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ABSTRACT OF DISSERTATION

James Robert Monaghan

The Graduate School University of Kentucky 2009

PHYSIOLOGICAL GENOMICS OF SPINAL CORD AND LIMB REGENERATION IN A SALAMANDER, THE MEXICAN AXOLOTL

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

> By James Robert Monaghan Lexington, KY Director: Dr. S. Randal Voss, Associate Professor of Biology Lexington, KY

2009

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PHYSIOLOGICAL GENOMICS OF SPINAL CORD AND LIMB REGENERATION IN A SALAMANDER, THE MEXICAN AXOLOTL

Salamanders have a remarkable ability to regenerate complex body parts including the limb, tail, and central nervous system. Although salamander regeneration has been studied for several hundred years, molecular-level studies have been limited to a relatively few important transcription factors and signaling molecules that are highly conserved among animals. Physiological genomic approaches were used here to investigate spinal cord and limb regeneration. Chapter 2 reports that hundreds of gene expression changes were identified during spinal cord regeneration, showing that a diverse injury response is activated in concert with extracellular matrix remodeling mechanisms during the early acute phase of natural spinal cord regeneration. Chapter 3 presents results that identify the salamander ortholog of mammalian Nogo-A, a gene known to inhibit mammalian nerve axon regeneration. Nogo-A gene expression was characterized during salamander development and adulthood in order to address the roles of Nogo-A in the nervous system. Chapters 4 and 5 use physiological genomic approaches to examine limb regeneration and why this process is dependent upon an intact nerve supply. Results presented in Chapter 4 showed that many processes regulated during early limb regeneration do not depend upon nerve-derived factors, but striking differences arise between innervated and denervated limbs by 14 days after amputation. Chapter 5 identified genes associated with peripheral nerve axon regeneration and identified gene candidates that may be secreted by nerves to support limb regeneration. Lastly, chapter 6 characterizes the expression of a developmentally important family of genes, matrix metalloproteinases, during tail regeneration. These results suggest that matrix metalloproteinases play multiple roles throughout the regeneration process. Primarily, this dissertation presents data from the first genomic studies of salamander regeneration. The results suggest genes such as matrix metalloproteinases, and molecular pathways such as the Wnt and FGF signaling pathways that can be exploited to enhance regenerative ability in humans.

KEYWORDS: Axolotl, regeneration, microarray, spinal cord, limb

James Robert Monaghan

April 1, 2009

PHYSIOLOGICAL GENOMICS OF SPINAL CORD AND LIMB REGENERATION IN A SALAMANDER, THE MEXICAN AXOLOTL

By

James Robert Monaghan

<u>S. Randal Voss, Ph.D.</u> Director of Dissertation

Brian C. Rymond, Ph.D. Director of Graduate Studies

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Lexington, KY

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CHAPTER 1

INTRODUCTION

"But if the above-mentioned animals (salamanders), either aquatic or amphibious, recover their legs, even when kept on dry ground, how comes it to pass, that other land animals, at least such as are commonly accounted perfect and are better known to us, are not endued with the same power?...Should the flattering expectation of obtaining this advantage for ourselves be considered entirely as chimerical?"

Lazzaro Spallanzani, 1768

This question, which has motivated and perplexed scientists for centuries, remains unanswered. Formal studies of salamander (Urodele) regeneration trace back at least to the 1760's, when Lazzaro Spallanzani and Charles Bonnet described the phenomenon of tail, limb, and eye regeneration in newts (Dinsmore, 1996). Since this time, it has become widely appreciated that the salamander's remarkable regenerative abilities are unparalleled among vertebrates. Much progress has been made in describing how salamander regeneration is accomplished at the morphological level. However, only recently have resources and technologies been developed to allow specific genes and gene functions to be studied in salamanders. In particular, microarray analysis, high-throughput sequencing, feasible transgenic and knockdown approaches, and bioinformatic databases are now available to re-address centuries old questions for the first time at the genomic level. The studies described in this dissertation address several salamander regeneration paradigms, including spinal cord regeneration, limb regeneration, wound healing, and the neural dependence of limb regeneration. The results of these studies provide some of the first unbiased insights into how salamanders re-grow a variety of tissues following injury. The hope is that someday we will soon be able to translate the secrets of salamander regeneration to clinical therapies in humans.

Spinal cord regeneration

The salamander spinal cord offers a promising model of central nervous system (CNS) regeneration because it is capable of functional recovery after transection, crush, and amputation injuries. However, it is unclear why salamanders have such a high capacity for spinal cord regeneration (SCR) while mammals do not. One difference between salamander and mammalian spinal cord injury (SCI) is that salamanders

regenerate large numbers of neurons from progenitor cells following SCI while mammals do not (Bareyre, 2008; Pinto and Götz, 2007; Ferretti et al., 2003; Chernoff et al., 2003). Results presented in Chapter 2 attempt to address this problem by comparing gene expression changes that take place during salamander SCR to gene expression changes that take place after mammalian SCI. It is also possible that salamanders can regrow nervous system tissue so readily because their neurons have a higher intrinsic capacity to regenerate axons than mammalian neurons. We attempt to identify genes associated with salamander axon regeneration by identifying up-regulated genes in sensory neurons during axon regeneration. Lastly, molecules known to inhibit regeneration in the mammalian CNS may not be present in the salamander CNS. We address this problem by identifying and characterizing the salamander ortholog of a gene that is inhibitory in mammals, Nogo-A. To develop the salamander model further, studies are needed to address these and other aspects of CNS repair, and from this information, determine the bases of a natural regenerative response.

A striking feature of salamander SCR is the activation of cells that line the central canal of the spinal cord (Egar and Singer, 1972; Nordlander and Singer, 1978). These cells have traditionally been called ependymoglia (Chernoff et al., 2003; Egar and Singer, 1972), but are structurally similar to mammalian radial glia seen during development (Holder et al., 1990; Mchedlishvili et al., 2007). Upon SCI, ependymoglia migrate to close off the exposed spinal cord lumen, proliferate, and eventually differentiate into new neurons (Norlander and Singer, 1978; Benraiss et al., 1999; Echeverri and Tanaka, 2002; Mchedlishvili et al., 2007). Ependymoglia-like cells seem to be critical in all animals capable of SCR including salamanders, zebrafish, anuran larvae, and lizard tails, although cell-tracking studies are severely lacking in all of these models (Reimer et al., 2008; Chernoff, 1996; Chernoff et al., 2003). In the future, it will be necessary to know what cell types ependymoglia are capable of differentiating into and the signaling mechanisms that regulate their activities. Experiments described in chapter 2 present some early efforts in identifying the early gene expression changes after SCI that may contribute to ependymoglia activation.

The extracellular environment of the injured mammalian spinal cord is thought to be inhibitory to regeneration. Investigations aimed to promote human SCR have mainly

focused on circumventing these inhibitory properties (Silver and Miller, 2004; Schwab, 2002; Horner and Gage, 2000). Several factors contribute to the hostile CNS environment including glutamate excitotoxicity, astrocytic gliosis, inhibitory myelin products, and toxic metabolic byproducts (Profyris et al., 2004 for review). Salamanders may be capable of SCR because they can naturally circumvent some or all of these injury byproducts. Indeed, a gliotic scar does not form after SCI, but the contribution of the other components is poorly understood. Inhibitory myelin products are thought to be major contributors to the inhibitory environment (Ramer et al., 2005). Nogo-A, or Reticulon 4A, in particular has received considerable attention as a target for clinical intervention (Buchli and Schwab, 2005; Freund et al., 2006, 2009). It has been proposed that salamanders may have a higher regenerative ability because Nogo-A is absent in the CNS (Schwab, 2004; Oertle et al., 2003; Klinger et al., 2004; Chernoff et al., 2003). There is also evidence that other inhibitory myelin breakdown products, specifically myelinassociated glycoprotein, and the inhibitory molecule Tenascin-R are quickly eliminated from the injury site. We address the first hypothesis in Chapter 3 by providing evidence that Nogo-A is present and expressed in the salamander CNS. This chapter concludes with a discussion of the possible roles of Nogo-A in the salamander nervous system.

The prospect of using information obtained from vertebrates capable of SCR for therapeutic treatments to SCI is appealing. This realization will be impossible without understanding how the salamander performs such feats. The results presented here address some of the most relevant questions related to these questions. Future studies will be needed to build upon these results using anatomical, functional, and genomic approaches to tease apart the complex process of building new neural tissue.

Limb Regeneration

The second model addressed in this dissertation is limb regeneration. Limb regeneration was chosen to study because it is the best characterized model of regeneration at the morphological level, but little gene expression data has been collected to date. The goal of our work is to fill this knowledge gap by performing physiological genomic techniques on regenerating limbs.

Limb regeneration is possibly the most extreme example of regeneration in vertebrates. It is an amazing feat to us humans that an animal can regenerate entire appendages that have been cut or bitten off. Hundreds of years of observation have given us a framework upon which we can build new understanding using the techniques and tools of modern biology. Below, I will review this framework and then describe how work presented in this dissertation builds upon this framework.

Amputated limbs recruit local cells to participate in regeneration. Following amputation, cells just proximal to the amputation surface are coaxed into migrating to the injury site, become highly proliferative, and form a mass of mesenchymal-type cells termed the blastema. The blastema is a self-organizing structure capable of re-growing into a new limb. The blastema is the fundamental element of a regenerating limb. Two of the major components needed to form a blastema are a functional wound epidermis, and an adequate supply of innervation at the amputation site. Understanding the role of each of these components are major focal areas in the field of regenerative biology.

Wound healing and the wound epidermis

Critical events necessary for limb regeneration take place only hours after limb amputation. A fibrin clot forms over the wound stump shortly after amputation. Basal keratinocytes proximal to the stump then lose their hemidesmosomes that connect them to the basal lamina and begin to migrate over the fibrin clot (Repesh and Overpriller, 1978, 1980; Norman and Schmidt, 1967). The keratinocytes meet in the wound epicenter and start to pile up within the first 24 hours. Preventing the formation of a healthy wound epithelium (WE) inhibits regeneration, demonstrating that the WE is essential to regeneration (Goss, 1956; Mescher, 1976; Tassava and Garling, 1979; Thornton, 1957; Lheureux, 1983). Once the WE is formed, continued migration of cells from the proximal epidermis and cell proliferation thicken the WE to create a specialized structure termed the apical epidermal cap (AEC).

The WE/AEC is thought to play multiple roles during regeneration. An unequivocal role of the WE is to phagocytose wound debris and either expel or dissolve it (Reviewed by Carlson, 2007). Singer and Salpeter (1961) showed that implantation of large and small debris are taken up by the WE into the intercellular space as well as inside

epidermal cells and extruded from the blastema. Extrusion of normal cellular debris from dying cells has also been clearly observed by microscopy (reviewed by Singer and Salpeter, 1961). A second hypothetical role of the WE/AEC is to promote histolysis of underlying tissues, which initiates dedifferentiation and blastema formation. Tissue histolysis does not take place if a functional WE is not formed (Adova and Feldt, 1939; Carlson, 1967; Reviewed by Singer and Salpeter, 1961 and Carlson, 2007). This histolytic property has been associated with matrix metalloproteinases (MMPs) including MMP3/10a and b, newt-specific MMPe, MMP9, and newt collagenase (Miyazaki et al., 1996; Kato et al., 2003; Yang et al., 1999; Vinarsky et al., 2005). Results presented in Chapter 6 support this hypothesis by showing that two collagenases, MMP9, and MMP3/10a are expressed in the WE during tail regeneration.

A third role of the WE/AEC may be to support blastemal cell growth. Biolly and Albert (1986; 1990) showed that culturing blastemal cells supplemented with crude protein extract isolated from WE can increase the mitotic index by 11x, which was significantly more mitogenic than either blastemal mesenchyme or nerve extract. There is strong evidence that fibroblast growth factors (FGFs) are produced by the wound epithelium to support blastema cell proliferation. These include the localization of FGFs 1, 2, 8, and 10 to the WE, the localization of FGF Receptor 1 to blastemal cells, and the observation that exogenous FGF can induce blastema cell proliferation when applied *in vitro* or *in vivo* (Biolly et al., 1991; Mullen et al., 1996; Han et al., 2001; Christensen et al., 2001; Campbell and Crews for review, 2008).

Nerve-dependency of limb regeneration

Amputated salamander limbs also need an adequate supply of nerves to regenerate. About 50 years after Spallanzanni described salamander limb regeneration, Todd (1823) showed that the limb does not regenerate when the nerve supply is compromised. His observations are clearly explained by the following quote:

[&]quot;If the sciatic nerve be intersected at the time of amputation, that part of the stump below the section of the nerve mortifies [necroses]...If the division of the nerve be made after the healing of the stump, reproduction [regeneration] is either retarded or entirely prevented."

Tweedy John Todd (1823) *J Sci Lit Arts* 16, 84-96.

Anatomical, immunological, and retrograde staining of neurons has shown that nerve fibers quickly grow from the severed end of brachial nerves into the injured tissues and WE (Singer, 1949; Thornton, 1970; Koussoulakos et al., 2003; Satoh et al., 2008). It is hypothesized that nerve fibers supply a factor or set of factors that support the proliferation of blastemal cells during the early phases of regeneration. This hypothesis is based upon the observation that spinal ganglia extracts are capable of supporting blastema cell proliferation *in vivo* and *in vitro* (Boilly and Albert, 1988; Globus and Vethamany-Globus, 1977; Mescher and Loh, 1981; Reviewed by Dinsmore and Mescher, 1998). Removing the nerve supply shortly before or during the first few weeks after amputation leads to a loss of cell proliferation and regenerative failure. Two critical questions need to be answered for us to understand the role of nerves during limb regeneration:

1) What is the neurotrophic factor (NTF) or NTFs secreted from regenerating peripheral nerve axons that support regeneration?

2) What are the cellular and molecular targets of the NTF(s) in the injured tissues?

The NTF(s) has not been identified to date. Multiple gene candidates have been proposed and supported experimentally including fibroblast growth factors (Mullen et al., 1996, but see Dungan et al., 2002; Satoh et al., 2008), substance P (Globus et al., 1991), neuregulin (Wang et al., 2000), and transferrin (Mescher et al., 1997), but no candidates have been accepted by the community as *the* neurotrophic factor. Specifically, Mullen et al. showed that FGF2 is expressed in sensory neurons and the wound epidermis. Implanting beads soaked in FGF2 could also rescue denervated limbs to regenerate, but similar experiments with FGF1 failed to rescue denervated limbs (Dungan et al., 2002). Substance P has been localized to neuronal cells and the regenerating limb, decreases abundance following denervation, and is mitogenic in cultured blastemal cells, but has not been tested for its ability to rescue denervated limbs (Globus et al., 1991). Neuregulin is expressed in dorsal root ganglia and can increase blastema cell proliferation in denervated limbs, but did not rescue regeneration in denervated limbs. Lastly, transferrin is expressed by sensory neurons, released from growth cones, and support blastemal cell growth *in vivo* and *in vitro* (Mescher et al., 1997). Although each of these candidates is promising, a

comprehensive study is needed to address whether these genes or a combination of them can be definitively identified as the neurotrophic factor.

Several criteria are known about the NTF(s) that may assist in narrowing down candidate molecules in future studies. It is hypothesized that sensory, motor, and sympathetic nerves all produce the NTF(s), but sensory neurons are the major contributors of the NTF because they contribute more axons to the blastema (Singer, 1978 for review; Koussoulakos et al., 2003). The NTF(s) is produced in the neuronal cell body, transported down the axon, possibly by fast axonal transport, and released at the synapse (Scadding, 1988; Wallace, 1972; Kiffmeyer et al., 1991). The NTF(s), and hence the stimulatory properties of axons, are increased following nerve injury or limb amputation (Singer, 1952; Maier et al., 1984; Boilly and Albert, 1988; Boilly and Bauduin, 1988). Lastly, the NTF(s) should not be produced in the blastema and down-regulation should mimic denervation. Limb regeneration should also be rescued with local expression of the NTF following denervation. These criteria should be kept in mind when determining the role of nerves during regeneration (Brockes, 1984).

The cellular and molecular targets of the NTF(s) are largely unknown. Recent experimental evidence support multiple cellular targets including the WE (Satoh et al., 2008), Schwann cells (Kumar et al., 2007), endothelial cells (Smith and Wolpert, 1975; Rageh et al., 2002), and blastemal cells (Maden, 1978; Vethamany-Globus, 1978). It will be important to find out whether the loss of blastemal cell proliferation following denervation is directly or indirectly downstream of the NTF. Kumar et al., (2007) have recently identified a downstream molecular target [Newt Anterior Gradient (nAG)] that may do the bidding of the NTF. nAG is a secreted mitogen of blastemal cells that is expressed in Schwann cells and skin glands shortly after injury. Denervation at the time of amputation abrogates expression in these cell types. Expression of nAG using DNA constructs in denervated limbs can rescue regeneration to the digit stage, identifying nAG as a likely downstream molecular target of the NTF. Alternatively, Satoh et al (2008) have proposed that the nerves target the WE, which then do the bidding of the NTF. A promising strategy for identifying the downstream nerve target is to sample differentially regulated genes between innervated and denervated limbs. mRNA localization of these differentially regulated genes with in situ hybridization would likely identify the

downstream targets of the nerves. We use physiological genomic strategies in chapter 4 to identify these differentially regulated genes and the possible downstream targets for nerves. Results from this chapter show that the nerve has little affect on the early events of limb regeneration, but has profound affects when the blastema begins to form. Chapter 5 attempts to identify the NTF as well as genes that are upstream of the NTF. In this experiment, we sampled differentially expressed genes in dorsal root ganglia following limb amputation. Several new gene candidates were identified that could be the NTF and transcription factors that may be associated with axon outgrowth of the peripheral nerve or induce expression of the NTF were identified. Overall, these experiments give the most detailed molecular description of how the NTF may work. The next steps will be to characterize both the upstream and downstream components of the NTF using *in situ* hybridization because knowing which cell types they are expressed is essential for us to understand how the NTF affects limb regeneration.

All of the work presented in this dissertation is descriptive in nature, but it is necessary for understanding how a salamander can regenerate a spinal cord or limb. Experiments of the past have suggested that both of these regeneration paradigms need cellular and molecular events to take place that are unique to the salamander. We attempt to identify these events by characterizing the expression pattern of thousands of genes simultaneously during regeneration and then compare these patterns to non-regenerating tissues. Overall, we found that both spinal cord (Chapter 2) and limb regeneration (Chapter 4) are global processes that involve thousands of genes. We also found that some gene expression signatures of the wound response are similar between regenerating spinal cord, non-regenerating mammalian spinal cords, regenerating limbs, and non-regenerating denervated limbs. The truly regeneration-specific gene expression patterns become apparent days after the injury takes place. Future experiments should focus on where these genes are expressed, how early do they become regeneration-specific, and are they necessary for regeneration to take place. The studies presented here lay the groundwork for researchers to address the role of these genes during regeneration and whether this knowledge can be translated into human therapies.

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CHAPTER 2

EARLY GENE EXPRESSION DURING NATURAL SPINAL CORD REGENERATION IN THE SALAMANDER *AMBYSTOMA MEXICANUM*

James R. Monaghan,* John A. Walker,* Robert Page,* Srikrishna Putta,* Christopher K. Beachy,† and S. Randal Voss* *Department of Biology & Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40506 †Department of Biology, Minot State University, Minot ND 58707

S. Randal Voss PhD, 741 South Limestone Avenue, Lexington, KY 40536

Telephone: 859-257-9888

Fax: 859-257-5737

E-mail: <u>srvoss@uky.edu</u>

Abbreviations used: EST, expressed sequence tag; FDR, false discovery rate; FWER, family-wise error rate; d, day; N, non-significant; U, up-regulated ; D, down-regulated; C, constant; QRT-PCR, quantitative real-time PCR; lgals1, galectin 1; ISH, *in situ* hybridization; DIG, digoxygenin; ECM, extracellular matrix; mmp, matrix metalloproteinase; sfrp2, secreted frizzled-related protein 2; apoE, apoliprotein E; ck18, cytokeratin 18; fst, follistatin; SHH, sonic hedgehog; BMP, bone morphogenic protein; FGF, fibroblast growth factor; PCA, principal component analysis

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Introduction

Salamanders have a remarkable ability to regenerate complex body parts including the limb, tail, lens, and CNS. Although salamander regeneration has been studied for several hundred years (Spallanzani, 1768; Müller, 1864), molecular-level studies have been limited to a relatively few important transcription factors and signaling molecules that are highly conserved among vertebrates, and in some cases metazoans (e.g. Schnapp *et al.* 2005; Christensen *et al.* 2002; Carlson *et al.* 2001; Caubit *et al.* 1997; Torok *et al.* 1999). Broader assessments of gene expression during salamander regeneration may identify mechanisms that can be exploited to enhance regenerative ability in humans.

Salamanders regenerate their spinal cords and regain full movement and function after tail amputation. Within a few hours of amputation, injury responses are initiated to increase cell survival and transform the tissue-damaged environment into one that is permissive for repair and subsequent regeneration. It is possible that the unrivaled regenerative ability of salamanders is due in part to this early injury response phase of regeneration, but very little is known about early response genes and associated biological processes. Most attention has been directed to understand cellular and developmental changes during the dramatic and conspicuous de-differentiation and re-patterning phases of regeneration. During de-differentiation, cells of mesodermal origin (muscle, dermal fibroblasts, and cartilage) re-enter the cell cycle and proliferate to form a mass called the blastema (Hay and Fischman, 1961). Blastemal cells subsequently re-differentiate into mesodermal tissues but apparently do not contribute to the regenerating spinal cord. Epithelial cells (ependymoglia) of the ependymal lining that surrounds the central canal of the spinal cord re-form neural tissues of the regenerating spinal cord (Nordlander and Singer, 1978). The signals that initiate and maintain the proliferative response of ependymoglia are largely unknown, however recent studies implicate some of the same highly conserved genes that are known to regulate the proliferation and differentiation of neural stem cells among vertebrates including sonic hedgehog, FGFs, epidermal growth factor, (O'Hara and Chernoff, 1994; Zhang et al. 2000, 2002; Schnapp et al. 2005). This suggests that some aspects of salamander spinal cord regeneration may be shared with organisms that have little or no potential for neural regeneration. Analyses of gene

expression in salamanders may point to key similarities and differences that are associated with regenerative ability.

We designed a custom Affymetrix GeneChip and performed the first microarray analysis of spinal cord regeneration in the Mexican axolotl (*Ambystoma mexicanum*). We sampled regenerating spinal cord tissue at five early time points after amputation and identified differentially expressed genes and temporal patterns of gene expression. We compared our lists of significantly regulated genes to lists that have been similarly compiled from microarray studies of spinal cord injury in rat. Our results highlight genes and gene expression patterns that are associated with the salamander's natural ability to regenerate spinal cord.

Materials and Methods

Animals, tissue collection, and RNA isolation

The handling and surgical manipulation of all salamanders was carried out according to the University of Kentucky Animal Care and Use guidelines (IACUC #00609L2003). The caudal 1/3 of the tail was amputated from 225 Mexican axolotl sibs (*mean snout-vent length* = 6.2 cm) from an inbred Voss laboratory strain. Spinal cord tissue was collected 1.0 mm rostral to the injury plane at the time of spinal cord transection (day 0), and also on 1, 3, 5, and 7 days post amputation. The tail blastema was removed prior to sampling however it is likely that some infiltrating blastemal cells were represented in the day 1-7 samples. Total RNA (*mean* = 1.7μ g) was extracted from pools of nine tissues for each of five replicates that were collected at each time point. Probe labeling, hybridization, and scanning for the 25 RNA samples were performed by a single staff member of the University of Kentucky Microarray Core Facility.

Development of a microarray platform

A custom *Ambystoma* Affymetrix GeneChip was designed from curated expressed sequence tag (EST)s assemblies for *A. mexicanum* and *A. t. tigrinum* as described in Page *et al.* (2006). These ESTs are enriched for genes expressed in neural and regenerating tissues (Putta *et al.* 2004). Briefly, the array contains 4,844 total probe sets, 254 of which are controls or replicate probe sets. Of the remaining 4,590 probe sets, all but 188

correspond to unique *A. mexicanum* contigs, of which 2,960 are significantly identical in nucleotide composition (e-7; BLASTX) to a human sequence in the non-redundant, RefSeq protein database. Significant salamander-human blast hits were considered gene orthologs in our analyses and we assumed that salamander-human orthologs have similar gene functions or ontologies. Raw data files can be obtained at <u>www.ambystoma.org</u>.

Quality Control and Low Level Analyses.

