesis that correlate with the initial optic primordia (Seo et al., 1998a; Seo et al., 1998b). Loss-of-function of any one of these genes does not cause any obvious developmental defect probably due to the overlapping expression territories and possible functional redundancy between six3-related genes. Morpholino-mediated knockdown of both six3a and six3b, however,
showed impaired cell proliferation and thus reduced size of forebrain in zebrafish embryos (Ando et al., 2005), and knocking down \textit{six7} on a \textit{six3b} null background resulted in brain asymmetry defects (Inbal et al., 2007).

Although the function of \textit{six3} during early embryonic development has been investigated extensively, its role in adult tissue regeneration is not known. In my microarray analysis of isolated Müller glia from regenerating zebrafish retinas, only one of the three \textit{six3}-related genes, \textit{six3b}, was up-regulated in the injury-activated Müller cells. Therefore, functional validation of \textit{six3b} in zebrafish photoreceptor regeneration will not only shed light on the molecular genetic pathways that initiate a neurogenic program in zebrafish Müller glia following retinal injury, but also elucidate the distinct functional roles of the various zebrafish \textit{six3} homologs when the fish are challenged to regenerate retinal neurons.

\section*{Methods}

\textbf{Zebrafish}

Zebrafish lines \textit{Tg(gfap:GFP)mi2002} (Bernados and Raymond, 2006) and \textit{six3b\textsuperscript{vu87/+}} (kindly provided by L. Solnica-Krezel) (Inbal et al., 2007) were maintained according to standard rearing protocols. The Committee on Use and Care of Animals in Research at the University of Michigan approved all procedures using animals. Adult fish (3-month to 1-year old) were used for all experiments. To generate \textit{six3b\textsuperscript{vu87/vu87}} mutants, heterozygous carriers were crossed, embryos raised to adulthood, and homozygous mutants identified through genotyping (Inbal et al., 2007). For the light lesion, zebrafish
were exposed for 20-30 minutes to a fiber optic light source of ultra-high-intensity light (~120,000 lux) as described (Bernados et al., 2007).

qRT-PCR

To prepare RNA samples used for qRT-PCR, retinas were dissected from dark-adapted \textit{Tg(gfap:GFP)mi2002} zebrafish at 8, 16, 24 and 36 hpl and non-light-treated controls (0 hpl). Tissues were minced with a razor blade and dissociated by enzymatic digestion with 16 U/ml papain (Worthington), 0.2 U/ml dispase (Worthington) (Nelson et al., 2003) in phosphate buffered saline (PBS) at pH 6.5 for 30 minutes at 28°C and triturated. Cells were pelleted at 6000 rpm for 3 minutes, resuspended in 1 mg/ml papain inhibitor (Worthington), 100 μg/ml DNase I (Sigma-Aldrich) with 2 mM MgCl$_2$ in PBS at pH 7.4 for 10 minutes at room temperature and then put on ice. GFP$^+$ Müller glia were isolated on a Vantage SE cell sorter (BD Biosciences). Gating was based on cell size and fluorescence intensity, with parameters set by reference to a control sample of dissociated retinal cells from wildtype zebrafish. At each sample time, retinas from three or four fish were pooled for cell dissociation and cell sorting. Total RNA was extracted and purified from 1-2 x 10$^5$ freshly sorted GFP$^+$ cells using the RNAqueous-4PCR kit (Ambion). The interval between retinal isolation and cell lysis was ~2.5 hours. The quality and quantity of RNA were assessed with a 2100 BioAnalyzer (Agilent Technologies). Three biological replicates were prepared for each time interval.

For qRT-PCR, total RNA was reverse transcribed and linear amplified with the Ovation Biotin Labeling System (NuGEN). All real-time PCR reactions were carried out in duplicate with iQ SYBR Green Supermix (BioRad) on a iCycler iQ real-time PCR
detection system (BioRad). The standard curve method was used to determine levels of expression of the genes of interest relative to \textit{gpia} \textit{(glucose phosphate isomerase a)} and relative fold changes in gene expression after lesion. Sequences of the gene-specific primer pairs used are as follows: \textit{gpia}: F 5’-TCCAAGGAAACAAGCCAAGC-3’, R 5’-TTCCACATCACACCCTGCAC-3’; \textit{six3a}: F 5’-ACTGGCTCAAGCCACTGGAC-3’, R 5’-GCATGCCATTCTGCCCTATT-3’; \textit{six3b}: F 5’-CCAATCCGAGCAAGAAAAGG-3’, R 5’-CAGACTGCTTTTGCCCGAGTC-3’.

\textit{in situ} hybridization

Eyes from adult \textit{Tg(gfap:GFP)mi2002} fish were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and prepared for cryosectioning. For \textit{in situ} hybridization on cryosections, digoxigenin (DIG)-labeled cRNA probes for \textit{six3a} (plasmid kindly provided by A. Fjose) (Seo et al., 1998a), \textit{six3b} (plasmid kindly provided by A. Fjose) (Seo et al., 1998a), and \textit{six7} (IMAGE clone ID: 4200307) were prepared and hybridized at 5 μg/ml as described (Raymond et al., 2006).

Immunohistochemistry

Immunohistochemistry on cryosections was performed as described previously (Bernardos et al., 2007). Primary antibodies used included: anti-GFP (rabbit, 1:500; Invitrogen); anti-PCNA (mouse, 1:1000; Sigma-Aldrich); zpr-1 (mouse, 1:400; Zebrafish International Resource Center, ZIRC). Secondary antibodies included: preabsorbed anti-mouse or anti-rabbit cyanine 3 (Cy3), Cy5, and FITC (1:100; Jackson ImmunoResearch).
Imaging

Fluorescent microscopy was performed with an AxioImager epifluorescent compound microscope equipped with an AxioCam mRM digital camera and an ApoTome (Carl Zeiss Microimaging) to generate optical sections. Images were processed with Adobe PhotoShop (Adobe Systems) as described previously (Bernardos et al., 2007). All adjustments were applied to the entire image.