We used the Bioconductor package affy (<u>www.bioconductor.org</u>) that is available for the statistical programming environment R (www.r-project.org) to perform quality control and preprocessing procedures at the individual probe level (Bolstad et al. 2005a). These procedures included: (1) generating matrices of M versus A plots for all replicate arrays, (2) investigating measures of central tendency, measures of dispersion, and the distributions of all 25 arrays via boxplots and histograms, (3) viewing images of the log₂ (intensity) values for each array to check for spatial artifacts, and (4) viewing an RNA degradation plot (Bolstad et al. 2005b) that allows for visualization of the 3' RNA labeling bias across all arrays simultaneously. In addition, we used ArrayAssist Lite software (Stratagene, La Jolla, CA) and the MAS5.0 algorithm to assess several quality control measures that are recommended by Affymetrix (www.affymetrix.com) such as average background (mean = 61.5, range = 55-81.5), noise (mean = 4.23, range = 2.89-6.94), and percent present (mean = 84.7% range = 81.2-87.0%). This high number of present probe sets likely reflects the biased selection of regeneration-associated genes and high quality contigs for probe set design. Next, the repeatability of probe set estimates of hybridization intensity was evaluated between arrays. We examined the correlation of hybridization intensities across all probe sets among the biological replicates for each regeneration time point (mean r = 0.994; range r = 0.983-0.998). These results demonstrate that we were able to obtain a high level of repeatability. We processed our data similarly to the methods of Choe et al. (2005) to determine a probe set intensity value. Briefly, our processing method consisted of using the MAS 5.0 background correction algorithm, the quantiles algorithm for probe-level normalization, the MAS 5.0 algorithm for perfect match/mismatch correction, the median polish algorithm for expression summary generation, and a loess normalization at the probe set level using the

GoldenSpike package for R (Choe *et al.* 2005; <u>www.ccr. buffalo.edu/ halfon/ spike/</u> index.html).

Detection of differentially expressed genes and data filtration.

Microarray platforms may not accurately or precisely quantify genes with low intensity values (Choe et al. 2005; Draghici et al. 2006). Because low intensity genes contribute to the multiple testing problem that is inherent to all microarray studies, we filtered 1,203 probe sets whose mean intensity across all 25 arrays were smaller than or equal to the mean of the lowest quartiles across all arrays (mean = 6.44, SD = 0.09; data presented on a log₂ scale). Probe sets (3641) were then tested for differential expression via a one-way fixed effect linear model (intensity = day sampled) using the F_s test of Cui et al. (2005) and J/MAANOVA software (www.jax.org/ staff/ churchill/ labsite/ software/anova/index.html). Initially, we adjusted for multiple testing by setting the false discovery rate (FDR) to 0.01 using the step-up algorithm of Benjamini and Hochberg (1995). As is shown in Figure 2.1a, upon performing this FDR correction, 2771 probe sets of the 3,641 probe sets tested (76.11%) were selected as differentially expressed. We then took a more conservative approach to our first pass at selecting differentially expressed genes by setting the family-wise error rate (FWER) to 0.01. Upon adjusting the FWER to 0.01, 1,273 of the 3,641 genes tested (34.96%) were selected as differentially expressed (Figure 2.1b). In order to identify a smaller subset of probe sets, we prioritized probe sets that were selected as differentially expressed that exhibited: (1) a 2-fold change at any time point relative to day zero and (2) F_s values that were in the upper 50% of these 1,273 genes ($F_s > 28.36$), leading to a total of 376 probe sets. The intensity values of three probe sets pairs designed for the same contigs as well as probe sets corresponding to the same human gene were combined, yielding a final short list of 360 unique genes (Table 2.1).

Candidate gene lists may differ when different preprocessing algorithms are used to identify statistically significant genes from oligonucleotide microarrays (Millenaar *et al.* 2006). To address this concern, we compared the 376 candidate probe set list above to a 646 probe set list that was generated using only the robust Robust-Multiarray Averaging algorithm (Irizarry *et al.* 2003), One-way ANOVA (FDR = 0.01), and a 2-fold change criterion. Only 11 of the 376 candidate probe sets (2.9%) were unique, indicating that our methodology for identifying candidate genes is largely concordant with other statistical approaches.

Identification of gene expression patterns

We used the following criteria to define temporal gene expression patterns for the 360 genes that met statistical and fold-level criteria. For each gene we assigned a score to qualify the mRNA abundance at each post-amputation sample time (d1, d3, d5, and d7). A gene received a score of non-significant (N) for each sample time that mRNA abundance was < 2 fold deviant of the d0 estimate. We refer to the d0 estimate as the baseline estimate of mRNA abundance. A gene received a score of up-regulated (U) or downregulated (D) for the first post-amputation sample time that mRNA abundance deviated by \geq 2 fold from baseline. For subsequent sample times, each gene received one of three possible scores: C, U, or D. A score of constant (C) was assigned if the fold level estimate was < 2 fold deviant of the previous U or D estimate (C was never assigned after N), and > 2-fold deviant from baseline. A score of U or D was assigned if mRNA abundance deviated again by ≥ 2 fold. Using this scoring system, a gene received a score of U, D, or N for d1, and U, D, N, or C for d3, 5, and 7. A complete breakdown of the 360 changed genes is shown in Table 2.2. To annotate genes, we used multiple databases (GO, KEGG, IHOP, OMIM, etc.) and searched the literature for information about the expression and functions of each gene that we identified as significant in our study. We biased our annotations to emphasize possible gene functions that have been described in regeneration and spinal cord injury research fields.

Identification of genes expressed differently between salamander regeneration and rat spinal cord injury

A bioinformatics approach was used to identify gene orthologs that are expressed similarly or differently after salamander tail amputation versus rat spinal cord injury. We used current (May 2006) human Entrez Gene ID's that were assigned to each annotated probe set on the *Ambystoma* GeneChip to identify all presumptive salamander orthologs on RatU34A, B, and C GeneChips. To accomplish this cross-referencing task, we used Resourcerer (Tsai *et al.* 2001), a database that allows orthologous genes to be identified among species-specific microarray resources. This yielded a list of 1,036 probe sets between the *Ambystoma* and RatU34 GeneChips that presumably correspond to 662 unique, orthologous genes. We compared the expression pattern of each of these genes using results from this study and published studies that profiled gene expression after SCI in rat, using RatU34 GeneChips (Song *et al.* 2001; Carmel *et al.* 2001; Aimone *et al.* 2004; De Biase *et al.* 2005). De Biase *et al.* (2005) provide a table that compares specific details of these rat SCI microarray studies. The rat studies used thoracic T8-10 contusion models (MASCIS, OSU, and weight drop methods) and tissues were sampled at and flanking the impact site during the first 48 hours post-injury; Aimone *et al.* (2004) also sampled 7 and 35 days after injury. For each gene, we qualified gene expression as either significantly up, significantly down, or non-significant. We used statistical and fold-level criteria (FWER < 0.01; > 2-fold change) to score salamander genes for these criteria. If a gene was reported as significantly regulated in the rat studies, we recorded it as such; otherwise we recorded it as non-significant.

Quantitative Real-Time PCR (QRT-PCR)

Ten genes from the microarray experiment were selected for validation by QRT-PCR. Genes were selected based on technical and biological rationale. Technically, we wanted to validate genes that yielded a broad range of relative fold change estimates by microarray analysis and included both possible directions of differential expression. These genes also exhibited a range of hybridization intensity values; for example, the average intensity value of *hairy enhancer of split 1* ranked among the bottom 37% of all probe sets while *galectin 1* (*lgals1*) ranked among the top 95%. Biologically, we selected genes that are of interest in regenerative biology and spinal cord injury research fields. A BioRad iScript cDNA synthesis kit (Hercules, CA) was used to synthesize cDNA templates from three d0 and d3 RNA samples from microarray analysis. Primers were designed with Primer3 (Rozen and Skaletsky, 2000) and used to amplify DNA fragments from the same gene regions that were used to design corresponding GeneChip probe sets (Table 2.3). Reactions included cDNA that was synthesized from 10ng total RNA, 300nM primers, and iQ SYBR-Green real-time PCR mix and run on a BioRad I Cycler QRT-PCR system (BioRad). The three replicates were normalized against a gene that showed no significant gene expression change in the microarray experiment (*glyceraldehyde-3-phosphate dehydrogenase*, MC01187). PCR efficiencies for each primer were incorporated into the relative fold change calculations according to Pfaffl (2001). Student's t-tests were performed using the three normalized biological replicates for d0 and d3 samples.

In situ hybridization (ISH)

Digoxygenin (DIG)-labeled RNA probe production and ISH were performed as described by Hirota *et al.* (1992) with minor modifications. RNA probes were synthesized by *in vitro* transcription using 300-350 base pair PCR products as template and included SP6 or T3 RNA polymerase promoters appended to the 5' ends (Table 2.3). PCR products were cleaned using Qiagen PCR purification columns before performing *in vitro* transcription. Axolotl tissues were collected three days after tail amputation and fixed at 4°C in 1x PBS, 4% paraformaldehyde overnight. Bone was decalcified by incubating the tissue in 500 mM EDTA (pH8.0), 1xPBS for at least two days, cryoprotected overnight in 30% sucrose, and sectioned to 16µm using a Microm 500HM cryostat. Hybridization, washing, and colorimetric detection with NBT/BCIP were performed on a Tecan Genesis Workstation 200 liquid handling robot with a Genepaint[®] hybridization station (Zurich, Switzerland). Microscopy was performed using an Olympus IX81 microscope and images were acquired with an Olympus DP70 camera.

Results

Histology of the spinal cord during the first week after tail amputation

We performed histology on tails collected at day 1, day 3, day 5, and day 7 to relate our experiment to previous morphological descriptions of urodele spinal cord and tail regeneration (Piatt, 1955; Stensaas, 1983; Iten and Bryant, 1976). Upon amputation of the salamander tail, the spinal cord regresses approximately 0.5 mm rostral to the amputation plane and a clot, including a large number of leukocytes, forms at the wound site (Figure 2.2A; Iten and Bryant, 1976; Jones *et al.*, 1993). By day 7, the clot is replaced by a mesenchymous cell-mass called the blastema and the blastema forms while there is extensive extracellular matrix remodeling and bone degeneration (arrows, Figure 2.2A, C, E, G). Also throughout the first week, cell death occurs rostral to the injury plane and cell proliferation of inflammatory cells is apparent, but little cell division is observed among ependymal cells (Figure 2.2B; Zhang *et al.*, 2003; Stensaas, 1983). By day 3, rostral axons begin to degenerate and ependymal cells migrate to close off the lumen of the spinal cord, thus creating a terminal bulb (Figure 2.2B, C, D, E; Egar and Singer, 1972; data not shown). Ependyma become highly proliferative by day 7, increasing ependymal tube thickness and extending the tube along the length of the regenerating tail (Zhang *et al.*, 2000). Following the first week of regeneration, ependymal cells differentiate into new CNS neurons and peripheral ganglia, reconnecting the spinal cord to the body periphery and recovering function (Koussoulakos *et al.*, 1999).

Identification of differentially expressed genes and gene expression patterns

We identified 360 probe sets as detecting significantly different mRNA abundances between d0 and another time point (d1-7), using statistical and fold change thresholds (ANOVA p < 0.01; FWER of 0.01; F_s > 28.36; > 2-fold). More than half of these probe sets (n = 210) correspond to salamander sequences (genes) that show high sequence identify to a presumptive human protein-coding locus; the remainder correspond to anonymous EST contigs that do not align to human genes. In comparison to d0 (baseline) mRNA levels, most genes exhibited significantly different mRNA abundances at two or more post-amputation time points. This temporal variability did not yield an extensive list of gene expression patterns. Although a total of 100 different gene expression patterns were possible under our scoring system, only 32 different patterns were observed and over 85% of all genes were classified into ten categories (Table 2.1; Table 2.2). Eight of the top ten categories identified groups of genes in which mRNA abundance increased or decreased at a particular time point, and afterward the level remained constant through d7. Transcript levels for a few genes did increase or decrease by > 2 fold among post-amputation time points, however only three genes yielded a temporal expression profile that deviated significantly from baseline in both up- and down-regulated directions during the seven day period (UNND; Table 2.1). Thus, the majority of the gene expression profiles that we examined consisted of a single, significant deviation from base line levels followed by relatively constant mRNA abundance. It is likely that many of the uniquely expressed genes at d7 are regulated at

later time points because only 43 of the 360 genes exhibited transcript levels at d7 that approximated baseline. Clearly, we only sampled the initial phases of a continuous gene expression program that extends beyond d7. However, our experiment does precisely sample discrete phases of gene expression variability during this temporal process. For example, Figure 2.3 illustrates that gene expression profiles of samples collected at d1 are much more similar to each other than samples collected at d0, as well as d3, 5, and 7. Below, we describe major gene expression patterns in greater detail. We also highlight some of the genes and gene functions that were found in each of the major gene expression categories. Finally, we compare the expression of salamander genes to presumptive rat orthologs that have been examined in microarray studies of spinal cord injury.

Gene expression patterns

Overall, a greater number of genes were up-regulated above baseline during the 7day period (n = 238 compared to n = 125). The majority (n = 134) were significantly upregulated at the first sample time after amputation (d1) and half of these genes (UCCC: n = 64) registered constant mRNA abundances above baseline at all subsequent postamputation sample times (d3, d5, and d7). A substantial number of the d1 up-regulated genes showed decreasing mRNA abundances at later sample points. Some of these genes yielded mRNA abundances at d3 (UNNN: n = 20), d5 (UCNN: n = 5) or d7 (UCCN, UDNN: n = 5) that approximated d0 levels, while others remained above baseline (UDCC, UCDC, UNNU, UDDC: n = 22). The early group of up-regulated genes suggests that a diversity of regulatory pathways and biological processes are activated within the first 24 hours after tail amputation. In addition to genes that presumably function in wounding, stress, inflammation, and immunity, this group includes genes that function in tissue remodeling, apoptosis, ion transport, cell-cell interactions, cell migration, vitamin B economy, lipid metabolism, and cytoskeleton dynamics (Table 2.1). Several different regulatory networks are implicated directly or indirectly among these d1 up-regulated responses, including MAPK, WNT, v-MYC, TNF, v-YES, RAS, and TGF-beta.

Other groups of genes were up-regulated for the first time at d3, 5, and 7 (n = 104). The majority of the d3 and d5 genes maintained high, constant mRNA levels at subsequent time points (NUCC: n = 24, NNUC: n = 44). Gene functions that were observed among d1 up-regulated genes were also represented among d3-7 up-regulated genes. However, the distribution of genes among these functional categories was very different. In particular, fewer injury response genes and a greater number of extracellular matrix (ECM) and cytoskeleton-associated genes were observed compared to d1 upregulated genes. Also, a greater number of cell cycle related genes were observed (e.g. NNUC, *cell cycle* = 14) as well as genes that presumably function in DNA replication, metabolism, chromatin assembly, and cytokinesis. These results suggest that the regeneration gene expression program transitions during the first seven days from an injury responsive phase to one that is defined primarily by the up-regulation of genes that function in cell division. Throughout both injury response and cell proliferation phases, genes that function in tissue remodeling are significantly regulated.

Relative to the total number of up-regulated genes, a much smaller number of genes (n = 125) were down-regulated significantly below baseline levels during regeneration. In contrast to the up-regulated gene set, very few of these genes were downregulated at d1 (DCCC, DCNN, DNNN, DCDC = 12). Also, the magnitude of the foldlevel changes was generally lower than those measured for significantly up-regulated genes (mean of maximum up-regulated fold changes = 6.61; down-regulated = -3.07). The largest number of down-regulated genes was observed at d3 (n = 45) and this was followed by additional groups of down-regulated genes at d5 (n = 29) and d7 (n = 35). In general, many genes with neural related functions were down-regulated, including those that function in ion transport, glutamate metabolism, glutamate binding, neuroprotection, neurotransmission, neurogenesis, and lipid metabolism. Several functional categories that were observed among up-regulated genes were also observed among down-regulated genes, including apoptosis, cytoskeleton, ECM, signal transduction, and heat shock (Table 2.1). The overall pattern indicates that fewer genes are down-regulated during the first seven days of regeneration, and down-regulated genes show significantly lower mRNA abundances at d3, after the early up-regulation of genes at d1.

Some gene expression patterns were more complicated than linear, directional responses, involving changes in mRNA abundance that fluctuated both above and below the baseline. Some of these genes with complex expression patterns (UDCC, UCDC,

NUCN, UDDC, NDCD, UUCD) may function in the regulation of biological processes during regeneration. These include genes that function in ECM remodeling (*matrix metalloproteinase [mmp] 1, mmp13, tissue inhibitor of metalloproteinase 1 [timp1]*), coagulation (*tissue factor pathway inhibitor 2*), vitamin B transport (*intrinsic factor, transcobalamin 1*), cell proliferation (*v-Ha-ras viral oncogene, hypothetical protein FLJ20303*), transcriptional regulation (*jun-b proto-oncogene*), and cell signaling (*latent TGF beta binding protein; chromosome 8 orf 4; secreted frizzled-related protein 2 [sfrp2*]).

Gene expression after spinal cord injury: salamander verses rat

To identify similarities and differences between the salamander and mammalian spinal cord injury response, we compared our gene expression results to published results from the rat spinal cord microarray literature. Specifically, we compared the expression of 662 presumptive rat–salamander orthologous genes that are represented on both the *Ambystoma* and rat Affymetrix GeneChips. The resulting list of gene orthologs represents an unbiased sampling of ~24,000 transcripts on the rat arrays and 4,590 transcripts on the salamander array. Although the majority of gene orthologs were not significantly regulated (n = 553), we identified many similar and dissimilar gene expression responses between these organisms. Eleven genes are up-regulated in both species, with no common genes down-regulated. There were 46 and 41 uniquely up-regulated genes in the salamander and rat, respectively. Overall, the majority of dissimilarities between the rat and salamander injury response were changes in one animal and not the other (n = 126) rather than opposite gene expression changes between animals (n = 2; Table 2.5).

Quantitative Real-time QRT-PCR

Using QRT-PCR, we estimated fold change between d0 and d3 for ten genes from the microarray experiment (Table 2.4). All of the transcripts that met statistical and fold level criteria from the microarray analysis registered significant differences in mRNA abundance by QRT-PCR (6/10). Overall, we were able to verify nine of the ten gene changes in the correct direction with close agreement in most cases. We only failed to replicate the microarray estimate for *sox3*, which was not significant by QRT-PCR and
registered such a low fold change that it was excluded from the short list of microarray gene candidates. Thus, for all genes that met our stringent statistical and fold level criteria, and three genes that did not, QRT-PCR validated microarray estimates of gene expression with very good precision.

Spatial analysis of mRNAs using in situ hybridization

Ten genes that were significantly regulated during spinal cord regeneration were examined further by ISH. We probed tissues that were collected three days after tail amputation to localize expression among cell types that were within 1 mm from the end of the regenerating spinal cord. Figure 2.4 illustrates the diverse patterns of spatial expression observed with hybridization found in cells resembling ependymoglia, neurons, and immune cells. All genes but *fibroblast growth factor binding protein* used for *in situ* hybridization were up-regulated after injury. Assuming that these genes are not expressed in uninjured tissue, these results show that the general increase in mRNA abundance as determined from microarray analysis can be replicated and localized to specific cell populations and tissues of the spinal cord using ISH.

Discussion

We built a custom Affymetrix GeneChip and profiled gene expression during the early phases of natural spinal cord regeneration in a salamander model (*Ambystoma mexicanum*). Our results show that regeneration involves significant changes in mRNA abundance for many genes that are represented on the array. The overall list of 1,273 genes that met a very stringent statistical criterion is available as a new resource for regeneration and spinal cord injury research fields (www.ambystoma.org). The large number of genes on this list, which were identified using a custom microarray with enriched gene content, shows that thousands of genes are significantly regulated during the first few days of natural spinal cord regeneration. We used additional statistical and fold change criteria to sample a smaller sub-group of candidate genes to describe gene expression patterns and biological functions. The presumptive functions of this smaller list of genes suggest the operation of many biological processes that change temporally during spinal cord regeneration. Below we discuss up- and down-regulated genes and gene

functions that may be important in the regenerative response. In particular, we compare our gene expression results to several studies that have examined rat spinal cord injuries using microarrays.

Up-regulated gene responses

Similar gene expression changes are often observed after tissue injury, regardless of the type of injury or specific tissue type examined. With respect to CNS tissue injury in mammals, an early acute phase is characterized in part by the expression of transcription factors and immune response genes. Our study identified several genes that change by d1 in salamander spinal cord that are also expressed during the mammalian CNS acute injury response (Vazquez-Chona et al. 2005; Bareyre and Schwab, 2003). These include jun-B proto-oncogene, interferon regulatory factor 1, heme oxygenase 1, and apolipoprotein E (apoE). Overall, many of our d1 and d3 up-regulated genes encode proteins that participate in immune response functions, including lymphocyte, platelet and monocyte activation, macrophage differentiation and migration, cell adhesion, thrombosis, coagulation, inflammation, oxidative and metabolic stress, and apoptosis. In addition to immune response genes, we also observed up-regulation of genes that function in transport and binding of vitamin B and lipids, and ECM remodeling. While processes like vitamin B homeostasis have received little attention in regeneration and injury fields (Bauer, 1998), lipid turnover and MMP activity is well documented to be associated with regeneration (Vance et al. 2000; Vinarsky et al. 2005). In A. mexicanum, MMP1, MMP2, and MMP9 activity is associated with proliferating ependymal cells after 2-3 weeks of regeneration (Chernoff, 2000). Our study shows that mmps 1, 3, 9, 13, 27, and timp1 are all highly up-regulated by 24 hours after injury, which is the first association of mmps 13 and 27 with regeneration in urodeles. MMPs are also up-regulated in rodents after SCI and high levels appears to contribute to secondary injury (Noble, et al., 2002; Wells et al., 2003). Although application of MMP inhibitors may increase functional recovery after SCI, our results emphasize the beneficial effects of MMPs and the need to quantify the timing and amount of their delivery; clearly, MMP up-regulation and high MMP transcript levels after spinal cord amputation are characteristic of natural regeneration in A. *mexicanum*. In general, our results show a robust and diverse gene expression response is

activated during the acute phase of natural spinal cord regeneration, and this response includes genes whose functions are thought deleterious to recovery after SCI in mammals.

The majority of the early-activated genes were up-regulated throughout the first seven days, extending into a subsequent phase of cell cycle-related gene expression at d5. The accumulation of mRNAs that increase during the first seven days of regeneration suggests a temporal change toward biological processes that are associated with cell division. Many genes up-regulated at d5 (NNUC) are associated with mitotic cell cycle regulation including four genes involved in the G2/M transition and six associated with mitosis (Table 2.1). These gene expression changes maybe associated with the early proliferation of blastemal and ependymal cell populations, which are known to expand after the first week of regeneration (Lo et al. 1993; Zhang et al. 2003). Cell cycle-related genes are also up-regulated early after rat spinal cord injury, but the functions of these genes are associated primarily with S-phase and DNA repair and expressed in damaged or apoptotic neurons, not proliferating cells (Di Giovanni et al. 2003). Thus, within a few days after spinal cord injury, cell-cycle gene expression is biased towards cell death pathways in mammals but cell survival and proliferation pathways in salamanders. This observation could explain the cell proliferation of neural progenitors in the salamander, which is likely necessary for the spinal cord to regenerate.

Down-regulated gene responses

In comparison to up-regulated genes, there were fewer down-regulated genes and most showed gradual changes over time. Multiple genes were down-regulated whose products are associated with neural functions, including axon guidance, ion transport, glutamate metabolism, neuroprotection, and neurotransmitter signaling. Changes in neural-related gene expression patterns may reflect the damage or loss of neural cell types verses the survival, infiltration, and proliferation of other cell types. This explanation has been advanced to explain the down-regulation of genes after mammalian spinal cord injury, where there can be extensive tissue damage and cell loss (Profyris *et al.* 2004). Indeed, even in the regenerating salamander, there is local spinal cord tissue loss after injury (Figure 2.2B; Zhang *et al.* 2003; Stensaas, 1983). Thus, in both mammals and salamanders, many of the down-regulated gene expression patterns may reflect the

stochastic nature of cell survival at the injury site. However, we did observe significant down-regulation of several genes that are associated with glutamate metabolism and transport, that are up-regulated after CNS injury in mammals. This suggests the possibility that some genes are actively and uniquely repressed during salamander regeneration. It is also possible that the down-regulation of these genes is associated with the switch from neuronal signaling to neuronal axon outgrowth.