Results

**six3b is up-regulated immediately in injury-activated Müller glia after intense light treatment**

Although probe sets for all three zebrafish *six3* homologs are present on the microarray chip, only *six3b* showed a significant expression change in my gene profiling study of isolated Müller glia from regenerating zebrafish retinas (Qin et al., 2009). To confirm expression data from the microarray analysis, I did qRT-PCR and *in situ* hybridization to define the expression timing and patterns of all three *six3*-related genes during the early stages of photoreceptor regeneration.

qRT-PCR was performed using RNA samples of isolated Müller glia collected at the same time points as in the microarray study and data from both analyses were plotted on the same chart (Fig. 3.1). For *six3a*, microarray data suggested that it was down-regulated during the first 36 hours of photoreceptor regeneration, although it was not identified as a gene whose expression changed significantly in the analysis as the largest fold change (FC) observed (36 hpl, log₂FC = -0.98) was a bit smaller than the FC cutoff.
of -2 (log₂FC = -1). Consistent with the microarray data, qRT-PCR results of *six3a* expression at the same time points showed that its expression was decreased in the Müller cells. The largest fold change was at 16 hpl (log₂FC = -1.64) (Fig. 3.1A). For *six3b*, both microarray and qRT-PCR results suggested that it was up-regulated in the Müller cells as early as 8 hpl, and this increased expression persisted until 36 hpl. The largest fold change was seen at 24 hpl (log₂FC = 2.86 from microarray; log₂FC = 3.46 from qRT-PCR) (Fig. 3.1B). In spite of trying many different primer sets, I could not find a good pair of primers for *six7*, which would not give non-specific amplification in qRT-PCR (data not shown). This is probably because of the extremely low level of *six7* expression in Müller glia and their mitotic progeny, as corroborated by the *in situ* hybridization data described below.

For *in situ* hybridization, I first confirmed my antisense cRNA probes for *six3a*, *six3b*, and *six7* by examining their expression patterns during early embryonic development with whole-mount zebrafish embryos and comparing results with data in the Zebrafish Information Network (ZFIN) database (data not shown). To define expression patterns of all three *six3*-related genes in the adult zebrafish retina, retinal sections from *Tg(gfap:GFP)mi2002* fish were used to localize expression in Müller cells and their mitotic progeny after lesion. In the normal adult zebrafish retina, *six3a* transcripts were present in the ganglion cells and in cells of the inner nuclear layer. Higher levels of *six3a* expression were observed in the inner part of the inner nuclear layer compared with the outer part (Fig. 3.2A-C). Since Müller glia nuclei are located in the inner part of the inner nuclear layer, I took high magnification images to find out if the cells expressing higher levels of *six3a* were Müller cells. Although cells in the inner nuclear layer are tightly
packed together, the peri-nuclear in situ signals of six3a appeared to mostly associate with round nuclei (likely amacrine cell nuclei), but not with the polygonal nuclei of GFP+ Müller glia (Fig. 3.3). At 24 and 48 hpl, the expression level and pattern of six3a did not seem to change much within the lesioned area (Fig. 3.2D-I).

Like six3a, mRNA transcripts of six3b were detected in the ganglion cells and cells in the inner nuclear layer in the unlesioned retina. The difference is that higher levels of six3b expression were present in the outer part of the inner nuclear layer instead of the inner part (Fig. 3.4A-C). Cells expressing higher levels of six3b were presumably bipolar cells based on their localization and nuclear morphology. Co-localization analysis with the gfap:GFP transgene showed that most six3b in situ signals in the inner part of the inner nuclear layer were not associated with GFP+ Müller cells (Fig. 3.5). At 24 hpl, expression of six3b was still confined within the ganglion cell layer and inner nuclear layer. It was difficult, however, to appreciate the up-regulation of six3b in injury-activated Müller glia within the lesioned region because of its strong expression in other retinal cell types (Fig. 3.4D-F). This could probably explain why six3b had not been identified in previously published microarray studies of retinal regeneration using RNA samples from whole retinas (Cameron et al., 2005; Kassen et al., 2007). At 48 hpl, up-regulation of six3b within the lesioned area became more prominent as many six3b in situ signals were associated with radial-oriented groups of cells spanning the entire thickness of the inner nuclear layer that are reminiscent of Müller glia-derived neurogenic clusters (Fig. 3.4G-I). Indeed, a high magnification image taken at the boundary between the lesioned and unlesioned regions showed that six3b was only up-regulated in injury-activated, proliferating Müller glia and their progeny (labeled with faint GFP
fluorescence as it had been diluted after mitosis), but not in inactive Müller glia that did not re-enter the cell cycle and still retained high levels of GFP (Fig. 3.6).

In contrast, expression of *six7* was not observed anywhere in the normal adult zebrafish retina except in the photoreceptors. Specifically, *six7* transcripts were present in the cone photoreceptors and not in the rod photoreceptors (Fig. 3.7A-C). At 24 and 48 hpl, *in situ* signals of *six7* disappeared within the lesioned area as cone photoreceptors in this region had been damaged by the ultra-intense light treatment (Fig. 3.7D-I). This cellular specificity of *six7* expression suggested that it might be a marker for differentiated cone photoreceptors.

**Photoreceptor regeneration is not affected in *six3b* null mutants**

Expression data of all three zebrafish *six3*-related genes during the initial steps of photoreceptor regeneration showed that only *six3b* is up-regulated in the injury-activated stem cell population that proliferate and replace lost photoreceptors. This led me to hypothesize that *six3b* might be specifically required for zebrafish photoreceptor regeneration. To test this hypothesis, I used a mutant zebrafish line carrying a nonsense mutation in *six3b*, *six3b*\textsuperscript{vu87}. This mutation introduces a premature stop codon that results in a truncated protein lacking the entire homeodomain and part of the Six domain. *six3b*\textsuperscript{vu87} was identified through Targeting Induced Local Lesions IN Genomes (TILLING) (Draper et al., 2004; Wienholds and Plasterk, 2004), which combines a standard mutagenesis with a sensitive, high-throughput DNA screening technique to identify point mutations in a target gene. Although misexpression studies in early zebrafish embryos suggested that *six3b*\textsuperscript{vu87} is a null allele of *six3b*, there seems no
developmental defect in \( \text{six3b}^{vu87/vu87} \) homozygous mutants, likely due to functional redundancy between zebrafish \( \text{six3} \) homologs.

These adult viable \( \text{six3b}^{vu87/vu87} \) mutants provided a way to study the function of \( \text{six3b} \) during regeneration. To test if \( \text{six3b} \) is essential for photoreceptor regeneration, adult \( \text{six3b}^{vu87/vu87} \) mutants and wildtype siblings were light-lesioned and eyes collected at 2 and 14 days after lesion to examine the proliferative response of Müller glia and extent of regeneration, respectively. To my surprise, in both analyses, \( \text{six3b}^{vu87/vu87} \) mutants were comparable to their wildtype siblings. Within the lesioned area, Müller glia of \( \text{six3b}^{vu87/vu87} \) mutants proliferated normally at 2 dpl (Fig. 3.8A, B, see Table 3.1 for cell counts) and cone photoreceptors regenerated completely at 14 dpl (\( n = 4; \) Fig. 3.8C, D).

**Discussion**

The qRT-PCR and \textit{in situ} hybridization results presented here confirmed that expression of \( \text{six3b} \), a zebrafish homolog of the homeobox transcription factor \( \text{six3} \), is induced in injury-activated, proliferating Müller glia in the adult zebrafish retinas treated with our ultra-high-intensity light lesion. Along with the known function of \( \text{six3} \) during vertebrate forebrain and eye development (Oliver et al., 1996; Kobayashi et al., 1998; Loosli et al., 1999; Carl et al., 2002; Lagutin et al., 2003), these results suggested that \( \text{six3b} \) might play a central role in zebrafish Müller glia reprogramming and regeneration of retinal neurons. Functional assays using \( \text{six3b} \) null mutants, however, did not reveal any regeneration defect in these fish. One explanation could be other zebrafish \( \text{six3} \)-related genes, \( \text{six3a} \) and/or \( \text{six7} \), somehow compensate \( \text{six3b} \) loss-of-function in these
mutants, although in wildtype fish neither \textit{six3a} nor \textit{six7} is up-regulated in injury-activated stem cell population after lesion.

To test this hypothesis and to reveal any functional role of \textit{six3} during retinal regeneration, a morpholino injection and electroporation technique could be used to knock down expression of \textit{six3} homologs in the adult zebrafish retina. Simply, lissamine-tagged morpholino is injected into the vitreous and fish eyes are electroporated in a way so that the slightly positive-charged morpholino is directed to the dorsal retina. This technique has been successfully used to study the function of several genes during zebrafish photoreceptor regeneration (Thummel et al., 2008; Craig et al., 2010; Thummel et al., 2010). In these analyses, morpholino injection and electroporation resulted in minimum damage in the control morpholino-treated retinas, but blocked photoreceptor regeneration in the retinas treated with morpholino targeting the gene of interest. Morpholino sequences targeting both \textit{six3a} and \textit{six3b} (Ando et al., 2005; Sanek et al., 2009) or specifically \textit{six7} (Inbal et al., 2007) have been reported in previous studies. If \textit{six3a} or \textit{six7} functions redundantly with \textit{six3b} in the adult zebrafish retina, blocking expression of both \textit{six3a} and \textit{six3b} in the wildtype fish retina or knocking down \textit{six7} in the \textit{six3b} null fish retina will cause some regeneration defects. Further analyses using this morpholino injection and electroporation technique are needed to elucidate the function of \textit{six3}-related genes in zebrafish photoreceptor regeneration.