Identification of genes expressed differently between salamander regeneration and rat spinal cord injury

We compared genes that changed during early salamander spinal cord regeneration to gene lists that were compiled from microarray studies of spinal cord injury in rats. We acknowledge that this comparison is potentially confounded by several sources of variation including experimental, technical, statistical, tissue, and organismal differences. However, as we described above, some of the same genes that are expressed early after mammalian CNS injury are also up-regulated during spinal cord regeneration in salamander. If similar gene expression programs underlie homologous tissues, then comparisons of homologous tissues among distantly related organisms may filter conserved gene expression responses and help identify uniquely regulated genes. Some of the uniquely regulated genes from salamander are associated with regeneration in other organisms and tissues including amphibian limb regeneration (cytokeratin 18 [ck18], Corcoran and Ferretti, 1997; msx1, Beck et al. 2003; msx2, Carlson et al. 1998; and mmp9, Yang et al. 1999), fish tailfin regeneration (ck18 and periostin, Padhi et al. 2004), and annelid epimorphic regeneration (phosphoribosylaminoimidazole carboxylase, Myohara et al. 2006). Several other up-regulated genes are associated with mammalian liver regeneration, including *follistatin* (*fst*; Borgnon *et al.* 2005), *cystathionase* (Teshigawara et al. 1995), laminin alpha 1 (Kikkawa et al. 2005), transglutaminase 1 (Ohtake et al. 2006), and uncoupling protein 2 (Horimoto et al. 2004). Furthermore, eight cell cycle genes, a necessary process for true tissue regeneration, are present within this unique salamander gene list including cell division cycle 2, kinesin family member 11, and mitotic arrest deficient-like 1. Up-regulation of the same gene orthologs across multiple regeneration paradigms suggests that regeneration is definable across taxa and tissues by

distinct gene expression patterns. Further studies are needed to determine if a conserved group of genes function in molecular pathways that are required for regeneration.

Molecules that regulate morphogenic signaling

Morphogenic molecules such as sonic hedgehog (SHH), bone morphogenic proteins (BMPs), WNT factors, and fibroblast growth factors (FGFs) have been associated with regeneration because they establish positional identity, control cell proliferation, and regulate cell fate during development (Vergara et al. 2005; Whitehead et al. 2005; Schnapp et al. 2005; Niemann, 2006). In this study, we identified changed genes that code for extracellular molecules that participate in BMP, WNT, and FGF signaling. Fst, a protein that regulates dorsal-ventral patterning of the developing vertebrate CNS through BMP inhibition, is up-regulated during the first week of regeneration (Table 2.4; NUCC; Lee and Jessel, 1999). mRNAs for sfrp2, a secreted WNT antagonist that blocks ligand binding to frizzled receptors (Kawano and Kypta, 2003), is also highly up-regulated (Table 2.4; UUCD; Figure 2.4G-H). Furthermore, wnt5A demonstrates a 3.86 fold increase in expression (UCCC), suggesting a network of pro- and negative WNT signaling during regeneration. Lastly, fibroblast growth factor binding protein 1, a secreted molecule that sequesters FGF ligands from the ECM (Tassi et al. 2001) is down-regulated 6.42 fold at 24 hours (DCCC; Figure 2.4S-T). These large gene expression changes suggest that BMP, WNT, and FGF signaling pathways are all altered during early spinal cord regeneration. Further studies with each of these molecules and their corresponding binding substrates will be needed in order to assess their possible roles during regeneration.

Conclusion

The salamander's unique ability to regenerate complex body parts has long been recognized as an important model in developmental biology, however salamanders have received relatively little attention from researchers of mammalian spinal cord injury. Our study shows that genomic and bioinformatics resources are now available to associate gene expression changes with cellular and molecular aspects of natural spinal cord regeneration. The emerging salamander perspective on regeneration promises to extend existing research paradigms and may suggest novel therapies for CNS injury in humans.

Symbol	Function	Symbol Function	
UCCC = 64	Unk; n = 30	KIF11	cell cycle; M
MYO1B	actin binding	CCNB3	cell cycle; G2/M
TAGLN	actin binding	PLK1	cell cycle
ADFP	lipid metabolism	RFC2	cell cycle; S
TNFAIP8	anti-apoptotic	PCNA	cell cycle; S
ETHE1	anti-apoptotic	MCM7	cell cycle; S
LGALS1	apoptosis	STK6	cell cycle; M
GADD45G	apoptosis	CCNA2	cell cycle
GADD45B	apoptosis	CDC2	cell cycle
EFHD2	calcium binding	CHEK1	cell cycle
AGC1	unknown	MAD2L1	cell cycle; M
MYC	cell cycle	CDC20	cell cycle; M
CTSL	cysteine protease	AURKB	cell cycle; M
CTSK	cysteine protease	CDCA8	cell cycle; M
TUBB6	cytoskeleton	KRT18	cytoskeleton
FBP1	glycolysis	CALD1	cytoskeleton
B3GNT5	glycosylation	FEN1	DNA metabolism
TGFB1	growth factor	SLBP	mRNA processing
TYROBP	immune response	CKAP4	inflammation
SLC11A1	immune response	KPNA2	intracellular transport
MPEG1	immune response	VRK1	Kinase
CXCR4	immune response	COL12A1	ECM component
LGALS3BP	immune response	UCP2	neuroprotection
CYBB	immune response	CTSK	Protease
ANKRD1	injury response	OLFML2B	signal transduction
FTH1	iron homeostasis	CNIH4	Unknown
ATP6V0D1	proton transport	CCDC82	Unknown
C1ORF33	ribosomal	RPL38	ribosomal
MAP2K3	signal transduction	NDCC = 42	Unk; n = 22
MMP9	tissue remodeling	KRT7	cytoskeleton
MMP1	tissue remodeling	KIF21A	cytoskeleton
LYN	tyrosine kinase	SLC1A2	glutamate transport
TMEM49	unknown	SLC1A3	glutamate transport
FLJ2262	unknown	HSPA8	heat shock
WNT5A	WNT signaling	TTR	hormone transport
NNUC = 44	Unk; n = 14	SLC12A2	ion transport
GLRX	antioxidant	KCTD3	ion transport
LGALS3	carb. binding	FDPS	lipid metabolism
Table 2.1 contin	nued to right	Table 2.1 cont	tinued on next page

Symbol	Function	Symbol	Function
FAAH	lipid metabolism	NASP	histone transport
COL8A1	ECM component	COL2A1	ECM component
FBN2	ECM component	LAMA1	ECM component
GSTM4	metabolism	DAG1	ECM component
GRM3	neurotransmission	P2RY2	neuronal diff.
PADI3	protein metabolism	NUP107	nuclear transport
APCDD1	signal transduction	ANP32E	phosphotase inhib.
TJP1	signal transduction	PAICS	purine metabolism
APC	signal transduction	CTPS	pyrimidine bio.
MAPRE3	structural protein	SSB	mRNA processing
CRYAB	structural protein	EXOSC2	rRNA processing
NNND = 35	Unk; n = 17	RANBP1	signal transduction
PDCD4	apoptosis	TPRT	ubiquinone bio.
APP	apoptosis	C6ORF115	unknown
CCNI	cell cycle	CCDC58	unknown
SPTAN1	cytoskeleton	NNDC = 27	Unk; n = 8
SPTBN1	cytoskeleton	CDKN1C	negative cell prolif.
PYGM	glycogen metabolism	MLF1	cell differentiation
FABP7	lipid metabolism	KRT6L	cytoskeleton
CHPT1	lipid metabolism mitochondrial	ABLIM1	cytoskeleton glutamate
SLC25A4	transport	GLUD1	catabolism
GPM6B	neurogenesis	ATP1B3	ion transport
AHNAK	neurogenesis	FXYD3	ion transport
GSTM1	neuroprotection	PRKAG2	lipid metabolism
GSTP1	neuroprotection	COX4I2	metabolism
GABARAPL2	neurotransmission	BHMT	metabolism
PBP	protease inhibitor	PCBD	metabolism
CALCA	signal transduction	CKMT1A	metabolism
TRAPPC6B	transport	SERPINI1	neurogenesis
C110RF74	unknown	ABAT	neurotransmission
NNNU = 28	Unk; $n = 7$	TM4SF2	protein bio.
KIFC1	cell cycle; M	GNB5	signal transduction
CHC1	cell cycle; G2/M	POLR2L	transcription
RPA2	cell cycle	C6ORF110	unknown
MCM6	cell cycle; S	FAM79A	unknown
СТН	cysteine synthesis	NUCC = 24 Unk; n = 6	
CALD1	cytoskeleton	TAGLN	actin binding
Table 2.1 continued on right		Table 2.1 con	tinued on next page

Table 2.1 continued

Symbol	Function	Symbol	Function
ASAH1	apoptosis	PUS1	tRNA processing
UHRF1	cell cycle; S	FLJ36031	unknown
CDK4	cell cycle; G1/S	TGM1	injury response
KIAA0101	cell cycle; S	IFIH1	immune response
PPGB	cellular transport	UUCC = 12	Unk; n = 8
LGMN	cysteine protease	F13A1	wound healing
CSTB	protease inhibitor	MARCO	immune response
RRM2	DNA metabolism	FABP2	lipid metabolism
ANXA1	inflammation	GPNMB	negative cell prol.
THBS2	ECM component	UDCC = 10	Unk; n = 6
GLB1	metabolism	TFPI2	coagulation
ATP6V0E	proton transport	MMP1	tissue remodeling
RPL31	ribosomal	GIF	vitamin B transport
APOE	lipid metabolism	C8ORF4	WNT signaling
METTL2	ubiquinone biosynthesis	UCDC = 8	Unk; n = 3
CCDC43	unknown	HMOX1	heat shock
ANGPTL2	growth factor	MMP13	tissue remodeling
UNNN = 20	Unk; n = 7	MMP1	tissue remodeling
BYSL	cell adhesion	TIMP1	tissue remodeling
GLN3	cell cycle; G1/S	JUNB	transcription
DUSP1	heat shock	DCCC = 6	Unk; n = 5
IRF1	immune response	CYP2A13	metabolism
HSPA5	injury response	UCCN = 4	
SLC30A1	ion transport	LTB4DH	antioxidant
AGXT2L1	metabolism	TXNDC2	antioxidant
DKC1	ribosomal	TXN	antioxidant
RPS6KA1	signal transduction	BTBD3	protein binding
UNNN = 20	Unk; n = 7	DCNN = 4	Unk; n = 2
BYSL	cell adhesion	RGMA	axon guidance
GLN3	cell cycle; G1/S	FHL1	protein binding
DUSP1	heat shock	UCNN = 5	
IRF1	immune response	CES1	neuroprotection
HSPA5	injury response	USP2	protein breakdown
SLC30A1	ion transport	RAP2B	signal transduction
AGXT2L1	metabolism	SAT	polyamine homeostasis
DKC1	ribosomal	CD63	signal transduction
RPS6KA1 signal transduction		UNNU = 3	Unk; n = 2
Table 2.1 cont	tinued on right	Table 2.1 con	tinued on next page

Table 2.1 continued

Symbol	Function
NOL5A	ribosomal
NUCN = 3	
HRAS	cell proliferation
NSUN2	cell proliferation
LTBP1	TGF-beta signaling
UNND = 3	Unk; n = 3
NNUU = 2	
COL11A1	ECM component
POSTN	skeletal development
NUNN = 2	Unk; n = 2
UNUC = 2	Unk = 2
UDDC = 1	
TCN1	vitamin B transport
NDCD = 1	
AGR2	cell survival
NUCU = 1	
FLJ1472	unknown
UUCD = 1	
SFRP2	WNT signaling
UDNN = 1	
IL8RB	immune response
DCDC = 1	
KRT5	cytoskeleton
NDCC; DNN	NN; NDCN
NDNN; NND	DD; NNDN
Unk; $n = 1$	

Table 2.1 continued

Table 2.1. Gene symbols and functions for 360 changed salamander genes during the first week of spinal cord regeneration. Each column contains highlighted categories that describe gene expression patterns on day 1, day 3, day 5, and day 7 compared to basal gene expression (d0). Gene symbols are found under each category.

Pattern	Ν	Description	
UCCC	64	up D1, above baseline D1-7	
NNUC	44	baseline D1-3, up D5, above baseline D5-7	
NDCC	42	baseline D1, down D3, below baseline D3-7	
NNND	35	baseline D1-5, down D7	
NNNU	28	baseline D1-5, up D7	
NNDC	27	baseline D1-D3, down D5, below baseline D5-7	
NUCC	24	baseline D1, up D3, above baseline D3-7	
UNNN	20	up D1, baseline D3-D7	
UUCC	12	up D1 and D3, above baseline D1-7	
UDCC	11	up D1, down D3, above baseline D1-7	
UCDC	8	up D1, down D3, above baseline D1-7	
DCCC	6	down D1, below baseline to D1-7	
UCNN	5	up D1, above baseline D1-3, baseline D5-7	
UCCN	4	up D1, above baseline D1-5, baseline D7	
DCNN	4	down D1, below baseline D1-3, baseline D5-D7	
UNND	3	up D1, baseline D3-D5, below baseline D7	
UNNU	3	up D1 and D7, baseline D3-D5	
NUCN	3	baseline D1, up D3, above baseline D5, baseline D7	
NUNN	2	baseline D1, up D3, baseline D5-7	
NNUU	2	baseline D1-D3, up D5 and D7	
UNUC	2	up D1 and D5, baseline D3, above baseline D5-7	
DNNN	1	down D1, baseline D3-D7	
NDCD	1	baseline D1, down D3 and D5, below baseline D3-7	
NNDN	1	baseline D1-D3, down D5, baseline D7	
NNDD	1	baseline D1-D3, down D5 and D7	
NDNN	1	baseline D1, down D3, baseline D5-7	
NDCN	1	baseline D1, down D3, below baseline D5, baseline D7	
UUCD	1	up D1 and D3, down D7, above baseline D1-7	
UDNN	1	up D1, down D3, above baseline D1-3, baseline D5-7	
UDDC	1	up D1, down D3 and D5, above baseline D7	
NUCU	1	baseline D1, up D3 and D7, above baseline D3-7	
DCDC	1	down D1 and D5, below baseline D1-7	

Table 2.2. Distribution of gene expression patterns for 360 significantly regulated salamander genes during spinal cord regeneration.

Table 2.3. Primer sequences for QRT-PCR and in situ hybridization analysis. Forward ISH primers were appended with an SP6 RNA polymerase promoter and reverse primers with a T3 promoter at the 5' end (MC02287 and MC01277 are opposite). Promoter equences are SP6: 5'-ATT TAG GTG ACA CTA TAG AAG AG-3' and T3: 5'-AAT TAA CCC TCA CTA AAG GGA GA-3'.

Sal ID	Forward Primer	Reverse Primer		
MC03237	5'-GGCAAACTGCCTCTCCTT-3'	5'-CCTGTGGTTTTTCCCATGA-3'		
MC00341	5'-TTTCTGGACAGCCACTGC-3'	5'-TTTCTGGACAGCCACTGC-3'		
MC01765	5'-CCTGATGGGGGATCATCG-3'	5'-GTGCAGCCGGTACTTGTC-3'		
MC03278	5'-TGCATCAAAGCCAAGTCC-3'	5'-CCTCGGTTCACCTTGAAA-3'		
MC01583	5'-ATCCCGGAGAACAAGAGC-3'	5'-CATCCTTGAGCCAGAGCA-3'		
MC02459	5'-CAACGAGTGCATGAACGA-3'	5'-GCCAGACAGGTGGCCTA-3'		
MC01067	5'-TGAGACCAATGCCTTTGC-3'	5'- GAGCCCCAGAAGCAGAGT-3'		
MC01275	5'-GAGGCCAGAAAACCCAGA-3'	5'-CCGGTTTGGAAATTTCATC-3'		
MC01620	5'-TGGCCTGACCAGTAACGA-3'	5'-AAGTCCCATTCAGCACCA-3'		
MC02501	5'-TCCATCCATGTCCTCTGC-3'	5'-CTGTTTGCGATTGCATGA-3'		
MC01187	5'-CCAGGCGGCAGGTCAAGTCAAC-3'	5'-GTCGGCAAGGTCATCCCAGAGC-3'		
	in site behavior primers			

in situ hybridization primers

	In situ hybridization primers			
MC02287	5'-GCGCACGATGTCTTTCTGTA-3'	5'-GCGGTGGTACTCCAACTCAT-3'		
MC00365	5'- CGGCTTAGCCAGAAAATGAG-3'	5'- GTGGTTTCAGCAAAGCCAAT-3'		
MC00145	5'- GGCAAGATCATGGCTGAAAT-3'	5'- CAAGCCGCAGTATCATGTTG -3'		
MC02339	5'- TTTGGGAGCGAAGAAGAAAA-3'	5'- GAACGCTTGGTCTGGTAAGC-3'		
MC01583	5'-AAATTCCAATGCAAGTTGGG-3'	5'-ACGCCGTTCAGCTTGTAGAT-3'		
MC01706	5'-ACAACTCAGCCTGGACCATC-3'	5'- GTCTTTCACCCATTGCAGGT-3'		
MC00018	5'-ACATGGAGGACACCAAAAGC-3'	5'-AGCTCTGGAGTTCAGCTGGT-3'		
MC01275	5'-TATTAAGGGCCACGTTCCAG-3'	5'-TATGGAAGCCCTCCACAGAC-3'		
MC01182	5'-GTGCTGCAGGATGTCAAGAA-3'	5'-GCTGGTGTCTTCCTCTCTGG-3'		
MC01277	5'- AATCCAGCCACATCCTTCAC-3'	5'- GGCGCACCACATACACATAC-3'		

Table 2.4. Comparison of microarray versus real-time PCR estimates of fold change for ten genes that were quantified on d0 and d3. *In the list of 360 genes that met statistical and fold level criteria.

** Significant fold change difference between d0 and d3 according to real-time PCR (student's unpaired t-test, P < 0.05).

Sal ID	Gene Name	Microarray	Real Time
MC01620	SOX2	-1.7	-1.86**
MC02459	HES1	-2.15	-1.35
MC02501	SOX3	-1.92	1.14
MC03278	FST	3.09	3.45**
MC01765	CXCR4	3.17*	4.19**
MC01067	CD63	2.17*	4.77**
MC00341	TGFB1	3.69*	5.43**
MC01275	LGALS1	2.55*	10.5**
MC01583	SFRP2	17.95*	23.8**
MC03237	AGC1	56.84*	42.9**

Table 2.5					
Sal ID	Symbol	Sal ID	Symbol	Sal ID	Symbol
Up sal. and	rat; n=11	MC00222	GLB1	MC01940	ATIC
MC02474	LGALS3BP	MC01541	RGS10	MC00862	GSTA1
MC01211	HMOX1	MC02123	KIF11	MC04981	FKBP1A
MC01277	LGALS3	MC01119	СТН	MC03940	OTUB1
MC01006	ATP1B2	MC02344	GIF	MC01364	OCLN
MC01230	IRF1	MC02468	LAMA1	MC04214	DDIT4
MC03756	NIP7	MC01297	MAD2L1	D. rat, u	p sal; n=1
MC01354	GPNMB	MC02065	DAG1	MC02177	RAB11A
TC01086	TIMP1	MC04570	CRELD2	D. sal., N/	C rat; n=21
MC03344	CNIH4	MC01751	VRK1	MC00837	FAAH
MC04256	C8ORF4	MC02308	PLK1	MC00399	CYP2A13
MC00018	APOE	MC01068	CDC2	MC01099	COL8A1
Up sal, N/	C rat; n=46	MC00594	RRM2	MC02628	FXYD3
MC00197	TGM1	MC04538	VKORC1L1	MC00345	ADH1A
MC04407	SLC30A1	MC01709	TYROBP	MC02307	SERPINI1
MC00663	ARG2	MC05477	OLFML2B	TC00019	APC
MC02499	SGK	MC07609	MPS1	MC03555	MAST3
MC03162	LTB4DH	MC03420	ANKRD1	MC01037	BHMT
MC00337	LTBP1	MC02287	MMP9	MC00741	CSRP3
MC01521	RAC2	MC02182	ATP6V0D1	MC01503	PTPRR
MC00770	DKC1	gi 40809686	MSX2	MC04857	COX4I2
TC00995	RPA2	MC02833	POSTN	MC04494	MLF1
MC02792	NOL5A	MC00043	F13A1	MC01631	SPTBN1
MC03278	FST	D. rat, N/C	C sal.; n=18	TC00539	FABP7
MC01728	UCP2	MC01716	UBE2G1	MC05220	GSTM5
MC01639	SSB	MC02022	RNF14	MC04424	MID1IP1
gi 1139526	MSX1	MC02955	TMED2	MC00251	AK1
MC01194	GLRX	NP_002133	HOXA9	MC05114	C11orf74
MC04782	WDR75	MC00031	COL1A2	MC00841	FHL1
MC02823	PAICS	MC01720	UBE2L3	MC01620	SOX2
MC01405	PIM1	MC03866	LOC51255	Up rat, N/O	C sal.; n=41
MC02564	MPHOSPH10	MC00030	COL1A1	MC04731	HNRPD
MC01526	RANBP1	MC01092	COL2A1	MC04672	SEC13L1
MC04306	NUP107	MC00212	COL5A2	MC01058	CBR1
MC00145	KRT18	MC05202	YWHAZ	gi 5199142	LDHA
MC00535	RPL38	MC00423	IGF1R	MC01272	LDHB
Cont. in next column		Cont. in next	t column	Cont. on ne	xt page

Sal ID	Symbol	Sal ID	Symbol
MC03456	C9ORF10	MC00282	TP53
MC01484	PSMD8	Up rat, D	. sal.; n=1
MC04823	ELOF1	TC00219	APP
MC02100	HNRPAB		
MC00811	EIF4A1		
MC01697	TPT1		
MC00919	HSPB1		
MC00523	RPL28		
MC04974	HTRA3		
MC03977	NOLA2		
MC04373	RPL36A		
MC03396	CORO1C		
MC03842	HSPC152		
MC02234	GSTO1		
MC00196	TGFBI		
MC01786	DUSP11		
MC05214	ANXA11		
MC03882	FKBP11		
MC04970	LRRC42		
MC01722	UBE2N		
MC00545	RPS3		
MC02237	EIF4E2		
MC05309	EMP3		
MC01868	TRADD		
MC01933	ANXA7		
MC01392	PGD		
MC01095	COL4A1		
MC00332	GPX1		
MC00274	HEXB		
MC01711	RPS27A		
MC03904	CHRAC1		
MC03898	SMN1,SMN	12	
MC02876	HSPA8		
gi 49473435	NPY		
MC02061	CSPG3		
cont. in next of	column		

Table 2.5. Gene list for changed genes found on both the Ambystoma and rat U34 Affymetrix GeneChips. Genes were determined to be up-regulated, down-regulated, or not changed (N/C) according to the criteria set by each rat microarray study.

Figure 2.1. Volcano plots showing the number of genes selected as differentially expressed after spinal cord injury. False discovery rate was set to 0.01 (a) and the FWER set to 0.01 (b). Genes selected by each of these respective criteria are gray and non-selected genes are black.



Figure 2.2. Cross sections of regenerating spinal cords at days 1 (A-B), 3 (C-D), 5 (E-F), and 7 (G-H) days after tail amputation. Mayer's hematoxylin and Eosin were used to stain chromatin blue and cytoplasm shades of red. Photos were taken for sections within which the vertebrae were either fragmented or degenerating, and the central canal was clearly defined. A-B) At day 1, hemorrhaging is apparent, the white matter degenerates, and there are few neurons within the spinal cord grey matter. Leukocytes and erythrocytes are present near the injury plane (stained deep red) with apoptotic bodies (arrowheads) near the loosely interconnected ependymal cells (blue cells surrounding central canal; star). Bone degeneration is minimal (A: arrow) and there is proliferation of leukocytes at the injury site (B: arrow). Bone degeneration increases throughout the first week (A, C, E, G: arrows). E-H) A mesenchymous mass of cells (blastema) surrounds the regenerating spinal cord and ependymal cells project radial processes as the ependymal tube forms (D, F, H: arrows).



Figure 2.3. Two-dimensional plot of a principal component analysis (PCA) showing the relatedness of each GeneChip. JMP statistical software was used to perform PCA on 25 GeneChips. A Pearson's correlation coefficient matrix was made for 25 GeneChips using intensity values for 376 changed genes. Principal component 1 (PC1; 80.35% of the variation; eigenvalue 20.09) is displayed on the *x*-axis and Principal component 2 (PC2; 13.54% of the variation; eigenvalue 3.34) is displayed on the *y*-axis. The cumulative variation accounted for by PC1 and PC2 is 93.89%. Twenty-five principal components account for 100% of the variation in the dataset. Five biological replicate chips used for each of the five time points are enclosed by an oval to illustrate their close proximity. \blacklozenge : d0, n = 5; \blacksquare : d1, n = 5; \blacktriangle : d3, n = 5; \blacksquare : d5, n = 5; \blacksquare : d7, n = 5.