During early embryogenesis, all three zebrafish \textit{six3} homologs are expressed in progenitor cells that form the initial eye primordia (Seo et al., 1998a; Seo et al., 1998b). In the adult zebrafish retina, expression of each of these \textit{six3}-related genes is maintained in a different subset of differentiated neurons. This is not the only case where expression
of a progenitor marker continues in a group of differentiated cells later during zebrafish retinal development. Similar changes in expression pattern have been observed for other homeobox transcription factors. \textit{pax6}, a marker for multipotent retinal progenitors, is expressed in ganglion cells and amacrine cells in the differentiated zebrafish retina (Qin et al., 2009). Expression of \textit{crx} (cone-rod homeobox), a marker for late-stage neuronal progenitors in embryonic zebrafish retina, remains in differentiated photoreceptors and neurons in the outer part of the inner nuclear layer (presumably bipolar cells) in the adult retina (Shen and Raymond, 2004; Bernardos et al., 2007). The role of these homeobox transcription factors in differentiated retinal neurons is poorly understood. Future studies aiming at addressing this question might reveal a novel aspect of their function in maintaining neuronal properties in the adult zebrafish retina.
Figure 3.1. qRT-PCR validation of expression patterns of *six3a* and *six3b* during early stages of zebrafish photoreceptor regeneration. Expression fold changes of *six3a* (A) and *six3b* (B) in purified GFP$^+$ Müller cells detected by qRT-PCR (grey) and microarray (red). Error bars, standard error of the mean for three independent biological replicates.
**Figure 3.2.** Fluorescent in situ hybridization of *six3a* on retinal sections of *Tg(gfap:GFP)mi2002* zebrafish. (A-C) In the unlesioned retina, *six3a* transcripts are present in the ganglion cells and in cells of the inner nuclear layer. Higher levels of *six3a* expression are seen in the inner part of the inner nuclear layer compared with the outer part. At 24 hpl (D-F) and 48 hpl (G-I), the expression level and pattern of *six3a* do not seem to change much within the lesioned area. Autofluorescence in photoreceptor outer segments (arrow) and red blood cells (arrowhead). Asterisks, lesioned area (note the disrupted retinal pigment epithelium). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.
Figure 3.3. Expression of *six3a* in the normal adult zebrafish retina. The peri-nuclear *in situ* signals of *six3a* (magenta) appear to mostly associate with round nuclei, but not with the polygonal nuclei of GFP+ Müller glia (green). Arrows, *six3a*-expressing cells (note they are not GFP+). onl, outer nuclear layer; inl, inner nuclear layer.
**Figure 3.4.** Fluorescent *in situ* hybridization of *six3b* on retinal sections of Tg(gfap:GFP)mi2002 zebrafish. (A-C) mRNA transcripts of *six3b* are detected in the ganglion cells and cells in the inner nuclear layer in the unlesioned retina. Higher levels of *six3b* expression are seen in the outer part of the inner nuclear layer compared with the inner part. (D-F) At 24 hpl, expression of *six3b* is still confined within the ganglion cell layer and inner nuclear layer. It is difficult, however, to appreciate the up-regulation of *six3b* in injury-activated Müller glia within the lesioned region because of its strong expression in other retinal cell types. (G-I) At 48 hpl, up-regulation of *six3b* within the lesioned area becomes more prominent as many *six3b in situ* signals are associated with radial-oriented groups of cells spanning the entire thickness of the inner nuclear layer that are reminiscent of Müller glia-derived neurogenic clusters. Autofluorescence in photoreceptor outer segments (arrow) and red blood cells (arrowhead). Asterisks, lesioned area (note the disrupted retinal pigment epithelium). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.
Figure 3.5. **Expression of six3b in the normal adult zebrafish retina.** The peri-nuclear in situ signals of six3b (magenta) appear to mostly associate with round nuclei, but not with the polygonal nuclei of GFP+ Müller glia (green). Arrows, six3b-expressing cells (note they are not GFP+). onl, outer nuclear layer; inl, inner nuclear layer.
Figure 3.6. Expression of six3b at 48 hpl. At the boundary between the lesioned and unlesioned regions: expression of six3b (magenta), GFP⁺ Müller glia (green), and anti-PCNA (red). Note that six3b is only up-regulated in injury-activated, proliferating Müller glia and their progeny (arrows; PCNA⁺, faintly GFP⁺), but not in inactive Müller glia that do not re-enter the cell cycle and still retain high levels of GFP (arrowheads; PCNA⁻, strongly GFP⁺). onl, outer nuclear layer; inl, inner nuclear layer.
Figure 3.7. Fluorescent in situ hybridization of six7 on retinal sections of Tg(gfap:GFP)mi2002 zebrafish. (A-C) Expression of six7 is not detected anywhere in the unlesioned retina except in the photoreceptors. Specifically, six7 transcripts are present in the cone photoreceptors and not in the rod photoreceptors. At 24 hpl (D-F) and 48 hpl (G-I), in situ signals of six7 disappear within the lesioned area as cone photoreceptors in this region were damaged by the ultra-intense light treatment. Autofluorescence in red blood cells (arrowhead). Asterisks, lesioned area (note the disrupted retinal pigment epithelium). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.
Figure 3.8. Photoreceptor regeneration is not affected in the \textit{six3b}^{vu87/vu87} mutants. Within the lesioned area, Müller glia of \textit{six3b}^{vu87/vu87} mutants proliferate normally at 2 dpl (A, B) and cone photoreceptors regenerate completely at 14 dpl (C, D). PCNA, proliferating cell nuclear antigen; zpr-1, a specific marker for red-green double cones.
<table>
<thead>
<tr>
<th>Section</th>
<th>wt-1</th>
<th>wt-2</th>
<th>het-1</th>
<th>het-2</th>
<th>mut-1</th>
<th>mut-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>28</td>
<td>32</td>
<td>19</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>25</td>
<td>31</td>
<td>23</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>34</td>
<td>21</td>
<td>17</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>24</td>
<td>29</td>
<td>14</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>20</td>
<td>24</td>
<td>21</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>36</td>
<td>24</td>
<td>12</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>33</td>
<td>21</td>
<td>14</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>30</td>
<td>34</td>
<td>19</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>22</td>
<td>24</td>
<td>20</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>23</td>
<td>11</td>
<td>17</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>23</td>
<td>18</td>
<td>23</td>
<td>20</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>25</td>
<td>31</td>
<td>16</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td><strong>20.3</strong></td>
<td><strong>26.5</strong></td>
<td><strong>25.4</strong></td>
<td><strong>17.8</strong></td>
<td><strong>21.6</strong></td>
<td><strong>23.3</strong></td>
</tr>
</tbody>
</table>

Table 3.1. The number of PCNA⁺ cells per 100 μm on retinal sections of wildtype (wt), heterozygous (het), and \textit{six3b} \textsuperscript{vu87/vu87} mutant (mut) fish at 2 dpl. Two fish were counted for each genotype and eleven or twelve sections for each fish.
References


Bernados RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.


CHAPTER 4

FGF SIGNALING IN ZEBRAFISH PHOTORECEPTOR REGENERATION AND HOMEOSTASIS

Introduction

Rod and cone photoreceptors in adult zebrafish regenerate after damage, but little is known about the signaling pathways that mediate the regenerative responses in the retina. One candidate is the fibroblast growth factor (Fgf) signaling pathway.

Fgfs are a large family of secreted small polypeptides. Their binding to specific receptor tyrosine kinases in the cell membrane, Fgf receptors (Fgfrs), induces dimerization and activation of the receptors. Activation of downstream signaling pathways leads to cell proliferation, differentiation, migration, or survival depending on the cellular contexts (Turner and Grose, 2010). Fgf signaling has been implicated in many biological processes such as induction and patterning events during embryonic development (Crossley et al., 1996; Vogel et al., 1996; Zhu et al., 1996; Ohuchi et al., 1997; Martin, 1998; Reifers et al., 1998; Peters and Balling, 1999), tissue maintenance (Stone et al., 1999), wound healing (Ortega et al., 1998), and cancer pathogenesis (Turner and Grose, 2010).
Evidence for a role of Fgf signaling in regeneration first came from studies of amphibian limb regeneration: Components of Fgf signaling are present in the regenerating newt limbs (Boilly et al., 1991; Poulin et al., 1993; Zenjari et al., 1997) and \(fgf8\) expression is associated with successful hindlimb regeneration in *Xenopus* tadpoles (Christen and Slack, 1997); Functional inhibition of Fgf signaling by applying specific Fgfr inhibitors to *Xenopus* tadpoles blocks normal outgrowth during premetamorphic hindlimb regeneration (D’Jamoos et al., 1998), whereas gain-of-function analysis showed that regeneration of denervated axolotl limbs can be rescued by implanting Fgf2 beads into regenerates (Mullen et al., 1996).

Recently, studies of zebrafish appendage regeneration have provided more information on Fgfs’ function during specific stages of regeneration. In the regenerating zebrafish fin, expression of \(fgf20a\) can be detected at the epithelial-mesenchymal boundary as early as 1 hour post amputation and is maintained in the blastemal cells during blastema formation and regenerative outgrowth (Whitehead et al., 2005). Another Fgf ligand, \(fgf24\) (previously called \(wfgf\)), is expressed in the wound epidermis during regenerative outgrowth. The expression pattern of Fgfr subtype \(fgfr1\) is similar to that of \(fgf20a\) during early blastema formation, although \(fgfr1\) is also expressed in the basal epidermal layer during regenerative outgrowth. Functional characterization by treating fish with a specific Fgfr inhibitor (SU5402) revealed that Fgf signaling is required for both blastema formation and maintenance during fin regeneration (Poss et al., 2000). In addition, when Fgf signaling is blocked by expression of a dominant-negative form of \(fgfr1\) under the control of a heat shock promoter in a stable transgenic line, \(Tg(hsp70:dn-fgfr1)\), regeneration of amputated fins fails. Further analysis using these transgenic fish
suggested that Fgf signaling controls the level of blastemal proliferation and rate of regenerative outgrowth in a position-dependent manner, with both greater in the proximally amputated regenerates (Lee et al., 2005). Different from the above studies, in which the entire Fgf signaling was affected, mutant zebrafish carrying a missense mutation in one of the Fgf ligands, \textit{fgf20a}, have been identified in a forward genetic screen for temperature-sensitive mutants of fin regeneration. The associated mutation affects a highly conserved tyrosine residue that is thought to be involved in receptor binding. As a result, these mutant fish have defects in initiation of fin regeneration: they form abnormal wound epidermis and lack blastema (Whitehead et al., 2005).

Function of Fgf signaling during zebrafish heart regeneration has also been studied by examining expression patterns of Fgf components in regenerating hearts and by genetic manipulations of Fgf signaling using the transgenic line \textit{Tg(hsp70:dn-fgfr1)}. It was suggested that Fgf signaling in the Fgfr2 and Fgfr4-expressing epicardial tissue, most likely activated by Fgf17b released from the underlying myocardium, is necessary for the epithelial-to-mesenchymal transition (EMT) of epicardial cells. Inhibition of this signaling pathway blocks vasculature formation in the newly generated myocardium and completion of cardiac regeneration (Lepilina et al., 2006).

Although function of Fgf signaling has been suggested in several different regeneration models, it has not yet been studied during zebrafish photoreceptor regeneration. In this chapter, I investigate whether Fgf signaling is required for photoreceptor regeneration in adult zebrafish by using our ultra-intense light lesion paradigm and the \textit{Tg(hsp70:dn-fgfr1)} animals in which Fgf signaling can be experimentally manipulated upon heat-shock induction.
Methods

Zebrafish

Zebrafish lines Tg(hsp70:dn-fgfr1) (kindly provided by K. Poss) (Lee et al., 2005) and Tg(gfap:GFP)mi2002 (Bernardos and Raymond, 2006) were maintained according to standard rearing protocols. The Committee on Use and Care of Animals in Research at the University of Michigan approved all procedures using animals. Photoreceptors were destroyed in adult Tg(hsp70:dn-fgfr1) fish and wild type siblings by a 30-minute exposure to intense light (>100,000 lux), as described previously (Bernardos et al., 2007). To detect effects of Fgf signaling on photoreceptor regeneration, fish were maintained in an automated heating unit after lesion and exposed daily to heat shock (38°C for 1 hour) beginning the day after lesion (Lee et al., 2005).

BrdU injection

To label the proliferating progenitors, Tg(hsp70:dn-fgfr1) fish and wild type siblings were injected intraperitoneally with a 2.5 mg/ml solution of 5-bromo-2’-deoxyuridine (BrdU) in saline at 3 and 4 days post lesion (dpl).

Immunohistochemistry

At 14 dpl, Tg(hsp70:dn-fgfr1) fish and wild type siblings were euthanized and the eyes fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and prepared for cryosectioning and immunohistochemistry (Bernardos et al., 2007) with an antibody
against BrdU (rat anti-BrdU, 1:50; Accurate Chemical and Scientific Corporation), a cone specific monoclonal antibody for red-green double cones, zpr-1 (1:400; Zebrafish International Resource Center, ZIRC), Cy5 conjugated anti-rat IgG, and Cy3 conjugated anti-mouse IgG (1:100; Jackson ImmunoResearch). All sections were also stained with the nuclear marker, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich).

in situ hybridization

Eyes from adult Tg(gfap:GFP)mi2002 fish were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and prepared for cryosectioning. For in situ hybridization on cryosections, digoxigenin (DIG)-labeled cRNA probe for fgfr1 (plasmid kindly provided by K. Poss) was prepared and hybridized at 5 μg/ml as described (Raymond et al., 2006).

Imaging

Fluorescent microscopy was performed with an AxioImager epifluorescent compound microscope equipped with an AxioCam mRM digital camera (Carl Zeiss Microimaging). Images were processed with Adobe PhotoShop (Adobe Systems) as described previously (Bernardos et al., 2007). All adjustments were applied to the entire image.

Quantitative analysis

To quantify the effect of Fgf signaling on zebrafish photoreceptor regeneration, retinal cryosections through the dorsoventral axis in the plane of the optic disc from the
eyes of Tg(hsp70:dn-fgfr1) fish and wild type siblings were used for quantitative analysis and 10 samples (linear length = 100 µm) were analyzed within the lesioned region of each eye. This analysis was done ‘blind’: one person collected and assigned numbers to the eyes and another person did the cell counts and measurements without knowing the identity of the samples. Regions selected for analysis met the following criteria: the ganglion cell layer was a single row of cells and the inner nuclear layer had BrdU-retaining cells indicative of a lesion (Raymond et al., 2006). All zpr-1+ cells in these samples were counted and the thicknesses of the rod nuclear layer in the middle of these samples were measured. Unpaired Student’s t-test was used for statistical analysis.

**Results**

**hsp70:dn-fgfr1 transgene is expressed in the retina upon heat-shock induction**

The transgenic zebrafish line Tg(hsp70:dn-fgfr1) provides an excellent genetic tool to manipulate the level of Fgf signaling during regeneration. These fish harbor a transgene of a dominant negative fgfr1 fused with gfp under the control of a zebrafish heat shock promoter hsp70. Specifically, the tyrosine kinase domain of fgfr1 is replaced by the coding sequence of gfp. The resulting fusion protein is believed to form heterodimers with endogenous Fgfrs upon ligand binding and thus to block the downstream signaling of all Fgfr subtypes (Lee et al., 2005). To test if Fgf signaling is required for photoreceptor regeneration, adult Tg(hsp70:dn-fgfr1) fish and wildtype siblings were treated with the ultra-high-intensity light and subjected to daily heat shock
starting from the next day until 14 days later when eyes were collected and photoreceptor regeneration was assessed (Fig. 4.1).