Figure 2.4. *In situ* hybridizations of d3 and d0 axolotl spinal cords. *In situ* hybridizations were performed on d3 (A-R) and d0 (S-T) axolotl spinal cords using DIG-RNA probes that correspond to significantly regulated genes from the microarray analysis. Anti-sense probes are represented in columns 1 and 3, and sense control probes in columns 2 and 4. *ck18* (A-B), *mmp9* (C-D), *Annexin A1* (E-F), and *sfrp2* (G-H) transcripts are all present in ependymal cells near the end of the regenerating spinal cord. Inflammatory-like cells that are found in the degenerating white matter are positive for *ApoE* (I-J), *Ferritin-heavy polypeptide* (K-L), *lgals1* (M-N), and *lgals3* (O-P). *Thioredoxin* (Q-R) transcripts are present in cells resembling ependyma as well as neurons of the injured spinal cord. *Fibroblast growth factor binding protein 1* (S-T) is highly expressed in lateral ependyma and a subset of neurons in the uninjured spinal cord. Bar = 100 µm.



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CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF *NOGO-A* IN A NATURALLY REGENERATING SALAMANDER (*AMBYSTOMA MEXICANUM*)

James R. Monaghan¹ and S. Randal Voss¹ ¹Department of Biology, University of Kentucky, Lexington, KY 40506 **Running Title:** Nogo-A in the salamander

S. Randal Voss PhD, 741 South Limestone Avenue, Lexington, KY 40536 Telephone: 859-257-9888 Fax: 859-257-5737 E-mail: <u>srvoss@uky.edu</u>

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Introduction

Salamanders are capable of axon regeneration after spinal cord injury. They apparently perform this task by circumventing various inhibitory factors that have been shown to limit CNS axon regeneration in mammals. Several different experimental approaches using mammals show that CNS myelin inhibits the growth of axons that would allow partial functional recovery after injury (Review by Schwab and Bartholdi, 1996; Huang et al., 1999; David and Aguayo, 1981; Caroni and Schwab, 1988). A wellestablished family of inhibitory factors in mammals are CNS myelin-associated molecules including myelin-associated glycoprotein (MAG; Li et al., 1996), oligodendrocyte-myelin glycoprotein (OMgp; Wang et al., 2002), and Nogo-A (GrandPre et al., 2000; Chen et al., 2000). In contrast to mammals, axons of lower vertebrates such as fish (Gaze, 1970; Review by Martin et al., 1994) and salamanders (Stensaas, 1977) successfully traverse the lesion site, leading to full functional recovery. Several explanations for the fish and salamander's regenerative abilities have been proposed such as the possibility that inhibitory myelin-associated molecules are not present in fish and salamanders, they are cleared from the area early after injury, the membrane topology or expression is different, or they are not inhibitory to the growth of axons in anamniotes (Schwab, 2004; Oertle et al., 2003; Klinger et al., 2004).

Among the myelin-associated inhibitory molecules, Nogo-A has been best characterized. The *nogo* gene (i.e. *reticulon 4*) is a member of the reticulon gene family. Reticulon-like genes are found throughout eukaryotes (Oertle et al., 2003) and code for proteins possessing a highly conserved membrane-spanning region at the carboxy terminus called the reticulon homology domain (RHD). The reticulon proteins are primarily located within the endoplasmic reticulon with multiple possible functions including synaptic plasticity (McGee et al., 2005), vascularization (Acevedo et al., 2004), apoptosis (Tagami et al., 2000; Watari and Yutsudo, 2003), amyloid- β regulation (He et al., 2004), and vesicle trafficking (Steiner et al., 2004). Mammalian Nogo (*mNogo*) is of primary interest to human health because it codes for a protein isoform (Nogo-A) found on the cell surface of oligodendrocytes that contributes to the lack of axon regeneration in the mammalian CNS (Review by Schweigreiter and Bandtlow, 2006; Dodd et al., 2005). The *nogo* gene is processed to form three primary protein isoforms named Nogo-A, Nogo-

B, and Nogo-C. All three isoforms contain a common carboxyl end that contains a highly conserved reticulon homology domain. The Nogo-A isoform is the largest of the three because it contains a large exon (2400 base pairs) that codes for a domain found to be inhibitory to axon outgrowth. The Nogo-A isoform was first identified as being the antigen of a monoclonal antibody (IN-1) capable of neutralizing the inhibitory nature of mammalian CNS myelin (Caroni and Schwab, 1988; Chen et al., 2000; GrandPre et al., 2000). Since this time, the inhibition of Nogo-A and its downstream signaling mechanisms have become worthy candidates for clinical application to human CNS trauma (Buchli and Schwab, 2005; Freund et al., 2006, 2009). Furthermore, a role of Nogo-A in nervous system development other than myelin inhibition is starting to be recognized including peripheral nerve axon guidance, neural differentiation, neurite formation, and cortical development, (Brösamle and Halpern, 2008; Caltharp et al., 2007; Mingorance-Le Meur et al., 2007; Richard et al., 2005, 2009; O'neill et al., 2004).

Understanding the evolutionary history of the Nogo-A isoform may broaden our understanding of the normal roles Nogo-A has in the CNS. Recent work has shown that the Nogo-A isoform is present in frogs (Klinger et al., 2004) but not fish (Diekmann et al., 2005). The frog loses the ability to regenerate some CNS axons after metamorphosis, while fish maintain regenerative ability throughout life; a characteristic that may be possible because of the absence of Nogo-A. Furthermore, frog CNS myelin and not fish or salamander myelin are inhibitory to axon growth *in vitro* (Lang et al., 1995). The identification and characterization of this isoform in a vertebrate capable of CNS axon regeneration throughout life has yet to be observed.

Here we report on the identification of the amniotic ortholog of *Nogo-A* in the salamander *Ambystoma mexicanum* (*axNogo-A*). We demonstrate orthology via sequence similarity and genetic linkage analysis. We show that *axNogo-A* mRNAs are enriched in nervous system tissue throughout development. We also find that *axNogo-A* is expressed in the nervous system well before myelin formation, suggesting an early role in nervous system development. Thus, while the CNS of the Mexican axolotl is permissive to axon growth, it contains transcripts of *axNogo-A*, an inhibitory molecule that is structurally similar to *mNogo-A* orthologs from vertebrates that lack regenerative ability.

Methods and Materials

Animals and RNA extraction

Wild-type adult axolotls and albino embryos were obtained from the Ambystoma Genetic Stock Center (University of Kentucky) or the Voss lab and housed at 20-22°C in modified Holtfretter's solution. Staging was according to Beetshcen and Gautier classification (Armstrong and Malacinski, 1989). Animals were anesthetized using 0.01% benzocaine (Sigma) before tissue collection. Tissue extraction was performed from adults measuring 10-15 cm snout to cloaca and snap frozen in liquid nitrogen. RNA isolation was performed using TRIzol reagent according to manufacturer instructions, followed by an Rneasy column, quantified by spectrophotometry, and quality-assessed by formaldehyde-gel electrophoresis.

Identification and Mapping of axNogo-A

Primers 5'-CTC CAC GCC CAG AGA TTG-3' (F.1) and 5'-ACT GAG AGC AGG GCC AAT-3' (R.1) were used on axolotl brain and spinal cord for long distance PCR. ExTaq long distance polymerase (Takara) was used with PCR parameters: 94°C, 30 seconds, 65°C 1 minute, 72°C 5 minutes for 30 cycles. Nested PCR was performed using a 1:1000 dilution of the above PCR product and 5'-AGC CAC CCT TAG CAG GAG AC-3' (F.2) and 5'CCA ATG CAA TGT ACG CTG AC-3' (R.2). PCR parameters were 35 cycles at 94°C for 45 sec, 65°C for 45 sec, and 72°C for 1 min (fig 1b). PCR product was gel isolated using QIAquick gel isolation kit. A clone representing 1300bp of the 3' end was also isolated. Both were sequenced using Bigdye and assembled using Seqman II DNASTAR software. Mapping of *axNogo* was performed according to Smith et al. (2005) using the following primers: 5'-GAA GAC GAT GAA ACG ACT GAG AG-3', 5'-CCG CAG CGC CAG GTG ATG GTC GAG GAA-3', and 5'-GGC TTC TTC CTC TCC TCA AAA G-3'.

Sequence Alignment and Analysis

Rattus novegicus, Mus musculus, Homo sapien, Gallus gallus, and *Xenopus laevis* Nogo-A sequences are found at NCBI (NP_114019, NP_918943, NP_065393, NP_989697, and AAQ82646, respectively). Sequence assembly and open reading frame prediction of axolotl *nogo-A* was performed with DNASTAR. Sequence alignment was performed using CLUSTALW and shaded for similarity using BOXSHADE. Protein motifs were predicted using Motifscan (Pagni et al., 2007). Hydrophobic regions were predicted using Tmpred, TMHMM (Krogh et al., 2001), and DAS transmembrane prediction programs (Cservo et al., 1997).

RT-PCR

cDNA was produced using 1ug total RNA and the Bio-Rad ISCRIPT cDNA synthesis kit. Templates for PCR included 10ng cDNA. PCR primers for *nogo-A* were forward (5'-TGA TGG AAA AAC TGG GGA GA-3') and reverse (5'-GGG GAT GTA CGG AGT CTC AA-3') giving a product of 991bps. *EF-1a* primers are described elsewhere (Carlson et. Al., 2001). Primers have a modified T7 and T3 promoter added onto each for subsequent digoxygenin RNA probe production. *MBP* primers were forward (5'-TAA TAC GAC TCA CTA TAG GGA GGC CAG AAC CTT GGA ATC TGA-3') and reverse (5'-AAT TAA CCC TCA CTA AAG GGA GGA CAA CGG GGT TAT CCT CAA-3') giving a PCR product of 344bp. PCR parameters for *nogo-A* were 28 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec, 24 cycles using the same parameters for *EF-1a*, and 33 cycles at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec for *MBP*. Sul of PCR product was analyzed on a 2% gel and visualized using a Gel Logic 100 Imaging system. Relative PCR product was compared with the control *EF-1a*.

In situ Hybridization

PCR products produced using primers F.3 and R.3 was cloned into a Promega pGEM-T vector by the A-tailing procedure. *Nogo-A* digoxygenin RNA probe was produced using PCR templates produce from M13 forward and reverse primers and T7 or SP6 polymerase as described in Roche DIG-RNA labeling manual. Briefly, reactions were incubated 2 hours at 37°C, stopped by adding 2ul 200mM EDTA, ethanol precipitated, and quantified using formaldehyde-gel electrophoresis. Tissue samples were fixed in 4% paraformaldehyde/1XPBS overnight, decalcified in 0.5M EDTA/1xPBS for 4 days when bone was in the tissue, cryoprotected by immersion in 10% sucrose/1xPBS for 1hr, 20% sucrose/1xPBS, followed by 30% sucrose/1xPBS for 3hr to overnight. Tissues were then

mounted in OCT medium, sectioned at 16um, and stored at -80°C. Sections were processed, hybridized, and washed as described elsewhere (Hirota et. Al., 1992). Overnight incubation with NBT/BCIP gave sufficient signal for specific detection. Sections were mounted with Permount, imaged using an AX-80 Olympus microscope, and photographed using an Olympus DP-70 camera.

Results and Discussion

Cloning and identification of axNogo-A

It is of particular interest to understand the evolutionary history of the long exon found within *nogo*. It has been suggested that the *nogo-A* isoform is not present in amphibians or fish, which is in accordance with their ability to regenerate axons after CNS injury (Schwab, 2004). Indeed, the *nogo-A* isoform is not present in the teleost fish genome (Diekmann et al., 2005). In contrast, Klinger et al. has shown that the *nogo-A* isoform is present and expressed in the CNS of anuran, *Xenopus laevis* (2003). In this study, we unambiguously identify the mammalian *nogo* ortholog in the axolotl and show that the *axNogo-A* isoform is present and expressed.

We identified a 1577 base pair contig from the *Ambystoma* Expressed Sequence Tag Database (http://www.ambystoma.org) with high nucleotide sequence identity to human reticulon 4 (BLASTX; 2e-74). The contig contained the presumptive full-length coding sequence of the *rtn-4B/nogo-B* mammalian isoform; it lacked a large terminal exon characteristic of the *nogo-A* isoform (Figure 3.1a). To obtain the characteristic *nogo-A* exon, PCR was performed with primers F.1 and R.1, producing two fragments at 2625bp and 183bp. These products were sequenced to identify the *axNogo-A* large exon. The nested primers F.2 and R.2 were used to amplify the *axNogo-A* region yielding a 2349bp PCR product (Figure 3.1b). The resulting DNA sequence was assembled with the *nogo-B* contig to yield a 4024bp sequence with a predicted open reading frame of 1158 amino acids. The predicted protein is highly identical to Human RTN4-A/Nogo-A (BLASTP; 3e⁻¹⁵⁷). To provide additional evidence that our sequence was orthologous to amniote *nogo-A*, we mapped the sequence to linkage group 13 (LG13) in the *Ambystoma* genome (Smith et al., 2005). Few syntenic regions have been identified between LG13 and the human chromosomal region where *Nogo* is located (Hsa 2p16.3). Therefore, we compared

LG13 to the position of *nogo* in the chicken genome, which shows greater overall conservation in gene order to *Ambystoma*. Eleven *Ambystoma* LG13 contigs, including *axNogo-A*, map to chicken chromosome 3. This strongly suggests that *axNogo* is the ortholog of the amniote *nogo*. Thus, two separate lines of evidence, sequence similarity and gene mapping, indicate that the assembled contig is the amniote ortholog of *nogo-A* in Mexican axolotl (here after referred to as *axnogo-A*).

The presence of *nogo-A* in both urodele and anuran amphibians indicate that its origin is basal to their closest ancestor, believed to be during the Jurassic period approximately 200 million years ago (Zardoya and Meyer, 2001). A loss or gain of function in the inhibitory property of myelin must have occurred during the divergence of these organisms because adult anuran spinal cord myelin has inhibitory activity *in vitro*, while axolotl myelin is permissive to axon growth (Lang et al., 1995). It is also possible that the myelin isolated from the axolotl in Lang et al. was from a region that does not express inhibitory myelin molecules. We propose that it is not the presence or absence of the *nogo-A* isoform contributing to this loss or gain of function in these organisms. It is possible that inhibition was gained independently in anurans or inhibitory myelin is a basal property to tetrapods, which is lost in urodele amphibians. It would be interesting to pursue the evolution of *nogo-A* and inhibitory myelin further in lobe-finned vertebrates as this would better resolve the ancestral amphibian condition.

Comparison of vertebrate Nogo-A Protein Sequences

Several conserved domains were found between salamanders and other vertebrates. The most striking resemblance is within the RHD. The RHD is located at the carboxyl end and is much more similar than the 970 aa amino end of the rat Nogo-A predicted protein sequence (87% compared to 34%, respectively). The RHD contains two >34 aa predicted transmembrane domains around a conserved 66 aa domain, the extracellular Nogo-66 domain that inhibits neurite outgrowth through binding to Nogo receptor (NgR; Fournier et al., 2001). The RHD in teleost fish are also highly similar and seem to have similar transmembrane regions, suggesting similar orientation and function (Diekmann et al., 2005). Interestingly, a mammalian NgR ortholog is also present in fish (Klinger et al., 2004) and morpholino knockdown of NgR or the Nogo-66 region causes

defects in peripheral nervous system axon outgrowth to the head and lateral line (Brösamle and Halpern, 2008). These results suggest that Nogo-66/NgR signaling is a conserved pathway in vertebrates that regulates axon growth during development. A second conserved motif in the RHD is a dilysine endoplasmic reticulum retention motif, giving indication of the primarily intracellular location of Nogo proteins (Chen et al., 2000; Grandpre et al., 2000; Van der Haar et al., 2003). The intracellular role of Nogo-A is poorly understood in any vertebrate.

The ~800 aa Nogo-A-specific region (NAS) is more divergent with similarity ranging from 32-36% between all vertebrates analyzed (Figure 3.2). The carboxyl 24 aa of human NAS binds to NgR through an Isoleucine found at human amino acid 995 (Hu et. Al. 2005). All other animals examined, except the frog, have a Valine at this position (Figure 3.2). A highly inhibitory region found within the rat NAS termed Δ 20 (Oertle et al., 2003) shows higher similarity (45%) between rat and axolotl compared to the entire NAS (34%). This suggests that the salamander Δ 20 region may also have inhibitory properties similar to the rat Δ 20 domain. It will be important to identify whether the amphibian Δ 20 domain has inhibitory properties in order to know whether Δ 20 inhibition was a basal character of Nogo-A or a recently derived character in mammals.

A second unknown receptor binds within the first 172 amino acids of the rat nogo-A/B region inhibiting fibroblast spreading (Oertle et al., 2003). This region is also responsible for migration of endothelial and vascular smooth muscle cells *in vitro* and vascular remodeling *in vivo* (Acevedo et al., 2004). A large proline-rich region is predicted in both amphibians and mammals within this Nogo-A/B amino region. There are multiple SH3-ligand motifs found within the proline-rich region suggesting proteinprotein interactions. It is conceivable that this region is responsible for binding to some of these proteins and receptors in mammals as well as amphibians, mediating similar signaling. Overall, low sequence similarity within this region is seen except for the proline-rich motifs.

Nogo-A expression

To examine how *nogo-A* expression changes during development, we performed RT-PCR using RNA isolated from whole embryos. Strong *axNogo-A* expression started

between stages 27 and 36 (Figure 3.3b). The tail bud embryo starts to enlarge during this developmental window, which correlates with the differentiation of neuronal circuits in the spinal cord. Twitching also starts during these stages meaning that motor neurons have innervated the musculature of the embryo (Armstrong and Malacinski, 1989). To illustrate the independent regulation of nogo-A from the onset of myelination, RT-PCR analysis was performed using primers specific to the myelin marker, *myelin basic protein (MBP)*. axNogo-A expression starts well before the onset of MBP transcription around hatching (Figure 3.3), suggesting that Nogo-A has a role in development before oligodendrocyte differentiation and myelination. Expression of Nogo-A in neurons before the onset of myelination has been observed in chickens (Caltharp et al., 2007; O'Niell et al., 2004), frogs (Klinger et al., 2003), rodents (Huber et al., 2002; Tozaki et al., 2002; Mingorance et al., 2004), and humans (O'Niell et al., 2004). Nogo-A knockout mice show mild deficits in neuronal migration during cortical development (Mingorance-Le Meur et al., 2007) and increased neuronal plasticity (Montani et al., 2009). The expression of Nogo-A in neurons before myelination in multiple tetrapods strongly supports a conserved neuron-specific role during development. To define the localization of axNogo-A during development, whole-mount in situ hybridization was performed on animals at developmental stages 36 and 38 (Figure 3.4), stages when Nogo-A mRNA is expressed (Figure 3.3). axNogo-A mRNAs localized to multiple areas of the CNS including the developing brain, spinal cord, and eye. Expression was also evident in a specific location near the developing spinal ganglia. It is unknown if the developmental role of axNogo-A is related to Nogo-66/NGR or the NAR. Morpholino knockdown studies of all Nogo isoforms compared to the Nogo-A isoform should resolve the developmental function of Nogo-A. Regardless, the broad expression of Nogo-A in the developing nervous system of axolotls and other vertebrates strongly suggests a conserved role in early nervous system formation.

The adult distribution of Nogo-A was examined using RT-PCR and *in situ* hybridization. RT-PCR showed that *axNogo-A* mRNA was most abundant in brain, eye, spinal cord and heart tissues (Figure 3.3a). These results are consistent with observations found in other amphibians and mammals (Figure 3.3a; Klinger et. Al., 2004; Oertle et al., 2003). *In situ* hybridization localized mRNAs to neurons and oligodendrocytes according to location in the brain, spinal cord, and dorsal root ganglia (Figure 3.5 and 3.6). Some

neuronal populations had much stronger staining than others. To localize these regions in the brain, darkly stained cells were highlighted on cross sections of the adult brain (Figure 3.5). Heavily stained regions corresponded to the medial pallium in the telencephalon, habenula in the epithalamus, outer neurons of the optic tectum, and neurons of the hindbrain. AxNogo-A transcripts were also localized to areas of differentiated neurons throughout the spinal cord (data not shown), with strongest expression in the ventro-lateral portion of the grey matter (motor neurons). Strong staining was also observed in neurons of the dorsal root ganglia, as was observed in mammals (Huber et al., 2002). The conservation in expression between salamanders and mammals suggest a conserved role in neuronal development.

An interesting finding of the study was that Nogo-A transcripts were localized to cells located in regions of oligodendrocytes. Figure 3.5 and 3.6 show that MBP and nogo-A staining do not overlap in the forebrain, but are expressed in cells located in the same regions as oligodendrocytes in the hindbrain and spinal cord. No Nogo-A staining was observed in cranial nerves expressing high levels of MBP, suggesting that Nogo-A is not expressed in Schwann cells. The large number of Nogo-A positive cells located in the white matter of the CNS strongly suggest that axNogo-A is expressed in oligodendrocytes in some portions of the adult CNS. This staining pattern was surprising because IN-1 antibody, an antibody that binds to the amino arm of Nogo-A (Fiedler et al., 2002), was found to stain myelinated axon tracts in X. laevis, but was not observed in axolotl myelin (Lang et al., 1995). This suggested that Nogo-A is not expressed in axolotl oligodendrocytes, explaining the growth permissive nature of axolotl myelin. It may be that IN-1 immunoreactivity is independent of the expression of Nogo-A mRNA and more related to a conformation found at the cell surface, as suggested elsewhere (Klinger et al., 2004). It is also possible that this cell surface conformation is necessary for inhibitory activity in vivo. Understanding what this conformation may be and how it interacts with other CNS components is necessary to design more effective therapeutics against the inhibitory activity.

One hypothesis why the axolotl possesses such high CNS regenerative capacity is that Nogo-A is down regulated following injury. Figure 3.7E shows that mRNA levels decline only slightly during the first week of injury (Monaghan et al., 2007). Some

neurons can be seen expressing Nogo-A at the injury plane following tail amputation (Figure 3.7F and G). A similar gene expression pattern is observed after spinal cord lesions in rats suggesting that Nogo-A is not a lesion-induced inhibitor of axon growth (Huber et al., 2002). A second hypothesis of how the axolotl can circumvent myelin inhibitors is that Nogo-A protein is cleared from the injury site following injury. This hypothesis is supported by the observation that tenascin-R and MAG, both potent inhibitors of neurite outgrowth in mammals, are cleared from an optic nerve injury lesion within 8 days after injury in newts (Becker et al., 1999). Future studies are needed to address the role of Nogo-A in neurons and why salamander myelin is not inhibitory even though it likely contains Nogo-A.

Conclusions

We identified the ortholog of human *reticulon 4/nogo* in the Mexican Axolotl (*Ambystoma mexicanum*). We illustrate that the long exon that is diagnostic of *nogo-A* and encodes a highly inhibitory region in humans, is also coded for in the axolotl. We predict protein structure and possible motifs, and characterize developmental and adult mRNA expression. This is the first identification and characterization of *nogo-A* in a vertebrate capable of functional regeneration of spinal cord axons throughout life.

Figure 3.1 A) Cartoon of the predicted axolotl Nogo-A protein. Grey shading represents the Nogo-A-specific portion of the protein. Black shading represents the two inhibitory regions as predicted through homology with rat Nogo-A. Arrows indicate the location of primers designed in the study. B) Gel electrophoresis of the nested RT-PCR product as produced using primers F.1/R.1 followed by F.2/R.2.



Figure 3.2. Predicted protein sequence alignment and motif comparison of several tetrapod vertebrate Nogo-A proteins. Black shading represents 100% homology and grey shading 100% similarity. Predicted transmembrane regions are underlined with dark, black line. Presumptive inhibitory regions, human isoleucine at aa 995, and endoplasmic reticulon-retention motifs are boxed. The nogo-A specific region is indicated with black arrowheads. Predicted glutamic acid-rich regions are underlined with a thin grey line and proline-rich regions with multiple carrots ($^{\wedge\wedge\wedge\wedge}$).

Axolotl Rat Human Mouse Chicken frog	1 COPPTORPSING COPPAGENERPEPPOROVAVELELEEDODITERREWEEDORCOCVLDLIGGAGDFSHEPFNARFLUGSISHAAD FINA 1 TE-OSSIVE TDSFREPFARYOFVIPUEDEEDEDETERFEDERDEDEELEVIEWRAACISAANVPA-AAAFLDFSSIVAFANDEADEVIEWEENDE 1 TE-OSSIVE A	KPAWEESA OFSWERSP OFSWERSP OFSWERSP RGAWKDSL
Axolotl Rat Human Mouse Chicken frog	106 PESTOPSAPTEETVPPORAPTARVITPLAPLAPASPPRPEIARRACP CONTENAPEARS POLICIES	VASILHES FPSULLE FPSULLE FPSULFE FIAUPLD FASULLOS
Axolotl Rat Human Mouse Chicken frog	203 SATEPLISKOVYPNOJLADSELPOPPLAGDLOBELGOVILESTS DALIVENPDTRANLOGOTE OPALAVPLKSETLIVHTANEKLS SEEGEPLHOIAAPKHO 220 AATT LISKOTVSTKORTICKLÄNVSSEGTI DTLIKLSKELEERATNFFVAR-DLEPSDEVSE OSSFKOSFKOSFKOSKLUVENTKEVIVASKOKD-LV-SAALH 232 AATT LISKOTSKORTICKLÄNVSTUPPTOLIOUVSENSKUSEKATLIIA-DLEPSDEVSE OSSFKOSFKOSFKOSSKULVENTKEVIVASKOKD-LV-SAALH 216 AATT LISKOTVSTKORTICKLÄNVSTUTTET DTLIKLSKELEERATNFFVAR-SEESVESE SESSFKOSFKOSFKOSSKULVENTKEVIVASKOKD-LV-SAALH 22 AATT LISKOTSKORTICKLÄNVSTUPTOLIATIOTI DTLIKLSKELEERATNFFVAR-SESVESSE SESSFKOSFKOSSKULVENTKEVIVASKOKD-LV-SAALH 23 AATT LISKOTSKORTICKLÄNVSTUPTOLIPACITI DTLIKLSKELEERATNFFVAR-SESVESSE SESSFKOSFKOSSKULVENTKEVIVASKOKD-LV-SAALH 24 AATT LISKOTSKORTICKLÄNVSTUPTOLIPACITI DTLIKLSKELEERATNFFVAR-SESVESSE SESTESSE SAALVENTKEVIVASKOKD-LV-SAALH 25 AATT LISKOTSKORTICKLÄNVSTUPTOLAATSLORVAN VAN VAN VAN VAN VAN VAN VAN VAN VAN	PVLFDEPF POESP OOELPTAL POESPATL SPETPEDL P
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Figure 3.3. RT-PCR of Nogo-A in adult tissues and development. A) *Nogo-A* in adult tissues compared to expression in adult human and *X. laevis* tissues. The top band is the specific Nogo-A PCR product. The bottom band is the control EF-1 alpha PCR product. The + and – found below indicate expression in humans and Xenopus modified from (Klinger et al., 2004) B) Expression of Nogo-A during development. Staging was according to Beetshcen and Gautier classification. C) Expression of EF-1 control gene during development. D) Expression of myelin basic protein mRNA during development. Notice that MBP is not expressed until hatching. H = heart, B = brain, E = eye, Sp = spleen, Sk = skin, G = gill, SC = spinal cord, Lu = lung, M = muscle, Li = liver.



Figure 3.4. Whole mount in situ hybridization of stage 38 embryos using an antisense probe against *axNogo-A*. Three different embryos are represented. The embryo on the left is viewed from the side, middle embryo is viewed from above, and right embryo viewed from the front. Purple staining represents locations of Nogo-A mRNA. Arrows indicate positive staining in locations of the possible lateral line ganglia. Arrowheads indicate the eye premordia.



Figure 3.5. Tracing of MBP and Nogo-A staining throughout the brain. The left hemisphere represents positive staining of cells probed with anti-MBP. The right hemisphere represents cells with strong expression of *axNogo-A*. Some light staining is not presented in the Nogo-stained sections because it was found in most neuron-containing regions. The images correspond from the middle of the telencephalon (A) to the hindbrain (E). Dots represent positive cells. The black areas on the outside of the left hemisphere are deeply stained cranial nerves. No staining was observed in cranial nerves for Nogo-A. Close-up images can be seen in Figure 3.6.


Figure 3.6. Positive staining for Nogo-A and MBP in the brain represented in Figure **3.5.** A, C, and E represent antisense Nogo-A probe staining. B, D, and F represent sense Nogo-A probe staining. G and I represent antisense MBP probe staining. H and J represent sense MBP probe staining. Sections correspond to the top image in Figure 3.5 (A, B, G, and H), second from bottom image in Figure 3.5 (C, D, I, and J), and bottom image in Figure 3.5 (E and F). No MBP staining was seen in the medial pallium of the telencephalon. Scale bar = 200um



Figure 3.7. Nogo-A and MBP positive staining in spinal cord and DRG tissue. A) cells with positive Nogo-A staining. The arrow indicates possible motor neurons. Notice the location of possible oligodendrocytes in the white matter. B) Positive MBP staining in the adult spinal cord. C) Positive Nogo-A staining in an uninjured adult tail. Arrows highlight possible motor neurons and the star indicates a dorsal root ganglia. D) Gene expression pattern of Nogo-A in the spinal cord following tail amputation (Monaghan et al., 2007). Minimal, but significant down-regulation was observed. E and F show Nogo-A staining in regenerating tails 7 days following amputation. Positive staining is still observed in motor neurons (arrows) and DRGs (stars).



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CHAPTER 4

MICROARRAY AND CDNA SEQUENCE ANALYSIS OF TRANSCRIPTION DURING NERVE-DEPENDENT LIMB REGENERATION

James R Monaghan¹, Leonard G Epp², Srikrishna Putta¹, Robert B Page¹, John A Walker¹, Chris K Beachy³, Wei Zhu⁴, Gerald M Pao⁴, Inder M Verma⁴, Tony Hunter⁴, Susan V Bryant⁵, David M Gardiner⁵, Tim T Harkins⁶, and Stephen R Voss¹

¹ Department of Biology and Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40506

² Department of Biology, Mount Union College, Alliance, OH 44601

³ Department of Biology, Minot State University, Minot, SD

⁴ The Salk Institute for Biological Studies, La Jolla, CA 92037

⁵ Department of Developmental and Cell Biology, and The Developmental Biology Center, University of California Irvine, Irvine, CA 92697

⁶ Roche Applied Science, Indianapolis, IN 46250

*Corresponding author

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Introduction

Salamanders are fascinating vertebrate organisms because they routinely regenerate complex tissues. In only a few weeks after losing a piece of limb to a hungry predator or scalpel-welding scientist, a salamander perfectly reforms the missing structure. In the early history of salamander regeneration research, scientists innovated elegant experimental designs to probe the anatomical basis of regeneration (Dinsmore, 1991). More recently and in parallel with the discovery of conserved, regulatory genes and developmental pathways among metazoans, scientists have focused attention on candidate molecules and signaling pathways whose functions were deduced first from studies of model organisms. In particular, much research has been devoted to understanding aspects of limb regeneration associated with wound healing that recapitulate limb development; this strategy has yielded many useful insights and molecular probes (Carlson et al., 2001; Christensen et al., 2001, 2002; Endo et al., 2004; Kumar et al., 2004; Mercader et al., 2005; Schnapp et al., 2005; Levesque et al., 2007; Satoh et al., 2007, 2008; Theodosiou et al., 2007; Villiard et al., 2007; Ghosh et al., 2008). Although it is clear that key regulatory molecules play important roles in the development of all organisms, it is not clear that a framework for understanding regeneration can be constructed using a generic and limited molecular toolkit. There is a need to go beyond candidate molecules and use unbiased approaches to characterize the molecular complexity underlying salamander regeneration.

Recent research resource development for the Mexican axolotl now allows classic regeneration experiments to be re-examined with powerful and unbiased genomic approaches. One particularly elegant experiment performed almost two hundred years ago showed that salamander limb regeneration requires the presence of peripheral nerves. Todd found in 1823 (Todd, 1823) that limb regeneration does not occur if the sciatic nerve of the hindlimb is severed shortly before or immediately after, a more distal limb amputation. Subsequent research showed that the brachial nerves entering the forelimb are required to promote limb outgrowth and patterning of a new limb (Singer, 1952, 1978). Within a few days after limb amputation, cells proximal to the amputation plane dedifferentiate and accumulate to form a blastema. Blastema cells proliferate and progressively differentiate during regeneration to give rise to all mesodermal structures of a typical vertebrate limb (Bryant et al., 2002). Upon amputation, nerve fibers in the

vicinity of the amputation plane extend into the blastema and play a supportive role in cell proliferation (Mescher, 1996; Satoh et al., 2008). Transection of the spinal nerves that enter the limb in the axolotl leads to a decrease in cycling cells and there is resorption of distal tissues of the amputated limb. Histological and cell proliferation analyses suggest that early cellular events are similar between denervated and innervated limbs, but denervated limbs are incapable of blastema formation because they do not support significant cell proliferation and outgrowth (Bryant et al., 1971; Mescher and Tassava, 1975; Geraudie and Singer, 1981; Olsen et al., 1984a, b; Barger and Tassava, 1985).

Although limb regeneration is a complex developmental process, nerve dependency and other aspects of regeneration have often been conceptualized as having a simple molecular basis, involving relatively few regulatory factors. For example, Ferretti and Brockes (1991) hypothesized that in the absence of nerves, Schwann cells produce an inhibitory factor that prevents blastema cell proliferation. This mechanism is supported by experimental results although the hypothetical factor has not been identified (Irvin and Tassava, 1998; Tassava and Olsen-Winner, 2003). Alternative mechanisms for nerve dependency have also been proposed for several factors with growth promoting effects (Brockes and Kintner, 1986; Smith et al., 1995; Mullen et al., 1996; Mescher et al., 1997; Wang et al., 2000). A recent study identified newt anterior gradient protein (nAG) as a blastema cell growth-promoting factor in vitro, whose over-expression was sufficient to rescue regeneration of denervated and amputated limbs in vivo (Kumar et al., 2007). Most recently, nerve-dependent expression of the transcription factor *sp9* has been identified as an early marker of dedifferentiation of the wound epithelium and the initiation of limb regeneration (Satoh et al., 2008). While considerable progress has been made in investigating the functions of candidate regulatory factors and signaling pathways, a broader systems-level perspective is needed to understand why multiple aspects of limb regeneration are dependent upon the presence of a nerve.

Genomic tools are now available that allow global characterization of the regeneration process in salamanders. Expressed sequence tag (EST) information has facilitated the development of an *Ambystoma* salamander Affymetrix microarray platform (Monaghan et al., 2007; Page et al., 2007; Stewart et al., 2008). This platform and a high-throughput 454 cDNA sequencing approach were used in this study to compare transcript

abundance among uninjured limbs, regenerating limbs, and limbs denervated at the time of amputation. The results show that innervated (NR) and denervated (DL) limbs exhibited similar (but not identical) gene expression patterns at 5 days post amputation (dpa) but then diverged as a blastema formed under the influence of nerves. The results are discussed within the context of previous studies of nerve-dependency, highlighting specific genes and biological processes that are associated with blastema formation and outgrowth, and more generally, the salamander's unparalleled ability to regenerate limbs.

Materials and Methods

Animal procedures

Mexican axolotls were obtained from the *Ambystoma* Genetic Stock Center at the University of Kentucky. Siblings were reared individually under *ab libitum* conditions to 60-70 mm snout to vent length. The 3rd, 4th, and 5th spinal nerves that enter the left limb were severed at the brachial plexus behind the shoulder. Denervated left and innervated right limbs were amputated at mid-stylopod and allowed to regenerate for 5 and 14 days. Animal care and use procedures were approved by the University of Kentucky Internal Animal Care and Use Committee.

Histology

Limbs were collected at 5 and 14 dpa and fixed in 4% paraformaldehyde, 1x PBS overnight at 4°C. Tissues were cryoprotected in sucrose, embedded in TissueTek, and sectioned at 16µm. Eosin Y and Gill's hematoxylin #2 (Sigma-Aldrich, St. Louis, MO, USA) were used to stain cytoplasm and nuclei. DIC and brightfield images were taken on an Olympus AX80 microscope (Center Valley, PA, USA).

RNA extraction and microarray analysis

DL (left) and NR (right) limbs were collected approximately 1 mm proximal to the amputation plane 5 dpa and 14 dpa. Nine animals were used for each time point and limbs were pooled into three groups of three. Left and right limbs were paired within animals when possible in making the pools for the 5 and 14 dpa time points. The day 0 pools were created using only the right limbs of 9 different individuals that were collected within

minutes following limb amputation. RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) followed by RNeasy minicolumns (Qiagen, Valencia, CA, USA). RNA quality was assessed by spectrophotometry using a Nanodrop ND-1000 (Nanodrop, Wilmington, DE, USA) and run on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The Ambystoma microarray platform was produced by the Voss lab and Affymetrix and has been described elsewhere (Page et al., 2007; Monaghan et al., 2007). Total RNA was used to produce cRNA probes for GeneChip hybridizations (Affymetrix, Santa Clara, CA, USA) at the University of Kentucky Microarray Core Facility according to standard Affymetrix protocols. Probe level quality control analyses were performed as described in (Monaghan et al., 2007). Data processing and statistical analysis was performed using the Affy Bioconductor package for the R statistical environment (Bolstad et al., 2005). Background correction, normalization, and probe set summarization were performed via the robust multi-array average (RMA) algorithm of (Irizarry et al., 2003). Correlation matrices (Pearson's r) for replicate GeneChips at the probe-set level were produced to assess correlation between GeneChips (minimum r = 0.9787, maximum r = 0.9952). Probe-sets were removed if mean signal intensity was less than the mean of the lowest quartile across all 15 GeneChips (mean \pm standard deviation = 7.748 \pm 0.031). Some microarray technologies may provide unreliable hybridization estimates for lowly expressed genes (Draghici et al., 2006). For this reason, a stringent cut-off was applied that removed the bottom quartile of genes for significance testing. Probe-set filtering yielded 3656 probe-sets for significance testing.

Microarray analysis

The limma package (Smyth, 2004, 2005) available from Bioconductor was used to conduct three analyses to statistically identify differentially expressed genes. In the first analysis, linear models were fit to each gene. These models used coefficients to denote each of the five treatments by sampling time combinations. The following coefficients were contrasted: Day 0 versus NR 5 dpa (NR5), Day 0 versus DL 5 dpa (DL5), Day 0 versus NR 14 dpa (NR14), Day 0 versus DL 14 dpa (DL14), NR5 versus NR14, and DL5 versus DL14. The other two analyses were equivalent to paired t-tests and compared NR5 versus DL5, and NR14 versus DL14. Multiple testing was corrected using an FDR cutoff

of 0.05 and then a fold-change filter (\geq 1.5-fold change) was implemented to derive final gene lists. All microarray data are available at (www.ambystoma.org). The identity of differentially expressed salamander transcripts was inferred from presumptive human orthologs. Orthology was assumed for all salamander transcripts that exhibited significant sequence similarity to protein coding sequences from human RefSeq and nr databases (BLASTx, e < 1 x 10⁻⁷). K-means gene clustering was performed using the Genesis software package (Sturn et al., 2002). Presumptive human-salamander orthologs were further annotated using GO terms and tools provided by the Database for Annotation, Visualization, and Integrated Discovery (Dennis et al., 2003). Significantly overrepresented GO terms (EASE score p < 0.01) were identified for specific treatment/sampling time combinations. Certain GO terms were excluded from the results if similar information was represented by a similar GO term. The null expectation for GO term representation was obtained by assigning GO terms to 3271 EST contigs from *Ambystoma* ESTdb.

454 cDNA Sequence Analysis

The same total RNA samples that were used in the microarray analysis were used to produce cDNA templates for 454 pyro-sequencing. cDNA libraries were generated for Day 0, NR5, NR14, DL5, and DL14 RNA samples using the Super SMART cDNA Synthesis protocol (Clontech, Mountain View, CA). Single stranded cDNA template was amplified using the Advantage 2 PCR Kit (Clontech) and size selected according to manufacturer's instructions. cDNAs were sequenced using the Genome Sequencer FLX System (Roche Applied Science, Indianapolis, IN). SeqClean was used for vector/poor quality trimming, bacterial contaminant screening, and identification of A. mexicanum mitochondrial DNA and rDNA sequences (http:// compbio.dfci.Harvard.edu/ tgi/software/). Retained sequences were pre-clustered using PaCE and then assembled using CAP3 with a 90% sequence similarity threshold (Kalyanaraman et al., 2003; Huang and Madan, 1999). Contigs (including singletons) were searched using BLAST algorithms against the Ambystoma ESTdb, human and nr RefSeq databases, and Xenopus laevis and X. tropicalis Unigene sets. Annotated queries that returned a significant BLAST hit were assigned the gene identifier of the best matching subject sequence. All of these new 454

sequence reads have been submitted to the Short Read Archive (SRA) at the National Center for Biotechnology Information (NCBI), accession SRA004195.2. New 454 Sequences were assembled with previous EST data and are available at Sal-Site (www.ambystoma.org).

The number of times each 454 DNA sequence read matched a unique EST contig from the Ambystoma ESTdb was recorded, and these count data were used to estimate mRNA abundances for presumptive axolotl genes. The following method was used to identify differentially expressed genes among 10,275 contigs that were sampled ≥ 5 times across all five cDNA libraries. First, 5000 random draws were taken from a multinomial distribution to derive expected count data for each gene (k = 5 cDNA libraries, n = the sum of counts across all libraries for a given gene, and p_1 , p_2 , p_3 , p_4 , and p_5 = expected proportion of counts per library given unequal sampling among cDNA libraries). Then, γ^2 statistics were calculated, on a gene-by-gene basis, for each of these random draws. Pvalues for each gene were estimated by calculating the proportion of randomized χ^2 statistics that were \geq to the χ^2 statistic associated with the observed data. Contigs with Pvalues ≤ 0.001 were considered differentially expressed. Upon examination of this dataset, it was noticed that 589 of the significant EST contigs were uniquely derived from different cDNA libraries and these tended to form small (≤ 250 bp) contigs that did not match previous sequences in the Ambystoma ESTdb. These sequences were considered cloning/sequencing artifacts and removed from the dataset. This yielded a final dataset of 1150 significant genes. GO analyses were performed as described above with the exception that human default GO term frequencies were used to establish null expectations (EASE score p < 0.02).

Results

Morphology and histology of denervated and innervated limbs

Histological staining verified our experimental procedures for creating innervated and denervated limbs on the same individual. Previous studies found few morphological or histological differences between innervated and denervated limbs during the first few days of regeneration (Singer, 1978; Deck, 1961; Carlone and Mescher, 1985). Consistent with these observations, denervated and innervated limbs at 5 dpa (NR5 and DL5) were histologically indistinguishable (Figure 4.1a, 4.1c). Histological staining (H & E) showed hemorrhaging directly beneath the epithelium containing Leydig cells, squamous cells, and basal keratinocytes (Figure 4.1b, 4.1d). All 5 dpa limbs resembled the wound healing phase or early phase of dedifferentiation according to the staging of (Tank et al., 1976). A blastema was visible on innervated limbs 14 dpa (NR14) and histological staining showed an accumulation of blastemal cells under the wound epithelium. These structures were not observed in denervated limbs at 14 dpa (DL14; Figure 4.1e-h). These results indicate that blastema formation and outgrowth only occurred in NR limbs. An apical thickening of the epithelium characterized the distal end of NR14 limbs and this layer consisted of keratinocytes and Leydig cells. These histological traits indicate that 14 dpa limbs in this experiment corresponded to the early to mid bud stage of limb regeneration (Tank et al., 1976). Immunological staining using RT-97 was performed to detect the presence or absence of nerve axons at 5 and 14 days after injury. At both time-points, neurofilament staining was positive in the NR limbs, but negative in DL limbs. In pilot experiments, we determined that >20 days is required for nerves to re-innervate limbs after denervation surgery (data not shown). Thus, DL and NR limbs were created successfully and histology showed that 5 and 14 dpa time points correspond to wound-healing and early-mid bud phases of regeneration, respectively.

Transcription during normal limb regeneration: Deviations of NR limbs from baseline

At both 5 and 14 dpa, mRNA levels for hundreds of genes were significantly different from baseline levels of genes expressed in whole limbs at Day 0 (Table 4.1). Many of the same genes (n = 215; up = 111; down = 104) were identified as significant in NR5 and NR14 limbs; the deviation from baseline was in the same direction for all but one of these genes (*fabp2*). Four matrix metalloproteinases (*mmp1*, *mmp3/10a*, *mmp9*, and *mmp13*) that are known to function during wound healing in many organisms were upregulated at both 5 and 14 dpa (Figure 4.2), while collagens (*col4a1*, *col4a2*, *col8a1*, *col9a3*, and *col11a1*; Figure 4.3) and muscle specific genes (Figure 4.4) were downregulated (Table 4.1). Upregulation of collagen catabolism genes coupled with downregulation of collagen structural genes suggests that transcriptional activation and

repression are integrated to efficiently remodel the extracellular environment of damaged tissues. Downregulation of muscle genes at both time points suggests that the differentiated muscle gene expression phenotype changes by 5 dpa, and changes more dramatically by 14 dpa.

Although many genes exhibited similar deviations from baseline at 5 and 14 dpa, unique gene expression changes were identified at each time point (Table 4.1; Supplemental file 1 and 2). The unique NR5 genes were associated with gene ontology (GO) terms that implicate extracellular protein changes and signal transduction pathways of the early wounding response. These terms included response to stimulus, signal transduction, extracellular region, and ion transport. The unique NR14 genes were associated with GO terms that implicate cell division and DNA metabolism including *ccnd2*, *ccnb1*, *rrm1*, *rrm2*, *nasp*, *rrc1*, and *cdc20*. The unique gene expression changes that were identified at 5 and 14 dpa support the idea of temporal progression from an early wound-healing phase to a blastema outgrowth phase during normal limb regeneration.

Transcription within denervated limbs: Deviations of DL limbs from baseline

As was observed in NR limbs, hundreds of genes were identified as significant when comparing DL5 and DL14 mRNA levels to baseline levels measured at Day 0 (Supplemental Tables 1 and 2). Some of the same or similar GO terms that were associated with NR limbs were identified as significantly enriched in DL limbs (Table 4.1). This was not unexpected because both DL and NR limbs undergo tissue histolysis at the limb stump and carry out an early wound healing response (Figure 4.1a, 4.1b). For example, extracellular region and MMP genes were upregulated at 5 and 14 dpa, as was seen in NR limbs (Figure 4.2; Supplemental Table 1). As in NR limbs, muscle contraction (Figure 4.4), cytoplasmic, and collagen genes (Figure 4.3; *col4a1, col4a2, col8a1*) were downregulated in both DL5 and DL14 limbs (Supplemental Table 2). Moreover, 83% of the genes that were identified as significant in NR5 limbs were also significant (and in the same direction) in DL5 limbs. These results indicate that many transcriptional events are nerve-independent during early regeneration.

Denervated limbs are known to cease growth following limb amputation. Several groups of genes may explain this observation including the downregulation of genes

associated with the M phase of cell division, mitochondrial transcripts, and genes associated with glucose metabolism (Table 4.1; Supplemental Table 2). Furthermore, DL14 limbs were morphologically similar to DL5 limbs because denervation prevented blastema formation (Figure 4.1g, 4.1h). Consistent with this observation, many of the genes that were identified as upregulated in DL5 limbs were also identified as significant in DL14 limbs (69%). These genes are associated with the following GO terms: lysosome, response to stress, hydrolyse activity, signal transducer activity, and ion transport. Overall, these terms suggest protraction and expansion of wound-healing responses in DL14 limbs, as well as changes in cellular metabolism and cell division.

Transcript abundance differences between NR and DL limbs

In the preceding two sections, transcriptional patterns of NR and DL limbs were described relative to baseline levels at Day 0. Here, significant genes are reported between NR and DL limbs. In general, few significant changes in gene expression were observed when NR and DL transcripts were compared directly. Transcripts for 16 genes were more abundant in NR5 limbs and 17 were more abundant in DL5 limbs, and these differences were small in terms of fold-level change (< 2.82 fold difference between NR5 and DL5; Supplemental Table 3). Eighteen of these genes exhibited significant sequence similarity to human or salamander presumptive gene sequences; the others are unknown. The genes with significantly more transcripts in NR limbs are associated with intracellular (e.g. dnase113, uap1, acy3, nans, myl4) and extracellular functions, including membrane proteins (e.g. psca, umod, and emp1) and collagen binding (serpinh1). The genes with significantly more transcripts in DL limbs are associated with extracellular functions or the immune response (e.g. igll1, CD74, hmox1, neil1, marco, sftpd, mmp9, mrc1). All but one of the significant 5 dpa genes showed the same directional deviation at 14 dpa; myllwas 1.5 fold higher in the NR limb at 5 dpa, but 2.2 fold lower at 14 dpa. Thus, as was observed when comparing DL5 and NR5 mRNA abundances to baseline levels, relatively few gene expression differences were identified between NR and DL limbs at 5 dpa, and the magnitude of these differences was small. These results further support the idea that transcription during limb regeneration is predominantly nerve-independent at 5 dpa.