To confirm the $dn$-$fgfr1$ transgene is heat-inducible in the adult retina, I examined GFP expression in the transgenic fish after 2 days of daily heat shock. Strong GFP fluorescence was observed in all retinal cells, especially in the photoreceptor outer segments (Fig. 4.2). This is probably because the Dn-fgfr1-GFP fusion protein is targeted to the cell membrane as the endogenous Fgfrs are and the outer segment is a membrane-stacking structure.

**Cone and rod photoreceptor regeneration is differentially affected by Fgf signaling attenuation**

In response to the light treatment, Müller glia within the lesioned region re-enter the cell cycle, proliferate and give rise to radial clusters of neuronal progenitors. These progenitors continue to proliferate and migrate into the layer of damaged/dying photoreceptors (outer nuclear layer) where they differentiate to replace the missing cone and rod photoreceptors (Yurco and Cameron, 2005; Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Kassen et al., 2007). To label the proliferating progenitors and to identify the lesioned region at 14 dpl when regeneration is complete, light-lesioned, heat-shocked $Tg(hsp70:dn-fgfr1)$ fish and wildtype siblings were injected with BrdU at 3 and 4 days after lesion (Fig. 4.1).

Surprisingly, I found regeneration of cone and rod photoreceptors was differentially affected by Fgf signaling attenuation at 14 days after lesion (Fig. 4.3). Within the lesioned region, the number of red-green double cones in 100 µm of linear
length retina (cone density) in the $Tg(hsp70:dn-fgfr1)$ fish was 21.8 ± 0.5, not significantly different from that in the wildtype siblings, 22.1 ± 0.5 (n = 8, $P = 0.68$). However, the thickness of the rod nuclear layer (as an indicator of the number of rod photoreceptors) in the $Tg(hsp70:dn-fgfr1)$ fish was 10.9 ± 0.3 µm, and was about 30% less than that in the wildtype siblings, 15.3 ± 0.3 µm (n = 8, $P << 0.001$) (Fig. 4.4).

**Discussion**

The results presented here suggested that regeneration of rod and cone photoreceptors in adult zebrafish is regulated by different signaling pathways. Blocking Fgf signaling interfered with regeneration of rod photoreceptors but had no effect on regeneration of zpr-1$^+$ double cones. The cellular mechanism of the rod regeneration defect was not known. One hypothesis was that inhibition of Fgf signaling affects proliferation of rod precursors in the outer nuclear layer that give rise to differentiated rods; another hypothesis was that although new rods are made after lesion, Fgf signaling is required for the survival of differentiated rod photoreceptors.

Further characterization of this rod phenotype in collaboration with Prof. Kenneth Poss’s laboratory at Duke University and Prof. David Hyde’s laboratory at University of Notre Dame provided evidence in supportive of a trophic effect of Fgf signaling on zebrafish rod photoreceptors: when $Tg(hsp70:dn-fgfr1)$ fish and wildtype siblings were heat-shocked daily without light lesion, as early as 10 days after heat shock started, degeneration of rod outer segments and apoptosis of rod photoreceptors were observed in the transgenics, but not in the wildtypes. As a result, proliferation of rod precursors in the
outer nuclear layer of the Tg(hsp70:dn-fgfr1) retinas was up-regulated. In contrast, cone photoreceptors in both the transgenic and wildtype retinas were largely unaffected (unpublished results, personal communication).

Fgf signaling has been reported to play a key role in maintaining mammalian photoreceptor homeostasis (Stone et al., 1999). Therefore, these data may suggest a conserved neuroprotective function of Fgf signaling in the zebrafish retina. What remains unclear is how specificity is achieved in zebrafish. Out of the four Fgfr subtypes, only fgfr1 is expressed in the adult zebrafish retina (unpublished results, personal communication), and it is expressed in both rod and cone photoreceptors (Fig. 4.5). How can rod and cone photoreceptors respond differently to the presence of an Fgf ligand? Zebrafish have more than twenty Fgfs, which is the ligand(s) that mediates the function in rod photoreceptor survival and homeostasis? Future studies aimed at manipulating individual components of Fgf signaling in a cell-specific manner will be needed to answer these questions.
Figure 4.1. Experimental design. *Tg(hsp70:dn-fgfr1)* fish and wildtype siblings were subjected to lesion, heat shock, and BrdU injections according to the above timeline.
Figure 4.2. Retinal section from adult Tg(hsp70:dn-fgfr1) fish. Dn-fgfr1-GFP fusion protein is expressed in the retina after heat shock, especially in the photoreceptors. pr, photoreceptor processes; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer.
Figure 4.3. Inhibition of Fgf signaling reduces rod but not cone regeneration.
*Tg(hsp70:dn-fgfr1)* fish (A, C) and wild type siblings (B, D) were heat-shocked daily for 14 days after intense light treatment. zpr-1, a specific marker for red-green double cones. Arrows, regenerated cones (BrdU$^+$/zpr-1$^+$). See Fig. 4.4 for quantification.
Figure 4.4. Quantification of photoreceptor regeneration at 14 dpl. Error bars, standard error of the mean; *, p<<0.001, unpaired Student’s t-test.
Figure 4.5. Expression of *fgfr1* in the intact, normal adult zebrafish retina. *fgfr1* transcripts are detected in the outer nuclear layer (both rod and cone photoreceptors) and inner nuclear layer (inl). (A) *fgfr1* in situ, (B) overlay with DAPI. gcl, ganglion cell layer.
References


Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.


CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Summary of results

My dissertation research is focused on the regeneration of photoreceptors in the adult zebrafish retina, using a light lesion paradigm. Regeneration of retinal neurons in the adult zebrafish depends on injury-induced activation of retinal stem cells, the Müller glial cells (Bernardos et al., 2007). To uncover the molecular genetic program that initiates the regenerative response, I performed an unbiased, genome-wide expression profiling analysis of isolated Müller glia from untreated and light-damaged retinas during the early stages of photoreceptor regeneration by using a transgenic zebrafish line in which Müller glia are fluorescent-tagged (Bernardos and Raymond, 2006). This novel cell-specific analysis focused on genes with significantly altered expression levels in the stem cell population while excluding general retinal injury-responsive factors. From this analysis, I identified a list of candidate genes whose function during photoreceptor regeneration could be further characterized.