Whereas only 33 transcriptional differences were observed between morphologically similar DL and NR limbs at 5 dpa, 282 differences were detected at 14 dpa (Supplemental Table 4). K-means cluster analysis of these genes with significant human protein hits highlight three clusters wherein genes exhibited similar patterns of expression (Figure 4.5). Genes in Cluster 1 presented expression patterns with transcript abundances above baseline in NR14 limbs and abundances below baseline levels in DL14 limbs. Genes associated with cell cycling, a well-established characteristic of blastemal cells, were highly enriched in Cluster 1 (n=26). Over 50% of the genes in Cluster 1 are predicted to localize to the nucleus including several transcriptional regulators (*msx2, id3, tmpo, atf5, rbm15, spen, parp1*, and *tardbp*). Thus, many of the genes in Cluster 1 have functions that are consistent with blastema formation and outgrowth in NR limbs.

Genes from Clusters 2 and 3 were generally expressed in the same direction between NR14 and DL14 limbs, however the magnitude of expression differed. Genes in Cluster 2 presented transcript abundances that generally exceeded baseline levels, with higher levels observed in DL14 limbs. This pattern suggests that most of these genes were activated in the same direction in the presence or absence of nerves, but denervation caused higher transcript abundances. Twenty two percent of the genes in Cluster 2 are associated with the GO term cellular response to stimulus (N =14) and a significant proportion localized to the lysosome (n=6), including *lgmn, ctsk, ctss, asah, atp6v0d1*, and *neu1*. Genes in Cluster 3 presented transcript abundances that were generally lower than baseline levels, with much lower levels observed in NR14 limbs. Given relatively few genes in this cluster, no biological process was identified as significantly enriched. However, inspection of the genes in Cluster 3 again supports the idea that some genes may function in muscle contraction (*actc1, myh7*, and *fhl1*) and tissue repair (*hsp27* and *hebp2*). In summary, mRNA levels for genes from Clusters 2 and 3 were quantitatively affected by the presence or absence of a nerve.

454 cDNA sequence analysis of nerve dependency

To further explore nerve dependency and generate an unbiased collection of molecular probes for regenerating limbs, we sequenced cDNAs derived from the same RNA samples that were used in the microarray analysis. Over 1.7×10^6 reads were

generated and this yielded approximately 90,000 - 230,000 high quality sequence reads for each limb treatment with an average of 215 base pairs in length (Table 4.2). More than half of the sequence reads correspond to mitochondrial transcripts and ribosomal RNA. This frequency of mtDNA transcripts (30%) approximates the number sampled in an earlier EST screen of the Ambystoma genome (Putta et al., 2004). The number of rRNA transcripts was higher than expected. Assembly of all high quality cDNA reads yielded 429,086 unique sequences. These sequences were assembled with previous EST contigs to produce 61,127 contigs containing at least two overlapping sequences. The distribution of contig lengths is shown in Figure 4.6. All contigs and singletons were searched against NCBI databases to identify significant similarity matches that would suggest presumptive gene identities. Ambystoma contigs and singletons yielded 25,446 significant hits to sequences in the human RefSeq database (BLASTx, $e < 1 \ge 10^{-7}$), including 9411 unique human genes. Figure 4.7 shows the distribution of percent coverage to predicted human RefSeq proteins. Interestingly, 7,130 Ambystoma queries that did not show significant amino acid sequence identity to a human reference sequence did show significant nucleotide identity to a Xenopus sequence. Assembly of new 454 cDNA sequences with existing EST contigs from the Ambystoma ESTdb more than doubled (3935 to 9411) the number of non-redundant human-A. mexicanum orthologous sequences. This increase in sequence content was even among ten GO functional categories that are relevant to salamander wound healing and regeneration (Table 4.3). Assuming that many of the anonymous 454 contigs and singletons (> 300,000) that were generated correspond to functional genes, significantly more than 10,000 different transcripts are expressed during the first two weeks of axolotl limb regeneration.

mRNA abundance estimates and gene discovery from 454 cDNA sequence data

The number of times that a non-redundant transcript was sampled by 454 cDNA sequencing was used to estimate mRNA abundances. The transcript counts for 1150 *Ambystoma* EST contigs (genes) differed significantly among limb cDNA pools that were created for each of the limb types (Supplemental Table 5). It was possible to assign a putative ortholog to 563 of these genes; the remaining genes were considered anonymous. This final list of genes was compared to the significant gene lists from the microarray

analysis. It was determined that for 271 of the 1150 significant genes from the 454 cDNA sequencing analysis, a portion of the gene sequence was represented by a probe set on the *Ambystoma* GeneChip. Of these, 104 genes were identified as significant by both methodologies and mRNA abundances for these genes were highly positively correlated (Supplemental Table 6; median Spearman's correlation = 0.87). The 167 genes found to be significant by 454 cDNA sequencing, but not by microarray analysis, were mostly characterized by low fold changes from baseline (median fold change as estimated from 454 cDNA sequencing counts = 1.75); or conversely, registered low hybridization intensities in the microarray analysis (median rank among 4844 probe sets = 343).

Gene functions that were identified as significantly enriched by microarray analysis were also identified as significant by 454 cDNA sequencing. For example, the muscle contraction GO term was identified as highly enriched and the underlying genes were similarly downregulated relative to baseline (Table 4.4; Figure 4.4; Supplemental Table 6). Also, genes sampled most often from NR14 limbs were associated with DNA metabolism, a biological process associated with cell cycling (Table 4.4), and transcripts for genes associated with cell proliferation and cell cycle progression (e.g. pcna, smc1, ctps, umod, psca, smc111, rad21) were either sampled more often among NR limbs or were only sampled from NR limbs (Supplemental Table 5). Thus, 454 cDNA sequencing also identified genes in NR limbs that are consistent with blastema formation. Several functional terms that were not identified by microarray analysis were identified as enriched by 454 cDNA sequencing. Transcripts for 40 genes associated with macromolecule metabolism were most abundant in NR5 limbs compared to other limbs. Transcripts for 12 genes associated with macromolecule catabolism were most abundant in DL5 limbs including mmp1, mmp3/10a, mmp3/10b, mmp9, and mmp13. This suggests that the presence or absence of nerves differentially affects transcriptional responses and regulation at 5 dpa. Also, 454 cDNA sequencing identified additional *psca*-like genes that were not represented on the GeneChip and these were also differentially expressed between NR5 and DL5 limbs. These and other examples suggest that 454 cDNA sequencing complimented the microarray analysis by providing deeper sampling of transcriptional programs and associated biological processes. This revealed more

candidate nerve-dependent gene expression changes at the earlier 5 dpa time point than was revealed by microarray analysis.

In addition to providing estimates of mRNA abundance, 454 cDNA sequencing also discovered new gene sequences for the Mexican axolotl. These include genes that are known to affect developmental processes in other vertebrate models: *notch* (1,2,3,4), *nrg1*, *bmp1*, *wnt4*, *ctnna1*, *btnna1*, *dkk1*, *axin 1*, *nrg1*, *fgf10*, *sirt* (1,2,5,6), *stats* (1, 2, 3, 5, 6), *sema4f*, *tf*, *sfrp* (1,2,5), *rara*, *rarg*, *rxr*, *pdgf*, *acvr1* (*IB*, *IIA*, *IIB*), *bmprI*, *bmprII*, *smad* (1, 2, 4, 5, 7, 9), *efna1*, *ntn1*, *slit2*, *slit3*, *robo1*, and *robo2*. Most of these genes were sampled less than 10 times and thus appear to be expressed at low levels. Other developmental genes that have not been previously associated with limb regeneration were sampled many more times than these candidates, and for the following examples, counts varied significantly among limb cDNA libraries. These include *mdk*, *fliI*, *tagln2*, *ddx5*, *umod*, and *cnot1* (Supplemental Table 5). Also, numerous retroelement-associated sequences were sampled differentially between DL and NR limbs (Table 4.5). Overall, the 454 sequencing approach verified the primary results from the microarray analysis and identified many new candidate genes and functional pathways that are associated with limb regeneration.

Discussion

Microarray analysis and 454 cDNA sequencing were used to identify nervedependent and independent gene expression changes during limb regeneration in the Mexican axolotl. The results show that limb regeneration is associated with thousands of transcriptional changes. Considerable similarity was observed between the DL and NR transcriptional programs at 5 and 14 dpa. For example, genes that are critical to wound healing were upregulated in both limb types (Table 4.6) while genes that are associated with muscle structure and function were downregulated (Figure 4.4). Many of the transcriptional changes that were observed at 5 dpa were also observed at 14 dpa. Thus, many aspects of early limb regeneration are accomplished in the absence of nerves. However, gene expression differences were identified between DL and NR limbs at 5 and 14 dpa. Many of the transcriptional differences correlated with blastema formation; cell numbers increased in NR limbs after 5 dpa and this yielded a distinct transcriptional signature of cell proliferation in NR14 limbs. Overall, this study identified genes that are associated with wound healing, early events of blastema formation, and subsequent blastema cell proliferation and outgrowth. Below, we discuss and expand upon these primary results and highlight genes whose functions appear to be important for understanding the basis of nerve dependency and limb regeneration.

Early wound healing response during limb regeneration

Previous studies have documented anatomical similarities between innervated and denervated limbs at early stages of regeneration (Bryant et al., 1971; Schotte and Butler, 1941; Thorton, 1953). This study shows that there are also many transcriptional similarities. This suggests that many aspects of the early wound-healing phase of limb regeneration are not dependent upon post-amputation, nerve-derived factors (Table 4.6). Instead, humoral immune and local tissue responses appear to be key. Many genes that are associated with wound healing and tissue repair, including stress, inflammation, cell survival, immunity, and extracellular matrix remodeling were upregulated from baseline in 5 dpa limbs (Figure 4.2; Table 4.6). It is probably no coincidence that essentially all of the early stress-associated genes that were previously identified as significantly regulated (using the same *Ambystoma* GeneChip) during early spinal cord regeneration (Monaghan et al., 2007), and during the innate immune response of axolotls to a deadly viral pathogen (Stewart et al., 2008), were also identified as significant in this study. Many of these genes appear to be expressed similarly in all vertebrates in response to stress, including *junb*, irfl, hmox1, apoE, mmps, ptx3, gal3, gadd45g, and tgfb. Additionally, significantly more "extracellular" genes were identified at 5 dpa than expected by chance, including genes that code for matrix remodeling proteins and secreted molecules whose functions are associated with growth factor binding, cell signaling, survival, death, adhesion, migration, and proliferation. The early wound healing response initiates local environmental changes of the injury site that are pivotal to subsequent phases of regeneration.

The 5 dpa time point was chosen in this study to identify critical nerve-dependent signaling events that are stimulated within the first few days of regeneration (see Satoh et al., 2008). Comparison of DL5 and NR5 data revealed few overall gene expression differences. The 5 dpa time point captured many transcriptional responses that are induced by injury/amputation but relatively few that are associated with known or suspected

neurotrophic signaling pathways. More comprehensive sampling and deeper sequencing is needed to detail early nerve-dependent transcriptional responses because several genes that are known to be nerve-responsive during the wound-healing phase were not identified in this study (e.g. *sp9*; *prrx1*, *tbx5*, Satoh et al., 2007, 2008).

Downregulation of genes associated with differentiated muscle

This study documented dramatic decreases in the relative abundance of mRNAs coding for skeletal muscle contractile proteins, including myosins, actins, actinins, titin, tropomyosins, and troponins. Many of these changes were also detected by 454 cDNA sequence analysis (Figure 4.4). This strong, muscle-specific transcriptional signature was observed because approximately half of uninjured forelimb nuclei in 7-9 cm axolotls, and likely more than half the cross-sectional area, derive from muscle (Tank and Holder, 1979). It is unlikely that the down-regulation of muscle genes is due to retraction of the muscle towards the shoulder because muscle transcripts are much more downregulated at 14 dpa than 5 dpa in both denervated and innervated limbs. Considering that limb tissue samples in this study included $\sim 1 \text{ mm}$ of un-damaged tissue proximal to the amputation plane, the results suggest that injury to skeletal muscle induces tissue-wide loss of muscle contractile transcripts. It is possible that the decrease in muscle transcripts is due to muscle wasting, caused by a lack of mechanical stress. It is interesting to speculate that this response maybe associated with the degeneration or cellularization of multinucleated muscle fibers into mononucleated cells, which occurs in both denervated and innervated amputated limbs (Tank et al., 1976; Hay, 1959; Petrosky et al., 1980; Lo et al., 1993; Kumar et al., 2000; Echeverri et al., 2001). It is interesting to note that muscle specific genes, including actc1, actn2, atp2a2, my1pf, tncc, tnni2, tnni3, are downregulated during early stages of mammalian skeletal muscle regeneration (Goetsch et al., 2003), and this is accomplished without blastema formation. Skeletal muscle regeneration in mammals and other vertebrates involves resident stem (satellite) cells (Morrison et al., 2006; Cameron et al., 1986). It is possible that muscle specific genes are downregulated as an integral step of a conserved, skeletal muscle regeneration program of vertebrates. Candidate transcriptional repressor genes were identified in this study including id3 (Iwasaki et al., 2008), tardbp (Buratti and Baralle, 2008), cnot (Winkler et al., 2006), msx1 (Kumar et al.,

2004; Schnapp and Tanaka, 2005) and *msx2*. It will be important in future studies to determine if the dramatic decrease in muscle transcripts is due to activation of muscle stem (satellite) cells, muscle loss, muscle dysfunction/wasting, or whether these transcriptional patterns have an active role in regeneration.

Genes associated with epigenetic reprogramming and genomic stability

Blastema formation requires a large number of progenitor cells derived from quiescent stem cells or differentiated cell types. It is generally known that reprogramming of differentiated cells is accompanied by epigenetic changes such as histone and DNA modifications (Costa and Shaw, 2007; Maherali et al., 2007). Few candidates have been identified previously as bringing about epigenetic changes necessary for cellular reprogramming during regeneration (Yakushiji et al., 2007; Palacios and Puri, 2006). This study identified several genes whose functions are associated with epigenetic phenomena, including chromatin remodeling, DNA methylation, and transcriptional regulation. These include *uhrf1* (Sharif et al., 2007), *lmnb2*, *parp1* (Guastafierro et al., 2008), *thymopoietin* (Dorner et al., 2007), and a gene with high sequence identity to SAM-dependent methyltransferases (Cluster_227434_Contig1; SRV_05867_a_at).

After cellular reprogramming and during limb outgrowth, blastemal cells undergo tremendous cell proliferation. During blastema cell proliferation, telomere lengths and overall genome stability must be maintained to prevent cell death. This study identified several candidate genes from NR14 limbs that are known to function in genome stability, telomere homeostasis, and DNA repair. These include *parp1* (Shrivastav et al., 2008), *hmgb2* (Thomas, 2001), *fen1* (Saharia et al., 2008), *aurka* (Yang et al., 2004), *aurkb* (Monaco et al., 2005), and *pif1*, a DNA helicase that maintains genome stability and binds to telomerase from yeast to humans (Mateyak et al., 2006). It is also important to note that many transcripts were identified from NR and DL limbs that code for retroelement components (Table 4.5; e.g. polyproteins, gag proteins, reverse transcriptases, and recombinases). Retrotransposons are normally transcriptionally silenced in differentiated somatic cells by epigenetic mechanisms, but become active upon changes in epigenetic status; these may also regulate nearby gene expression (Kano et al., 2007; Cropley and

Martin, 2007). It is unclear if upregulation of retroelement transcripts affects genome stability and/or is necessary for regeneration.

Genes associated with nerve dependent blastema outgrowth

In this study, blastemas were not observed in NR5 limbs or DL14 limbs, and only formed on NR14 limbs. Nerve dependent limb outgrowth occurs as a result of blastemal cell proliferation. Two early cell proliferation biomarkers, umod and psca, were identified as significantly different between NR5 and DL5 limbs. umod probably locates to the wound epithelium as it is upregulated in apical skin cells during thyroid hormone induced metamorphosis of axolotl epidermis (Page et al., 2009). It is possible that psca is a membrane receptor of blastema cells because it shows structural similarity to prod1, a surface protein that is implicated in proximal-distal positional identity of blastemal cells during newt limb regeneration (da Silva et al., 2002). Most of the cell proliferation biomarkers were identified at 14 dpa, when a blastema was present in NR limbs but absent in DL limbs. Thus, between 5 and 14 dpa, blastemal cells underwent considerable cell proliferation in the presence of nerves. Because the limb blastema continues to expand after 14 dpa, the blastema-specific genes that were identified in this study are probably transcribed at much higher levels at later time points. A clear signature of cell proliferation, including genes that function in the cell cycle, mitosis, and nucleotide synthesis, was observed in NR14 limbs. In contrast, these genes were slightly downregulated in denervated limbs at this time (Table 4.1; Figure 4.5). Thus, transcripts associated with cell proliferation are maintained at steady state (no increased proliferation) in NR and DL limbs for at least five days. This early, nerve-independent portion of the limb regeneration program may allow time for re-innervation of the injury site and production of nerve-derived molecules in sufficient quantity to initiate and sustain blastemal cell proliferation.

Multiple gene products have been hypothesized to be neurotrophic factors provided by nerves to sustain blastema cell proliferation. These include growth-promoting factors like fibroblast growth factors (Mullen et al., 1996; Satoh et al., 2008), substance P (Globus et al., 1991), neuregulin (Wang et al., 2000), and transferrin (Mescher et al., 1997). Transferrin and neuregulin were sampled by 454 sequencing, but were not identified as differentially expressed by our analysis. *fgf8* and *fgf10* were screened out of the microarray analysis due to low expression, but post-hoc analysis suggested that both are upregulated in NR14 limbs compared to control and DL14 limbs (Table 4.7). Expression of these molecules is known to be nerve-dependent in blastemas of *Xenopus* (Suzuki et al., 2005) and axolotls (Christensen et al., 2001, 2002; Han et al., 2001). Other molecules that have previously been associated with the blastema during limb regeneration were not included in our statistical analyses due to low hybridization intensity, but later found to be differentially expressed in NR14 limbs including hoxd10, hoxa13, hoxa11, and msx1 (Table 4.7). Recently, Kumar et al. (Kumar et al., 2007) identified a growth promoting extracellular ligand (newt anterior gradient; nAG) that rescues aspects of nerve-dependency of limb regeneration in newts. Probesets for *nAG* are represented on the *Ambystoma* GeneChip and transcripts for this gene were sampled by 454 cDNA sequencing. *nAG* mRNA was transcribed at a high level in all tissues, but did not differ significantly between NR, DL, or control limbs. It is possible that the effects of nAG and other neurotrophic candidates are associated with quantitative variation of mRNA transcript abundances over fine temporal and spatial scales. Such variation would have been missed in this analysis of three time points and sampling of mRNAs from heterogeneous tissues. Furthermore, it is possible that the increase in nAG immunoreactivity observed by Kumar et al. (2007) is regulated at the level of translation and would not be observed using microarray or sequencing approaches. Overall, we found that several, but not all, genes previously shown to be directly downstream of neurotrophic factors are expressed at higher mRNA levels in the blastema versus control and denervated samples.

Singer (1978) emphasized that the neurotrophic factor underlying nerve dependency was growth promoting and quantitative in effect. He showed that the trophic effect of nerves could be titred by surgically manipulating the number of nerves innervating the limb (Singer, 1952). Other lines of research showed that nerve-derived factors were not necessary for traversing cell cycle checkpoints, as an equivalent number of presumptive blastema cells are initially observed to enter S-phase in denervated and innervated limbs (Mescher and Tassava, 1975; Tassava and Bennett, 1974). Instead, the neurotrophic factor appears to be necessary to complete the cell cycle and this may be non-trivial considering the cost of replicating a large salamander genome. These studies suggest that nerves either directly or indirectly provide limiting macromolecules that are needed to accomplish cell division. According to this reasoning and assuming a correlation between transcript and protein abundance for specific mRNA species, transcripts associated with nerve dependency would be expected to exceed baseline levels in NR limbs during regeneration, and decrease in abundance in DL limbs. This study identified many human-axolotl presumptive orthologs and anonymous axolotl transcripts that showed this pattern. For example, *psat* codes for a protein that regulates the second enzymatic step of the phosphorylated pathway in mammals, which produces L-serine (de Koning et al., 2003). PSAT expression (protein and mRNA) is high among cell types with high rates of proliferation, including cancer cell lines (Martens et al., 2005; Vie et al., 2008). Strikingly, *psat* registered the largest mRNA abundance difference between NR14 and DL14 limbs among probesets on the Ambystoma GeneChip (8.2 fold change), and this result was verified by both 454 cDNA sequencing (NR14=20.1, DL14=3) and real-time PCR (8.37 fold change; data not shown). We also found that the third enzyme in the phosphorylated pathway, psph, was 3.58 fold higher in NR14 versus DL14 by real-time PCR (data not shown). PSAT and PSPH may have neurotrophic potential but more likely function to provide proliferating cells with a limiting, and conditionally important substance (L-serine) that is required for synthesis of macromolecules, amino acids, and purines that are needed to accomplish mitosis (de Koning et al., 2003). The fact that macromolecular synthesis and cell proliferation are both depressed in denervated limbs (Dresden, 1969; Lebowitz and Singer., 1970; Singer and Cason., 1972) supports the idea that PSAT activity is associated with nerve-derived factors that contribute to blastema outgrowth.

Comparison of microarray and cDNA sequencing approaches

Microarray analysis and 454 cDNA sequencing offer different advantages for dissecting complex biological processes. The strength of microarray analysis is the precision that this approach provides for estimating transcript abundances for a specific panel of genes. Such gene panels provide standardized markers for identifying shared and unique patterns of transcription among experiments. In turn, identification of such patterns provides systems-level insight. For example and as was discussed above, acrossexperiment comparisons of axolotl transcription helped distinguish general stress responses from local regenerative responses. A relatively conservative fold level threshold (> 1.5 fold) was used in this study to identify significant genes in the microarray analysis and lowly expressed genes (in the lowest quartile) were removed from consideration. These conservative approaches potentially exclude important genes but likely discover 'real' transcriptional differences between samples. A lower threshold could be applied to extract more information from the dataset and future studies would likely benefit by increasing replication of GeneChips to detect significant differences for lowly expressed genes. With respect to 454 cDNA sequencing, two goals were accomplished: gene discovery and estimation of transcript abundance. However, accomplishment of both goals required a trade-off in the allocation of resources toward deep sequencing versus replication. Sequencing resources were used to deeply sequence Day 0, NR, and DL cDNA libraries instead of shallowly sequencing replicate libraries for these time points. This strategy identified thousands of new gene sequences that will greatly enrich future regeneration studies. Future studies will benefit from experimental designs that replicate deep sequencing of cDNA libraries and this will be possible as sequencing costs decrease. Still, it was encouraging to find in this study that 38% of genes that were identified as differentially expressed by 454 cDNA sequencing and found on the microarray GeneChip were identified as significant by both of these technologies, and transcript abundances for these genes were highly positively correlated.

Conclusions

This study addressed the nerve-dependency of limb regeneration by characterizing downstream cellular events that are affected when an intact nerve supply is removed from an amputated salamander limb. Microarray analysis showed that the early wound response is largely nerve-independent, but transcriptional profiles diverge between denervated and innervated limbs when the innervated limb starts to regenerate. Pyro-sequencing supported these microarray results while substantially increasing sequence information from the salamander transcriptome. This study shows the utility of next-generation sequencing platforms for gaining transcriptome information (Emrich et al., 2007; Toth et

al., 2007; Vera et al., 2008). This new DNA sequence information will greatly enrich future regeneration studies using the axolotl.

 Table 4.1. Significant gene ontology terms for changed genes.

Upregulated		Downregulated			
NR5 and NR14 (N=111)	p-value	NR5 and 14 (N=104)	p-value		
extracellular region (N=14)	9.05E-07	muscle contraction (N=13)	1.81E-10		
collagen catabolism (N=5)	4.35E-06	cytoplasm (N=51)	8.41E-07		
		collagen (N=6)	4.05E-05		
		ion transport (N=12)	9.44E-06		
NR5 only (N=110)	p-value	NR5 only (N=49)	p-value		
extracellular region (N=15)	1.23E-05	extrinsic to membrane (N=3)	6.10E-03		
response to stimulus (N=17)	1.00E-02				
signal transduction (N=21)	2.89E-03				
Ion transport (N=9)	2.57E-03				
NR14 only (N=53)	p-value	NR14 only (N=93)	p-value		
DNA metabolic process (N=8)	4.41E-04	muscle contraction (N=18)	5.43E-19		
		calcium ion binding (N=11)	4.98E-04		
DL5 and DL14 (N=170)	p-value	DL5 and 14 (N=109)	p-value		
extracellular region (N=23)	6.64E-10	muscle contraction (N=15)	6.62E-13		
lysosome (N=11)	3.04E-08	cytoplasm (N=53)	1.46E-06		
ion transport (N=13)	1.42E-05	mitochondrial membrane (N=12)	3.49E-04		
hydrolase activity (N=26)	3.60E-05				
response to stress (N=21)	1.02E-03				
signal transduction (N=22)	5.40E-03				
collagen catabolism (N=5)	5.09E-05				
DL5 only (N=68)	p-value	DL5 only (N=58)	p-value		
extracellular region (N=9)	3.68E-04	extrinsic to membrane (N=3)	4.64E-03		
response to stimulus (N=12)	3.70E-03				
DL14 only (N=69)	p-value	DL14 only (N=111)	p-value		
extracellular region (N=7)	4.10E-03	muscle contraction (N=9)	4.26E-05		
immune response (N=5)	7.80E-03	mitochondrion (N=27)	6.71E-07		
		cytoplasmic (N=43)	2.58E-03		
		M phase (N=9)	1.40E-03		
		glucose metabolic process	2.97E-03		

Table 4.1. Significant gene ontology terms for changed genes. Significant genes were identified by microarray analysis and gene ontology terms were sampled more often than expected in NR5, NR14, DL5, and DL14 limbs. P-value is calculated by DAVID (Dennis et al., 2003).

Table 4.2. 454 DNA	sequence reads that were	generated for each	cDNA limb library.
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	Day 0	NR5	DL5	NR14	DL14	totals
Total Reads	312258	216281	220561	393012	578787	1720899
Filtered	19459	14390	18918	22679	88387	163833
mtRNA/rRNA	168954	111490	92136	171046	256684	800310
Final Reads	123845	90401	109507	199287	233716	756756

Table 4.3. Gene ontology breakdown of the 9411 salamander genes with presumptive human orthologs. The number of *Ambystoma* ESTdb contigs with gene ontology annotations before and after 454 DNA sequencing. The total is less than the sum of each category because several genes may belong to multiple categories. Bp = biological process.

Ontology	GO Level	Before 454 Sequencing	After 454 Sequencing
cell cycle	bp3	226	438
metabolism	bp2	1889	3982
cell motility	bp3	62	126
morphogenesis	bp2	109	272
development	bp1	342	795
response to stress	bp2	219	469
cell death	bp3	142	312
catalytic activity	bp1	1275	2855
transcription factor activity	bp2	112	344
kinase activity	bp4	159	456
		Total (3935)	Total (9411)

Table 4.4. Gene ontology results from 454 sequencing. Gene ontology analysis of 465 genes with presumptive human orthologs identified by 454 DNA sequencing. Only the most significant gene ontology category was selected for the table.

cDNA library	Most significant GO terms	e-value
Day 0 (N=127)	striated muscle contraction (N=17)	1.75E ⁻²⁷
NR5 (N=84)	macromolecule metabolism (N=40)	1.37E ⁻⁵
DL5 (N=59)	macromolecule catabolism (N=12)	6.21E ⁻⁸
NR14 (N=54)	DNA metabolism (N=6)	2.00E ⁻²
DL14 (N=43)	protein biosynthesis (N=14)	1.44E ⁻¹⁷

Table 4.5. Significantly changed retrovirus genes. Genes identified as differentially expressed by 454 sequencing analysis that have high sequence similarity to retrovirus genes.

Contig	Gene Name	NR5	DL5	NR14	DL 14	D0
10177_Contig1	rev. transcriptase-like	10	3	0	0	0
10435_Contig1	envelope polyprotein	19	8	0	2	0
10953_Contig8	end. retroviral family w	5	15	0	1	0
109875_Contig1	rev. transcriptase-like	0	0	10	0	0
110157_Contig5	hyp. protein LOC57523	7	20	42	6	0
126954_Contig17	polyprotein	12	22	32	6	8
12813_Contig1	zinc finger protein 9	14	37	67	20	37
154450_Contig16	retrotransposon-like 1	5	27	12	2	10
174946_Contig1	pol-like protein	0	0	12	0	0
185474_Contig140	helitron-like trans.	81	276	314	106	59
195627_Contig1	Gypsy 1 polyprot.	0	0	12	0	0
218328_Contig1	retrotransposon-like 1	0	0	13	0	0
2194_Contig1	pol polyprotein	17	0	0	0	0
23276_Contig1	chromobox homolog 1	14	0	0	0	0
23893_Contig1	NBB2750 hyp. protein	12	0	0	0	0
32735_Contig1	rev. transcriptase-like	12	0	0	0	0
331680_Contig1	lambda-recombinase	2	12	30	11	6
352970_Contig1	reverse transcriptase	0	0	0	0	12
39722_Contig1	rev. transcriptase	98	117	138	51	92
42238_Contig456	paternally expressed 10	41	3	10	3	12
42238_Contig60	paternally expressed 10	7	35	37	10	28
89909_Contig1	reverse transcriptase	0	12	0	0	0
94735_Contig1	lambda-recombinase	0	12	0	0	0

Probe ID	Symbol	NR5/C	NR14/C	DL5/C	DL14/C	function
SRV_01351_at	JUNB	4.6	2.35	4.74	3.46	SR
SRV_10702_at	CYBB	2.84	1.73	4.11	2.46	SR
SRV_00330_at	CYBB	2.39	1.58	3.46	2.23	SR
SRV_03054_at	MARCO	2	1.83	3.11	3.01	SR
SRV_00130_a_at	APOE	1.9	2.36	2.49	4.28	SR
SRV_00442_at	SLC11A1	1.95	1.58	2.76	3	SR
SRV_01821_at	TYROBP	1.98		2.45	2.65	SR
SRV_03023_a_at	GADD45G	1.96		2.29	2.01	SR
SRV_02588_a_at	LGALS3BP	1.91		2.37	1.59	SR
SRV_01177_a_at	ENTPD1	2.39		2.23	1.58	SR
SRV_02586_at	LGALS3BP	1.79		2.25	1.81	SR
SRV_11767_a_at	IFITM5	1.75	1.56	1.63		SR
SRV_02724_at	MAT2A	2.6	1.78	1.6		SR
SRV_01617_a_at	PTX3	4.3		3.78		SR
SRV_02002_at	CES1	2.36		3.01		SR
SRV_03221_a_at	DMBT1	1.81		2.5		SR
SRV_07036_at	IL1B	1.7		1.53		SR
SRV_02516_at	HSPA5	1.65				SR
SRV_02965_at	TFPI2	2.22				SR
SRV_12417_at	NDRG1	2.57				SR
AH_at	HSPA8	1.52	1.67			SR
SRV_01846_at	UMOD	2.27	2			SR
SRV_01294_at	FTH1			1.67	2.01	SR
SRV_01385_at	LECT2			3.93	7.38	SR
SRV_01840_at	UCP2			1.53	2.13	SR
SRV_11406_at	LYN			1.74	1.72	SR
SRV_11583_at	MAPK12			2.04	1.92	SR
SRV_07726_a_at	MPEG1			1.77	2.27	SR
SRV_04367_a_at	IGLL1				2.29	SR
SRV_04710_at	NEIL1				1.93	SR
SRV_10936_at	PTGDS				4.19	SR
SRV_00002_s_at	IGLL1				2.09	SR
SRV_00078_at	IGLL1				2.43	SR
SRV_01199_a_at	CLU				2.36	SR
SRV_02442_a_at	G1P2			2.56		SR
SRV_02611_a_at	SGK			1.57		SR
SRV_13637_a_at	IFITM3			1.51		SR
SRV_01323_a_at	HMOX1	10.05	5.13	18.24	8.53	А
SRV_00444_a_at	GPX1	1.67		1.51		А
SRV_01423_a_at	MMP1	26.65	2.94	37.91	11.41	ECM
SRV_01428_x_at	MMP10	22.86	2.87	30.96		ECM

Table 4.6 continued on next page

Table	4.6 continued					
Probe ID	Symbol	NR5/C	NR14/C	DL5/C	DL14/C	function
SRV_01430_at	MMP13	22.54	9.04	34.09	24.97	ECM
SRV_01425_at	MMP13	19.64	3.1	23.46	2.6	ECM
SRV_11420_at	MMP27	16.71		23.32	4.52	ECM
SRV_02399_a_at	MMP9	15.22	3.51	23.05	15.69	ECM
SRV_11423_at	MMP13	6.79		8.48	2.2	ECM
SRV_11417_a_at	MMP1	6.42		8.48		ECM
SRV_12016_at	MMP2		1.71		2.49	ECM
SRV_02241_at	MMP2		1.66		2.34	ECM
SRV_10485_at	MMP2				2.11	ECM
SRV_00453_a_at	TGFB1	2.4		2.32	1.93	GR
SRV_00328_at	CTSK	7.03	4.86	8.07	19.35	L
SRV_00327_a_at	CTSK	3.72	2.94	4.73	8.23	L
SRV_00326_a_at	CTSK	3.19	2.84	3.78	6.47	L
SRV_02600_a_at	LGMN	2.16	1.8	2.81	3.66	L
SRV_05534_at	RAB7B	1.91		1.99	1.83	L
SRV_02353_a_at	LITAF	1.88		2.29	1.75	L
SRV_01134_at	ATP6V1B2	1.83		2.24	1.96	L
SRV_02294_at	ATP6V0D1	1.73		2.33	2.73	L
SRV_14287_a_at	CTSL	1.66		2.23	2.03	L
SRV_02601_at	LGMN	1.66	1.58	2.09	2.44	L
SRV_05336_a_at	CTSL			1.75	1.69	L
SRV_00294_s_at	PPGB			1.67	2.12	L
SRV_01135_a_at	ATP6V0C			1.62		L
SRV_01242_a_at	CTSD			1.62	2.25	L
SRV_01559_a_at	PSAP			1.58	1.61	L
SRV_00355_at	NEU1			1.57	1.87	L
SRV_02141_at	ASAH1			1.56	2.15	L
SRV_09661_at	CTSC			1.52	1.99	L
SRV_01558_at	PSAP				2.71	L
SRV_00334_a_at	GLB1				1.64	L
SRV_10701_at	CTSK				1.58	L

Table 4.6. Significantly changed wound-healing genes. Fold-level change of upregulated genes that have functions associated with a wounding response (identified by microarray analysis). SR = stress response gene; A = antioxidant; ECM = extracellular matrix remodeling; GF = growth factor; L = lysosome.

Table 4.7 Lowly-abundant genes expressed in the blastema. NR14/C = Fold change ofNR14 compared to control. NR14/DL14 = Fold change of NR14 compared to DL14.

				NR14vsDL14 students t-test P
Probe ID	Gene	NR14/C	NR14/DL14	value
SRV_00099_at	FGF8	3.36	3.47	0.0052
SRV_00025_at	FGF10	2.32	1.79	0.0031
SRV_00107_at	HOXD10	3.52	2.77	0.0014
SRV_00102_at	HOXA13	2.26	2.57	0.0066
SRV_00091_at	HOXA11	2.00	1.71	0.0235
SRV_00011_at	MSX1	2.31	2.19	0.0068

Figure 4.1. Histology of innervated and denervated limbs at 5 and 14 dpa.

Eosin and hematoxylin staining of DL5 (A), NR5 (C), DL14 (E), and NR14 (G) limbs. Higher magnification inset pictures are provided for each image (B,D,F,H). Scale Bar A = $500 \ \mu m$; B = $50 \ \mu m$



Figure 4.2. Schematic of matrix metalloproteinase gene expression.

Matrix metalloproteinase gene expression is represented in each box. A) Microarray results are represented by fold change (FC) from day 0. B) Normalized counts are represented from the 454 cDNA sequencing experiment. Figures 2 - 4 were created using GenMAPP (Dahlquist et al., 2002). Figure 2 was modified from a MAPP originally created by Gladstone Institutes.



Figure 4.3. Schematic of collagen gene expression.

Collagen gene expression is represented in each box. A) Microarray results are represented by fold change (FC) from day 0. B) Normalized counts are represented from the 454 cDNA sequencing experiment.


Figure 4.4. Schematic of down-regulated muscle contraction genes.

Striated muscle contraction genes that were downregulated during limb regeneration. Each gene is represented by two boxes that denote proportional expression among Day 0, NR5, DL5, NR14, and DL14 samples. The left box reports hybridization intensity from the microarray experiment and the right box reports normalized count data from the 454 cDNA sequencing experiment. Figure 2 was created using GenMAPP (Dahlquist et al., 2002) and was modified from a MAPP originally created by Joanna Fong and Nathan Salomonis.





Figure 4.5. Clustering of genes identified as significant from the comparison of NR14 and DL14 limbs.

Fold change values are relative to baseline levels at Day 0. Blue coded genes are cell cycle associated; orange coded genes localize to the lysosome; green coded genes are associated with inflammatory responses; red coded genes are matrix metalloproteinase's; purple coded genes are associated with muscle. Genes coded with an * are associated with inflammation and localize to the lysosome.



Figure 4.6. Bar graph showing contig lengths.

Distribution of sequence lengths for each of the 61,127 contigs.



Figure 4.7. Bar graph showing percent coverage of human proteins.

The distribution of the percent coverage for each of the unique 9411 human proteins with presumptive salamander orthologs.



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CHAPTER 5

GENE EXPRESSION CHANGES IN DORSAL ROOT GANGLIA DURING SALAMANDER LIMB REGENERATION

James R. Monaghan,* Stephanie Tsai, Allison DeMaio, and S. Randal Voss* *Department of Biology & Spinal Cord, University of Kentucky, Lexington, KY 40506

S. Randal Voss PhD, 741 South Limestone Avenue, Lexington, KY 40536 Telephone: 859-257-9888 Fax: 859-257-5737 E-mail: <u>srvoss@uky.edu</u>

Introduction

Salamanders are the only tetrapods capable of regrowing limbs as adults. Following amputation of a limb, cells proximal to the amputation surface are recruited to form a mass of highly proliferative mesenchymal-type cells termed the blastema. The blastema is the fundamental and necessary element of a regenerating limb. It is a selforganizing structure that eventually re-differentiates into an almost perfect replica of the missing limb. By focusing on events that contribute to blastema formation, we aim to understand how the salamander can regenerate entire limbs.

Blastema formation is dependent upon an adequate supply of nerves. If the limb nerve supply is compromised, a blastema fails to form and hence limb regeneration. This phenomenon has been known since 1823 (Todd), but the role nerves play in blastema formation is still poorly understood. Nerves are intimately connected to the early blastema. Anatomical, immunological, and retrograde staining of neurons has shown that a large number of nerve fibers quickly grow into injured limb tissues and wound epithelium following amputation (Figure 5.1; Singer 1949; Thornton, 1970; Koussoulakos et al., 2003; Satoh et al., 2008). These nerve fibers supply a neurotrophic factor (NTF) or set of NTFs, independent of electrical transmission, that support the proliferation of blastemal cells during the early phases of regeneration (Singer, 1978 for review; Monaghan et al., 2009). Multiple gene candidates have been proposed to be the NTF including fibroblast growth factors (Mullen et al., 1996, but see Dungan et al., 2002; Satoh et al., 2008), substance P (Globus, 1991), neuregulin (Wang et al., 2000), and transferrin (Mescher, 1997), but none have fulfilled all the requirements to identify these molecules as the NTF. It is unclear whether these molecules do not completely rescue regeneration because their mitogenic properties are unrelated to the critical role nerves play during regeneration. Alternatively, these and other factors may act synergistically to support limb regeneration. Unbiased genomic approaches were used here to identify possible new gene candidates that may explain the vital role of nerves during salamander limb regeneration.

Several properties are known about the NTF(s) that may assist in identifying candidate molecules in future studies. The NTF(s) is produced in the neuronal cell body, transported down the axon, possibly by fast axonal transport, and released at the synapse (Scadding, 1988; Wallace, 1972; Kiffmeyer et al., 1991). The mitogenic affect of nerves is

increased following nerve injury or limb amputation, possibly due to an increase in NTF production (Singer, 1952; Maier et al., 1984; Boilly and Albert, 1988; Boilly and Bauduin, 1988). Lastly, the NTF(s) is produced by sympathetic, motor, and sensory nerves innervating the limb (Singer, 1952, 1974). A minimum threshold of these nerves is necessary for the limb to regenerate and can be met by innervation of only sensory axons. This is likely explained by the large contribution of sensory nerve axons to the early blastema (Singer, 1978; Koussoulakos et al., 2003).

Sensory nerves that innervate the salamander hindlimb originate from brachial nerves 15, 16, and 17. The cell bodies that supply the brachial nerve axons are located in dorsal root ganglia (DRG) found lateroventrally to the spinal cord (figure 2.7). The accessibility of DRGs to surgical removal and culture has made them useful for understanding the signaling involved in axon outgrowth. Furthermore, the substantial role sensory nerves play in blastema formation make DRGs an obvious choice for identifying candidates for the NTF(s). The aim of this study was to use microarray analysis to detect gene expression changes in DRGs during blastema formation in order to identify regeneration-associated genes (RAGs) as well as possible gene candidates for the NTF(s). We identified 109 genes as differentially regulated in DRGs during hindlimb blastema formation engrossing several aspects of neuronal physiology including axon outgrowth, mitogenic factors, and neuronal signaling. Many of the changed genes identified in this study have also been identified in mammalian models of peripheral nerve injury, suggesting a common transcriptional response to nerve injury. However, multiple RAGs were identified in this study that may be specific to salamander nerve regeneration. Several up-regulated genes were also localized to sensory neurons of the DRG suggesting that we sampled gene expression changes in sensory neurons during regeneration.

Materials and Methods

Animals, tissue collection, and RNA isolation

The handling and surgical manipulation of all salamanders was carried out according to the University of Kentucky Animal Care and Use guidelines (IACUC #00609L2003). Mexican axolotls were housed in the Voss lab on a 12 hour light/dark cycle and fed California blackworms (Lumbriculus variegates) *ad libitum*. Eight adult

animals were anesthetized in 0.01% Benzocaine (Sigma) and both hindlimbs amputated mid tibia/fibula. Limbs were allowed to regenerate for 14 days before right and left DRGs (15, 16, and 17) were collected and frozen on liquid nitrogen. Four biological replicates were made by pooling six DRGs isolated from two animals. Tissues were crushed under liquid nitrogen using a mortar and pestle, suspended in RNAlater (Qiagen), and further homogenized by running through a Qiashredder (Qiagen). A second group of eight animals had DRGs collected without limb amputation and the same tissue pooling scheme was performed. RNA was isolated using Trizol Reagent (Invitrogen) followed by RNeasy mini columns (Qiagen). RNA quality was assessed using an ND-1000 Spectrophotometer (Nanodrop; Wilmington, DE) and a Bioanalyzer 2100 (Agilent; Santa Clara, CA). Probe labeling, hybridization, and scanning for the eight RNA samples were performed by a single staff member of the University of Kentucky Microarray Core Facility.

Microarray analysis

Data processing and statistical analysis was performed using the Affy Bioconductor package for the R statistical environment (Bolstad et al., 2005). Background correction, normalization, and probe set summarization were performed via the robust multi-array average (RMA) algorithm of (Irizarry et al., 2003). The limma package (Smyth, 2008) available from Bioconductor was used to conduct the equivalent of unpaired t-tests between the control and regenerating groups. Genes were identified as differentially expressed if they passed a false discovery rate set at 5% and changed at least >1.5 fold from baseline.

Quantitative real-time PCR

Significantly changed genes and genes previously hypothesized to be the NTF were chosen for quantitative real-time PCR analysis. A BioRad iScript cDNA synthesis kit (Hercules, CA) was used to synthesize cDNA templates from three Day 0 and three Day 14 RNA samples collected from animals not used for microarray analysis. Primers were designed with Primer3 (Rozen and Skaletsky, 2000) to amplify 80-100 base pair PCR products. Reactions included cDNA that was synthesized from 10ng total RNA, 150nM primers, and SYBR Green Reaction Mix (Roche; Switzerland) and run on a

StepOne Real-Time PCR System (Applied Biosystems; Foster City, CA). The three replicates were normalized against a gene that showed no significant gene expression change in the microarray experiment (glyceraldehyde-3-phosphate dehydrogenase, MC01187). Fold change estimates were calculated using the $\Delta\Delta$ Ct methodology (Livak and Schmittgen, 2001). Real-time PCR primers used include: ANGPTL2 F, 5'- TCA AGC TCC TAC GCA AGG-3' and ANGPTL2 R 5'- CGG ATG ATT TCG TGG AGA-3'; ANKRD1 F 5'- TGG CGC AAA CCT AAC AGT-3' and ANKRD1 R 5'- TCC TTT GTG CCA TTC TGC-; CSRP F 5'- CAC GGT GTG GGA AGT CTG-3' and CSRP R 5'- TTC CCA CAA AAG GCA CAC-3'; GAP43 F 5'- GGC TAA CGG AGA GGC TGT-3' and GAP43 R 5'- TCG CAG ACG TGT CAG ATG-3'; GGF F 5'- ACA CCG ACC ATT GGA ACA-3' and GGF R 5'- AAG CAC TCG CCA CCA TT-3'; LECT3 F 5'- GGA GGG GTC CTC GTA CTG-3' and LECT3_R 5'- GGT TTC CGG TGA AAG GAG-3'; LEPTIN F 5'- TGC CAA AAA CCT CAC CAG-3' and LEPTIN R 5'- CTA AGC CGC TCA CCT TCA-3'; SODEFRIN F 5'- AAG GTT GCA GAC CCC AGT-3' and SODEFRIN R 5'- GAA GAA CCC CCA CAG TCC-3'; TRANSFERRIN_F 5'-GGC GTG TTA CGA CAC CAT-3'; TRANSFERRIN R 5'- CCT AAT GGC AAC GAG CTG-3'

In situ hybridization

In situ hybridization was performed as described by Hirota *et al.* (1992) with minor modifications. PCR products were used as probe templates ranging from 400-700 base pairs. Forward primers were appended with T3 (AAT TAA CCC TCA CTA AAG GGA G) and reverse primers with SP6 (ATT TAG GTG ACA CTA TAG AAG AG) RNA polymerase promoters. PCR products were cleaned using Qiagen PCR purification columns (Qiagen) before performing *in vitro* transcription using Roche DIG-labelling kits (Roche). Probes were cleaned up with Sigmaspin columns (Sigma) and diluted to 50 ng/ul in THE RNA Storage Solution (Roche). DRGs were collected from adult axolotls on Days 0, 14, and 28 dpa and fixed at 4°C in 1x PBS, 4% paraformaldehyde overnight. Tissues were sectioned to 16µm using a Microm 500HM cryostat. Differential interference contrast microscopy was performed using an Olympus AX80 microscope and images

were acquired with an Olympus DP70 camera. Primers (without RNA promoters) included: ANKRD1_F_ISH 5'-AAT TAA CCC TCA CTA AAG GGA GTT CCG TTC CGT CTC TTT GTC-3' and ANKRD1_R_ISH 5'-ATT TAG GTG ACA CTA TAG AAG AGG TAC AAG AGA ACC GCC TTG C-3'; HRAS_F_ISH 5'-AAT TAA CCC TCA CTA AAG GGA GCC GGG GAG TAA ACT TCT GAG-3' and HRAS_R_ISH 5'-ATT TAG GTG ACA CTA TAG AAG AGG CGT TGG CAA GTC ACA TTT-3'; SRV_02399_T3_S 5'-AAT TAA CCC TCA CTA AAG GGA GAG CGC ACG ATG TCT TTC TGT A-3' and SRV_02399_SP6_AS 5'-ATT TAG GTG ACA CTA TAG AAG AGG CGT TGA AAG AGG CGG TGG TAC TCC AAC TCA T-3'; MBP_5.1_ISH 5'-TAA TAC GAC TCA CTA TAG GGA GGC CAG AAC CTT GGA ATC TGA and MBP_3.1_ISH AAT TAA CCC TCA CTA CGG GGT TAT CCT CAA.

Results

Peripheral nerve regeneration in early blastemas

We chose 14 dpa for harvesting DRGs because this time point represented the early limb bud stage of regeneration. It is known that extensive innervation takes place during the early stages of blastema formation. In order to verify that axons are innervating the early blastema, immunohistochemical staining of axons using the antibody RT-97 was performed in limbs collected 14 dpa as well as 28 dpa. Figure 5.1 shows there is a large number of axons throughout the epidermis and blastema at 14 dpa. These results indicate that 14 dpa is an appropriate time point to harvest DRGs to detect mRNA changes associated with axon outgrowth and support of early blastema growth.