Among these candidate genes whose expression levels changed in Müller glia in response to injury were two that had previously been shown to be essential for
regeneration of the caudal fin and heart muscle in zebrafish. First, \textit{hspd1}, which encodes heat shock protein 60, is required to activate stem cells (Makino et al., 2005). Second, \textit{mps1}, a protein kinase involved in mitotic checkpoint regulation, is necessary for regulating mitosis in rapidly proliferating progenitors (Poss et al., 2002). My discovery raised the intriguing and surprising possibility that a common molecular program is triggered in response to injury to enable regeneration in tissues from distinct embryonic origins. Through genetic analyses of the known conditional (temperature-sensitive) zebrafish mutant lines of \textit{hspd1} and \textit{mps1}, I found that these two genes are similarly required for photoreceptor regeneration. These data provided a mechanistic link between the regeneration programs across a diverse array of tissues in zebrafish (Qin et al., 2009).

Another gene that I found up-regulated in my microarray dataset, \textit{six3b}, is one of the three \textit{six3}-related homeobox transcription factors in zebrafish. \textit{six3} is essential for forebrain and eye development in all vertebrates studied. In zebrafish, the three \textit{six3}-related genes, \textit{six3a}, \textit{six3b}, and \textit{six7}, exhibit similar expression patterns during early embryogenesis to delineate the initial eye primordia (Seo et al., 1998a; Seo et al., 1998b). Loss of function of any one of these three genes does not cause any obvious developmental defect likely due to functional redundancy between these genes (Ando et al., 2005; Inbal et al., 2007). Interestingly, however, only \textit{six3b} showed a significant change of expression level in my microarray study. In order to test if \textit{six3b} is specifically required for reprogramming Müller glia to regenerate retinal neurons, I performed qRT-PCR and \textit{in situ} hybridization to define the expression patterns of all three \textit{six3}-related genes during the early stages of photoreceptor regeneration—\textit{six3b}, but not \textit{six3a} and \textit{six7}, is up-regulated immediately in injury-activated Müller glia after light lesion.
Functional assays using *six3b* null mutants did not reveal a regeneration defect in these fish, so I speculated that other zebrafish *six3*-related genes, such as *six3a* and/or *six7*, may compensate *six3b* loss-of-function in these mutants. Further functional examination of the various zebrafish *six3* homologs during photoreceptor regeneration by morpholino-mediated knockdown will test this hypothesis.

Additionally, using a candidate approach, the role of a conserved developmental signaling pathway, Fgf signaling, during zebrafish photoreceptor regeneration was investigated in this dissertation. In collaboration with Prof. Kenneth Poss’s laboratory at Duke University, I tested the role of Fgf signaling during photoreceptor regeneration by utilizing a dominant-negative transgenic zebrafish line in which Fgf signaling is blocked after heat-shock induction (Lee et al., 2005) and assaying for cone photoreceptor regeneration following light lesion. Although regeneration of the cone photoreceptors was not affected by inhibition of Fgf signaling, I found significantly fewer rod photoreceptors in the transgenics compared with wildtype siblings after 14 days of heat-shock. Further characterization of this rod phenotype in collaboration with Prof. David Hyde’s laboratory at University of Notre Dame uncovered an unexpected differential requirement for Fgf signaling in survival and homeostasis of rod and cone photoreceptors in zebrafish: blocking Fgf signaling results in degeneration and subsequent apoptosis of rod photoreceptors, however, cone photoreceptors are largely unaffected.

Together, my studies not only provided genetic insights into the mechanisms of zebrafish photoreceptor regeneration, *e.g.*, how Müller glia are activated to form the regeneration substrate, but also furthered our understanding of the molecular program of injury-induced tissue regeneration in general.
Common molecular program of tissue regeneration in zebrafish

Functional analyses of the temperature-sensitive mutants of *hspd1* and *mps1* showed that these two genes are necessary for regeneration of diverse zebrafish tissues, indicating a common molecular program of tissue regeneration might be present in zebrafish. To identify possible additional players in this program, a comparative analysis of zebrafish regeneration transcriptomes was performed. The transcriptomes used in this analysis were microarray gene profiling datasets of (1) isolated Müller glia/progenitor population from light-lesioned retinas (Qin et al., 2009), (2) tissue from amputated caudal fins (Schebesta et al., 2006), and (3) tissue from surgically sectioned hearts (Lien et al., 2006). All of the three studies used the Affymetrix zebrafish genome array. It is noteworthy, however, that in my retinal regeneration study, I used RNA samples from isolated GFP<sup>+</sup> Müller glia and their mitotic progeny, while in the fin and heart regeneration studies, RNA samples were prepared from tissue regenerates containing heterogeneous cell populations. Genes in my dataset that are also differentially expressed during either fin regeneration or heart regeneration or during both processes were identified and listed in Table 2.1.

A total of 28 regeneration-associated genes were identified in this analysis. Gene ontology characterization of these genes showed they are mainly involved in four biological processes: (1) Stress response. In addition to *hspd1*, expression of two other stress response genes are increased during regeneration of zebrafish neural and mesodermal tissues—*heat shock 70-kDa protein 5 (hspa5)* and *calreticulin, like 2 (calrl2)*; (2) Immunoregulation. Many of these genes are associated with the innate
immune responses to tissue injury: cathepsin B, a (ctsb), cathepsin C (ctsc), clusterin (clu), matrix metalloproteinase 9 (mmp9), matrix metalloproteinase 14 beta (mmp14b), and tissue inhibitor of metalloproteinase 2 (timp2). Importantly, the mmp genes, which regulate the extracellular matrix (Hernandez-Barrantes et al., 2002), are among the most up-regulated genes during tissue regeneration in zebrafish. This is consistent with the fact that extensive tissue remodeling occurs during these processes. In amphibian limb regeneration, mmps have been shown to be essential for the disorganization of mesenchymal cells during blastema formation (Vinarsky et al., 2005). Several other genes in this category regulate the immune system by suppressing inflammatory cytokine signaling, including activating transcription factor 3 (atf3), LIM domain only 4 (Imo4), and suppressor of cytokine signaling 3b (socs3b). (3) Cell signaling. These genes are involved in conserved developmental signaling pathways: GLI-Kruppel family member GLI2a (gli2a) is a downstream mediator of the Hedgehog signaling pathway; insulin-like growth factor binding protein 3 (igfbp3) is a modulator of the IGF signaling; jagged 2 (jag2) is a member of the Serrate/Jagged family of Notch ligands; pdgfa is a ligand of the PDGF signaling, and it has been shown that PDGF signaling is required for DNA synthesis of cardiomyocytes during zebrafish heart regeneration (Lien et al., 2006); both TGFβ-induced (tgfbi) and TGFβ-induced factor homeobox 1 (tgf1) are components of the TGFβ signaling pathway. (4) Transcription regulation. Some of the transcription factors identified in this comparative analysis are implicated in regulation of progenitor cells, such as SRY-box-containing gene 4a (sox4a), SRY-box-containing gene 11b (sox11b) and zic family member 2 (odd-paired-like) b (zic2b).
The identification of these 28 genes shared by zebrafish neural (retinal) and mesodermal (fin or heart) regeneration provides further support for a common molecular program of injury-induced tissue regeneration in zebrafish and functional studies of these genes will be needed to address whether any of them are required for regeneration of distinct zebrafish tissues.

**HPE genes and tissue regeneration**

Unexpectedly, the human homologs of several genes found in the above comparative analysis of zebrafish regeneration transcriptomes have been identified as human disease genes implicated in holoprosencephaly (holo, whole; prosencephalon, forebrain; HPE), the most common human congenital disorder of forebrain development. HPE is thought to be caused by the failure of midline induction signals to instruct the forebrain to divide into two hemispheres, and it can result from the lack of either the signals or the ability to interpret the signals. HPE symptoms are highly variable and dependent on the severity of the disorder, symptoms range from cyclopia (a single median eye, the most severe form) to microforms of HPE (mild craniofacial anomalies). Mutations in nine human genes have been associated with HPE: *sonic hedgehog* (*SHH*), *ZIC2, SIX3, TGIF, patched (PTCH), GLI2, forkhead box H1 (FAST1); teratocarcinoma-derived growth factor 1 (TDGF1), and 7-dehydrocholesterol reductase (DHCR7)* (Cohen, 2006). Among these genes, several function in the classic Hedgehog signaling pathway, including *SHH* (ligand), *PTCH* (receptor), and *GLI2* (downstream transcription factor). Moreover, all of the remaining HPE-associated genes are involved in genetic pathways
that regulate the Hedgehog signaling directly and indirectly (Cohen, 2006; Geng et al., 2008; Jeong et al., 2008; Sanek et al., 2009).

The zebrafish homologs of three human HPE genes—gli2a, tgf1, and zic2b—were identified as common regeneration-associated genes. In addition, one of the zebrafish homologs of another human HPE gene, six3b, is rapidly induced in injury-activated Müller glia and their mitotic progeny during the early stages of retinal regeneration. Although it is not clear how pathogenesis of the human HPE disorder could be mechanistically linked to injury-induced tissue regeneration in zebrafish, it appears that the Hedgehog signaling might play a central role during both processes. Function of the Hedgehog signaling pathway during zebrafish tissue regeneration can be readily tested by treating fish with a specific inhibitor, cyclopamine (Miller and Yu, 2002). Another remarkable point is that although these HPE genes are involved in early embryonic development of the central nervous system, their function during regeneration does not seem to be neural-specific. Therefore, functional interrogation during tissue regeneration in zebrafish might reveal a new aspect of the biology of these highly conserved genes.

**Mammalian Müller glia and retinal regeneration**

The ultimate goal of studying regeneration in zebrafish is to apply the knowledge we obtain from zebrafish to humans so that we could coax the human body to regrow and repair lost and dysfunctional tissues and organs. Taking the retina as an example, more than 5% of the population in the Western world will become blind at some point in his or her life because of retinal degenerative conditions (Goldsmith and Harris, 2003). Age-
related macular degeneration (AMD), which is the progressive loss of photoreceptors and deterioration of vision in a central region of the retina called macula, accounts for about 12% of blindness in the United States (Margalit and Sadda, 2003). Currently, no treatment is available to replace degenerated retinal neurons and to restore compromised visual function.

Unlike their zebrafish counterparts, mammalian Müller glia undergo reactive gliosis in response to retinal damage and are not neurogenic. In the mammalian retina, Müller glia re-enter the cell cycle and up-regulate GFAP intermediate filaments, similar to the response in zebrafish, but instead of generating neurogenic clusters as in the zebrafish retina, the mammalian Müller glia proliferate to form a glial scar (Bringmann et al., 2006). Although neural regeneration in the adult mammalian retina is mostly abortive, recent studies have suggested that a latent neurogenic potential might be retained in the mammalian Müller cells. By treating the adult rat retina with a depolarizing neurotoxin, N-methyl-D-aspartate (NMDA), Ooto et al. observed that some Müller glial cells incorporated BrdU in response to the injury, and few of the BrdU + cells later expressed markers for bipolar cells and rod photoreceptors (Ooto et al., 2004). Another study described the mammalian Müller glia as “dormant neural stem cells” by analyzing a Müller glia-enriched cell culture system. In this study, the authors found Müller cells derived from postnatal (PN) days 10-21 rat retinas exhibited neural stem cell properties in culture. To test if mammalian Müller cells could generate retinal neurons in vivo, the authors isolated injury-activated Müller glia from neurotoxin and growth factor-treated retinas of PN 14 rats and transplanted them into PN 7 rat eyes. A week later, transplanted cells were detected expressing specific markers for ganglion cells, amacrine cells, and rod
photoreceptors. Based on these results, the authors concluded that the mammalian Müller glia also possess a neurogenic potential but are prevented from functioning as retinal stem cells perhaps due to the non-neurogenic environment in the adult mammalian retina (Das et al., 2006; Lamba et al., 2008; Lamba et al., 2009).

So what makes the mammalian Müller glia different from Müller glia in the zebrafish retina? A mouse Müller glia transcriptome has been published recently, and surprisingly, the retinal progenitor marker Pax6 was found to be expressed in mouse Müller cells (Roesch et al., 2008). A comparative analysis of the transcriptomes of the mouse and zebrafish Müller glia following retinal injury could be done to identify any intrinsic factors that could either limit the neurogenic potential of mammalian Müller glia or enhance the regenerative capacity of zebrafish Müller glia. Finding these intrinsic factors will be important for developing methods to stimulate human Müller glia to regenerate retinal neurons, which would be an important advance for treating retinal degenerative diseases.
References


Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.