Identification of differentially expressed genes and similarities to mammalian studies

To identify gene expression alterations in DRGs following limb amputation, custom axolotl Affymetrix microarrays were used to compare mRNA abundances between DRGs isolated from uninjured and regenerating limbs (14 dpa). 109 of 4844 probe sets were identified as significantly different in mRNA abundances between 0 and 14 dpa. Changes from baseline mRNA levels ranged from 29.45 fold up-regulation (*ankyrin repeat domain 1; ANKRD1*) to -2.39 fold down-regulation (*Na+/K+ transporting ATPase beta 2 protein*). The majority of genes exhibited subtle fold level changes with

only 11 genes changing > 5 fold up-regulation and only two genes >2 fold downregulation from baseline. 63% of the differentially regulated genes (n = 69) correspond to salamander sequences (genes) that show high sequence identify to a presumptive human protein-coding locus; the remainder correspond to anonymous EST contigs. Fold changes are summarized for up-regulated genes with presumptive human orthologs in Table 1 and down-regulated genes in Table 2. The genes found in Table 2 are potential candidates for participating in the re-growth of sensory neurons into an early blastema or as possible candidates for the elusive NTF(s).

In order to identify similarities and differences between salamander and mammalian peripheral nerve gene expresssion, we compared results from this study to studies performed on mice and rat DRGs after peripheral nerve injury. Of the 30 most upregulated salamander genes that have a presumptive human ortholog, almost half (n = 13) are up-regulated following mammalian peripheral nerve injury (Table 5.3). Three other genes are well-recognized regulators of neurite outgrowth in mammalian neurons (*PAK1* and *HRAS*). These results suggest that the transcriptional response following limb amputation in salamander DRGs is similar to the DRG response produced following mammalian peripheral nerve injury.

Down-regulated genes are less similar to the transcriptional response for mammalian peripheral nerve injury. The most striking similarity is that much fewer genes are downregulated than upregulated in both salamanders and mammals. Another similarity is the down-regulation of genes coding for NA+/K+ and calcium ion channels (Valder et al., 2003; Xiao et al., 2002). Although the changed genes are not identical, both Na+/K+ *transporting ATPase beta 2 protein* and *ATPase, class VI, type 11A* were down-regulated in salamander DRGs.

Several genes identified by microarray analysis were validated using real-time PCR on independent samples (Figure 5.2). In general, we found that genes identified as significant by microarray analysis were also found to change in the same direction by real-time PCR. *In situ* hybridization was used to localize gene transcripts within DRGs because gene candidates may be expressed in other cell types including Schwann cells, immune cells, and support satellite cells. We found that ANKRD1, MMP9, and HRAS

were all expressed in cells resembling neurons. A probe specific to myelin basic protein was used to identify Schwann cells and myelinated regions (Figure 5.3).

Similarities to retinal and spinal cord injury

To determine whether gene expression changes that take place following peripheral nerve injury are similar to transcriptional events following other nervous system injuries, we compared the gene list from this study to changed genes following salamander spinal cord injury (Monaghan et al., 2007) and optic nerve transection (Elizabeth Debski, unpublished). Table 5.4 contains 24 genes that were significantly regulated in all three injury models. This list includes genes shown to be up-regulated after mammalian peripheral nerve injury including *ankyrin repeat domain-containing 1*, *MMP9*, *GADD45* as well as genes involved in the mitogen-activated protein kinase (MAPK) signaling pathway including *H-RAS*, *C-MYC*, *PAK1*, and *GADD45*.

Potential candidates for nerve outgrowth and the neurotrophic factor(s)

Gene ontology analysis was performed on 69 unique genes in order to identify possible signaling pathways regulated in DRGs following limb amputation as well as possible NTF(s) candidates (Dennis et al., 2003). We found that the majority of differentially regulated genes were intracellularly located (n = 40). These intracellular gene products are involved in multiple signal transduction pathways and cellular processes associated with neuron growth included the MAPK signaling pathway (n = 6; p = 4.2E-3), apoptosis (n = 13; p = 7.4E-3), and transport (n = 18; p = 2.8E-2). Only eleven gene products were identified as being found extracellularly located, ten of which are predicted to be secreted (bolded in Tables 5.1 and 5.2). One salamander-specific gene, Sodefrin, is known to be secreted as a mating pheromone in some salamander species and is thus recognized as a secreted molecule in this study (Nakada et al., 2007).

Previous experiments have shown that the NTF(s) is made in the cell body, transported down axons to be secreted into the blastema, and may increase during blastema formation. We identified eight gene candidates that may fit this profile including *matrix metalloproteinase 9, sodefrin, angiopoietin-like 2, leptin, 26 serine protease, follistatin, leukocyte cell-derived chemotaxin 2, and latent transforming growth factor*

beta binding protein 3. Six of these gene products have also been shown to have mitogenic activity on certain mammalian cell types (Zhang et al., 2006; Hiraki et al., 1996; Mori et al., 1997; Koli et al., 2008; Jenne et al., 1991; Maumus et al., 2008; Edqvist et al., 2008). Unfortunately, the expression of secreted genes is often low and *in situ* hybridization failed on all but *MMP9. MMP9, sodefrin, ANGPL2*, and *leptin* are also upregulated following spinal cord injury and optic nerve transection. In order to address whether previously identified gene candidates increased expression in our injury model, we performed real-time PCR on neuregulin (GGF2) and transferrin. Both genes increased mRNA abundance in DRGs at 14 dpa (Figure 5.2). Singer hypothesized (1978) that all nerves are capable of supporting regeneration if in great enough quantities. These genes that are up-regulated in multiple injury paradigms make promising candidates for supporting salamander limb regeneration.

Discussion

The present study identified differentially regulated genes in DRGs during early blastema formation. The aim of the study was to identify regeneration-associated genes as well as possible candidates for the NTF(s). We compared the changed gene lists to changed genes in mammalian DRGs to identify an evolutionarily conserved set of genes that are associated with peripheral nerve regeneration. This comparison gave 12 genes that may be necessary for peripheral nerve regeneration in vertebrates. We also compared gene expression changes in the present study with differentially regulated genes following spinal cord injury and optic nerve transection. This analysis identified 24 genes associated with both central and peripheral nerve regeneration in the salamander. Interestingly, three of these genes, *ANKRD1*, *MMP9*, and *GADD45*, were also up-regulated in mammalian peripheral nerve injury. Lastly, eight up-regulated genes were predicted to be secreted from neurons, making them good candidates for the NTF. These genes will be important targets for functional analysis in order to understand the relationship between nerves and the early blastema.

One aim of the study was to identify possible genes responsible for inducing and supporting axon regeneration. Measuring mRNA level changes is appropriate for this task because *de novo* transcription is necessary for axon outgrowth of DRG neurons in

mammals and frogs (Plunet et al., 2002; Smith and Skene, 1997; Tonge et al., 2008). Two transcriptional regulators that were up-regulated more than 20 fold in this study, ANKRD1 and CGRP3, are also highly up regulated in rat DRGs for at least 14 days following sciatic nerve crush (Stam et al., 2007). Over-expression of ANKRD1, but not CGRP3, enhanced neurite outgrowth in vitro; knockdown of ANKRD1 had the opposite effect. These results indicate that ANKRD1 is involved in neurite outgrowth of rat neurons. ANKRD1 was not expressed in embryonic rat spinal cords or following a dorsal root nerve crush, an injury that does not induce substantial neurite outgrowth. This suggests that ANKRD1 expression is associated with neurite regeneration and not just neurite outgrowth. We found ANKRD1 to be up-regulated following salamander spinal cord injury, optic nerve transection, and peripheral nerve transection; all tissues that will eventually regenerate (Table 5.4). It is possible that ANKRD1 up-regulation regulates downstream transcriptional targets to promote axon regeneration in multiple injury paradigms. It is also possible that ANKRD1 up-regulation following spinal cord injury and optic nerve crush may be related to the uncommon regenerative abilities of the salamander CNS. These results justify further investigation of ANKRD1 during salamander regeneration.

Regeneration-associated genes, including GAP-43 (Bomze et al., 2001), RAR β (Corcoran et al., 2000, 2002), Cap23 (Bomze et al., 2001), α 7 integrin (Gardiner et al., 2005), and cJun (Raivich et al., 2004), have been identified in mammalian peripheral nerve injury models and shown to promote intrinsic growth ability. We found that GAP-43 and CAP23 significantly changed according to microarray analysis (p = 4.46E-5 and 2.0E-3, respectively), but did not meet fold level criteria (fold change = 1.46 and 1.18). The other RAGs above are not represented on the Ambystoma GeneChip. Real-time PCR showed that GAP-43 is up regulated and at 14 dpa, suggesting that the GAP43 probe-set underestimated mRNA level changes. GAP-43 and CAP23 are both highly expressed in uninjured DRGs (ranks 205 and 272 of 4844 probe sets), suggesting that salamander DRGs may express high levels of RAGs under normal conditions. Even though cJun is not included on the GeneChip, a closely related gene JunB was upregulated (fold change = 3.75; p = 3.35E-8). It is possible that JunB has a similar role to cJun because it has also been found to be up regulated in several mammalian peripheral nerve injury studies (Buschmann et al., 1998; Kenney and Kocsis, 1997). Fourteen up regulated genes were

associated with apoptosis. It is unknown whether programmed cell death takes place in DRGs following limb amputation in salamanders, but it is known that peripheral nerve injury in neonatal mice induces DRG neuron death (Himes and Tessler, 1989). Interestingly, sensory neuron cell death does not take place in adult mice, possibly due to up-regulation of HSP27 (Benn et al., 2002). It is possible that a similar protective mechanism takes place in salamander sensory neurons following limb amputation.

The comparison of changed genes between three nervous system injury models identified a list of 30 genes that changed in all three studies. Four of these genes, H-RAS, C-MYC, PAK1, and GADD45 are known to function in the mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK signaling pathway is activated by the binding of extracellular growth factors at the cell surface and leads to multiple cellular responses including cell proliferation, differentiation, and survival of neurons. The small GTPase, *H-RAS* is an up-stream component of the MAPK signal transduction cascade that is a major contributor to the survival of neurons through multiple signal transduction pathways including PI-3Kinase/AKT and MAPK pathways (Kaplan and Miller, 2000 for Review). One target of the MAPK pathway is the phosphorylation and activation of the transcription factor C-MYC. Phosphorylated C-MYC then transcriptionally activates a wide range of target genes that regulate cell behavior (Pelengaris et al., 2002). PAK1 (Smith et al., 2008) and GADD45 (Takekawa and Saito, 1998) are also involved in regulating MAPK signaling. PAK1 is upregulated in zebrafish retinal ganglion cells after optic axon injury (Veldman et al., 2007) and can control neurite outgrowth by regulating actin dynamics in neurons (Daniels et al., 1998; Rashid et al., 2001). The up-regulation of these genes in three nervous system regeneration models suggests that the MAPK pathway plays a role in nervous system regeneration in salamanders. The lack of upregulation of H-RAS, C-MYC, and PAK1 (data not shown) during limb regeneration supports the hypothesis that the role of these genes is specific to nervous system regeneration.

One criteria of the NTF(s) is that it is a secreted peptide or set of peptides (Singer, 1952). Co-culture experiments of injured and uninjured spinal ganglia with blastema cells suggest that this secreted peptide(s) increases in regrowing neurons (Boilly and Bauduin, 1988). Lastly, the NTF is thought to be found in all nervous tissue (Globus and Liversage,

1975; Globus and Vethamany-Globus, 1977; Kamrin and Singer, 1959; Singer et al., 1976; Carlone and Foret, 1979; Choo et al., 1978; Deck, 1971; Lebowitz and Singer, 1970). Several genes were identified as secreted and up-regulated in DRGs during blastema formation, spinal cord regeneration, and optic nerve transection including MMP9, Sodefrin, angiopoietin-like 2, and leptin (Table 3). MMP9 is likely not the NTF because it is highly expressed in all salamander injury models studied to date (Monaghan et al., 2009; Monaghan et al., 2007; Elizabeth Debski, personal communication; Cotter et al., 2008). It is possible that MMP9 activates growth factors or releases them from the blastema extracellular matrix (McCawley and Matrisian, 2001 for Review), but MMP9 activity is also known to promote neurite elongation (Shubayev and Myers, 2004).

A sequence (SRV 10508 a at) with 33% amino acid homology to the sodefrin precursor-like factor found in the salamander *Desmognathus monticola*, was upregulated 7.18 fold in DRGs and also upregulated in other nervous system injury models (Table 5.4). Sodefrin is a sex pheromone in plethodontid salamanders that is secreted from male glands and influences female behavior (Palmer et al., 2007; Kiemnec-Tyburczy, 2009). We find that the axolotl sodefrin contains a secretion signal sequence (TargetP; Emanuelsson et al., 2000) and contains a Phospholipase A2 (PLA2) inhibitor domain. PLA2s play roles in inflammatory pathways and disease (Touqui and Alaoui-El-Azher, 2001) and are found as toxic components of snake and insect venoms (Huang and Kini, 1997). PLA2 inhibitors are expressed in snakes to block toxic PLA2 enzyme activity (Dunn and Broady, 2001 for Review). It may be that sodefrin's role in limb regeneration is to decrease local inflammation by inhibiting PLA2 activity. There is support for sodefrin's role in the nerve dependence of limb regeneration, because it belongs to the Ly-6 family of proteins, the same protein family as prod1. Prod1 is the receptor to newt anterior gradient 2, a gene that can rescue regeneration in denervated limbs when overexpressed (Kumar et al., 2007). It will be interesting to see if either of these hypotheses is supported by functional analyses.

The gene product of *angiopoietin-like 2* (*ANGPTL2*) is a possible candidate for the NTF. *ANGPTL2* is a member of the angiopoietin-like family of orphan ligands that are involved in angiogenesis and lipid, glucose, and energy metabolism (Hato et al., 2008). Morpholino knockdown of *ANGPTL1* and *ANGPTL2* in zebrafish cause severe vascular

defects because they increase endothelial cell death during development (Kubota et al., 2005). Angiogenesis takes place early during blastema formation in newts, (Rageh et al., 2002), which is not surprising considering that all growing tissues, including developing limbs require oxygen and nutrients normally supplied by vasculature (Vargesson, 2003). Smith and Wolpert (1975) proposed that vascularization is necessary for regeneration to take place and that denervation inhibits vascularization of the regeneration stump. Tassava (personal communication) has since shown that angiogenesis does take place in denervated newt stumps, but further characterization is needed. It is possible that *ANGPTL2* is secreted by nerves into the early blastema and promotes angiogenesis. *ANGPTL2* has also been shown to be a potent stimulator of hematopoietic stem cell expansion *ex vivo* (Zhang et al., 2006). Furthermore, a related molecule, *ANGPTL6*, promotes mouse keratinocyte expansion *in vivo* and increases epidermal regeneration (Oike et al., 2003). It will be interesting to find whether *ANGPTL2* plays a mitogenic role in early blastema tissues.

The last of the up-regulated, secreted molecules in all nervous system injuries is leptin. Leptin is a major regulator of energy metabolism in mammals normally expressed in adipose tissue (Zhang et al., 1994). A closely related salamander species, the Tiger salamander, also shows leptin expression in nervous system tissue (Boswell et al., 2006). It is possible that leptin is secreted from peripheral nerves to regulate energy expenditure in the blastema. The probe set for leptin gives a very low signal in all studies, suggesting that leptin mRNA levels may be low. Regardless, it may have a very focused affect on the innervated blastema tissue.

In order to address whether previously identified NTF candidates change in our injury model, real-time PCR was performed on genes previously proposed to be the NTF including neuregulin-1 (Wang et al., 2000) and transferrin (Mescher, 1997). Both of these genes were up regulated in our injury model, supporting the hypothesis that these may be the NTF(s). In conclusion, this study indicates that changes in mRNA levels of at least 100 genes take place in DRGs following limb amputation. The results provide a set of new gene candidates for understanding the role of nerves during salamander limb regeneration. Intracellular genes such as ANKRD1, signaling pathways such as the

MAPK pathway, and extracellular candidates for the NTF(s) need to be followed up by experiments designed to assess the function of these genes during limb regeneration.

Probe ID	Human ortholog	FC	p-value		
SRV_03532_at	ANKRD1	29.45	6.83E-09		
SRV_02024_at	ADRP	21.76	1.21E-10		
SRV_00853_at	CGRP3	21.14	3.32E-08		
SRV_00344_at	Keratin 5	11.38	1.60E-07		
SRV_02399_a_at	MMP9	10.98	5.74E-06		
SRV_03023_a_at	GADD45γ	7.19	4.63E-08		
SRV_10508_a_at	SODEFRIN	7.18	2.66E-07		
SRV_01031_at	HSP27	4.4	4.55E-07		
SRV_01466_at	NMB	4	1.34E-04		
SRV_04693_a_at	DESMOCOLLIN 1	3.81	1.48E-08		
SRV_01351_at	JUN B	3.75	3.35E-08		
SRV_03250_a_at	ANGIOPOIETIN-LIKE 2	3.23	3.72E-10		
SRV_03753_at	GADD45β	2.97	6.29E-05		
SRV_01323_a_at	HMO1	2.95	3.19E-05		
SRV_00259_a_at	LEPTIN	2.77	1.90E-05		
SRV_05557_at	TMEM52	2.72	2.11E-05		
SRV_02773_at	26 SERINE PROTEASE	2.7	2.49E-05		
SRV_11456_at	HRAS	2.68	3.36E-08		
SRV_01488_at	PAK1	2.67	8.17E-08		
SRV_02611_a_at	GLUCOCORTICOID REG. KINASE	2.59	4.40E-06		
SRV_02866_a_at	TUMOR SUSCEPTIBILITY 101	2.56	1.28E-06		
SRV_02973_at	IGF-LIKE 2 MRNA BINDING 3	2.32	7.76E-05		
SRV_09706_at	GLUTAMINE SYNTHETASE	2.1	1.94E-05		
SRV_01307_a_at	GLUTAMINE SYNTHETASE	2.07	1.14E-03		
SRV_03871_a_at	SAR1	2.05	9.63E-08		
SRV_03246_at	6-PHOSPHOGLUCONOLACTONASE	2.04	1.93E-07		
SRV_03390_a_at	FOLLISTATIN	2.01	2.74E-03		
SRV_01385_at	LECT2	1.97	1.59E-03		
SRV_04964_a_at	TUBULIN, BETA 6	1.91	1.40E-05		
SRV_01467_a_at	GLYCOPROTEIN NMB	1.91	1.16E-04		
SRV_00140_at	CDK1C	1.78	9.70E-05		
SRV_05752_at	SYNCYTIN	1.76	1.67E-03		
SRV_05081_a_at	SORTING NEXIN 18	1.76	1.83E-05		
SRV_00332_a_at	G6PD	1.75	3.89E-06		
SRV_02600_a_at	LEGUMAIN	1.75	3.68E-05		
SRV_04911_at	TXNDC2	1.75	6.76E-07		
SRV_04735_at	NUCLEAR PROTEIN UKP68	1.71	2.83E-06		
SRV_04996_at	SOLUTE CARRIER FAMILY 7, 3	1.71	2.02E-03		
SRV_04524_at	SOLUTE CARRIER FAMILY 30, 1	1.71	2.48E-06		
Table 5.1 continued on next page					

Table 5.1. Up-regulated genes in DRGs following limb amputation.

Table 5.1 continued

Probe ID	Human ortholog	FC	p-value
SRV_03758_a_at	ARRESTIN DOMAIN CONTAINING 2	1.69	2.78E-05
SRV_02687_s_at	M6PRBP1	1.69	1.07E-05
SRV_04819_a_at	TRANSMEMBRANE PROTEIN 49	1.66	3.27E-04
SRV_04501_a_at	LATENT TGFB BINDING PROTEIN 3	1.6	2.14E-03
SRV_03383_a_at	PDCD61P	1.57	1.40E-06
SRV_09231_at	ADRP	1.55	3.65E-05
SRV_00867_a_at	DEFENDER AGAINST CELL DEATH 1	1.55	2.53E-06
SRV_01305_at	GLUTAMATE-CYSTEINE LIGASE	1.54	2.67E-04
SRV_00515_at	FLJ16008 PROTEIN	1.54	3.33E-03
SRV_03108_a_at	SPLICING FACTOR 2	1.54	1.09E-04
SRV_05100_at	GLUCURONOSYLTRANSFERASE P	1.54	1.56E-04
SRV_00744_a_at	ADRP	1.53	1.56E-03
SRV_01442_at	CMYC	1.51	3.13E-04
SRV_01651_a_at	RGS2	1.51	8.63E-04
SRV_02584_a_at	STATHMIN 1	1.51	1.04E-05

Probe ID	Human ortholog		p-value	
SRV_01118_a_at	ATPASE, NA+/K+ TRANSPORTING, $\beta 2$	-2.39	1.55E-06	
SRV_03357_at	CD69 ANTIGEN	-2.32	3.01E-03	
SRV_01275_at	FIBULIN 1	-1.96	7.50E-04	
SRV_02395_a_at	JUMONJI	-1.93	5.10E-05	
SRV_02948_a_at	FIBULIN 1	-1.79	9.16E-06	
SRV_04213_at	RHO GTPASE ACTIVATING 27	-1.78	7.70E-06	
SRV_02599_a_at	ATPASE, CLASS VI, TYPE 11A	-1.75	2.59E-05	
SRV_02792_at	SEMAPHORIN 3D	-1.71	3.84E-03	
SRV_02442_a_at	INTERFERON, ALPHA-INDUCIBLE	-1.67	9.07E-04	
SRV_05046_at	HORNERIN	-1.65	2.55E-03	
SRV_02421_at	PLASTIN 3	-1.6	1.85E-03	
SRV_01225_at	CRYSTALLIN, ALPHA B	-1.57	5.24E-05	
SRV_00038_at	KIT LIGAND	-1.55	8.19E-04	
SRV_01281_at	FK506 BINDING PROTEIN 4, 59KDA	-1.54	2.93E-03	
SRV_03517_a_at	TNF, ALPHA-INDUCED PROTEIN 8	-1.54	1.05E-03	
SRV_05108_a_at	PSAT1	-1.51	3.04E-03	

Table 5.2. Downregulated genes in DRGs following limb amputation.

Human			
Probe ID	ortholog	FC	Other studies
SRV_00853_at	CGRP3	21.14	Valder 2003 (U44948); Newton et al., 2000; Costigan et al., 2002; Xiao et al., 2002
SRV_03023_a_at	GADD45G	7.19	Tanabe et al., 2003; Befort et al., 2003; Nilsson et al., 2005;
SRV_03753_at	GADD45B	2.97	Tanabe et al., 2003
SRV_01466_at	NMB	4	Takeuchi et al., 2008
SRV_03532_at	ANKRD1	29.45	Stam et al., 2007; Boeshore et al., 2004; Costigan et al., 2002
SRV_02399_a_at	MMP9	10.98	Shubayev and Myers, 2004
SRV_03390_a_at	FOLLISTATIN	2.01	Nilsson et al., 2005
SRV_01323_a_at	HMO1	2.95	Magnusson et al., 1999; Takeuchi et al., 2008; Boeshore et al., 2004
SRV_01031_at	HSP27	4.4	Costigan et al., 2002; Tandrup et al., 2000; Lewis et al., 1999; Williams et al., 2006; Costigan et al., 1998; Xiao et al., 2002
SRV_04964_a_at	TUBULIN B6	1.91	costigan et al., 2002
SRV_01351_at	JUNB	3.75	Buschmann et al., 1998; Kenney and Kocsis, 1997
SRV_09706_at	GLUL	2.1	Boeshore et al., 2004

 Table 5.3. Upregulated genes that are also upregulated after mammalian peripheral nerve injury

		DRG	Retina	SC
Probe ID	Gene Name	FC	FC	FC
SRV_03532_at	ANKYRIN REPEAT DOMAIN 1	29.45	4.04	4.13
SRV_02024_at	AP1S3	21.76	2.97	2.74
SRV_08647_a_at	NO KNOWN ORTHOLOG	16.96	4.36	6.49
SRV_02399_a_at	MMP9	10.98	1.99	5.53
SRV_03023_a_at	GADD45γ	7.19	3.57	2.4
SRV_10508_a_at	SODEFRIN	7.18	21.53	1.75
SRV_09432_at	NO KNOWN ORTHOLOG	4.24	1.59	1.54
SRV_01466_at	GLYCOPROTEIN NMB	4	4.62	20.65
SRV_04693_a_at	DESMOCOLLIN 1	3.81	2.31	1.8
SRV_03250_a_at	ANGIOPOIETIN-LIKE 2	3.23	1.5	5.33
SRV_00259_a_at	LEPTIN	2.77	5.01	2.18
SRV_11456_at	H-RAS	2.68	2.34	2.05
SRV_01488_at	PAK1	2.67	2.28	1.68
SRV_09852_at	NO KNOWN ORTHOLOG	2.13	1.59	1.92
SRV_09045_at	NO KNOWN ORTHOLOG	1.94	1.55	4.88
SRV_04964_a_at	TUBULIN, BETA 6	1.91	3.84	3.2
SRV_01467_a_at	GLYCOPROTEIN NMB	1.91	3.92	7.54
SRV_04911_at	TXNDC2	1.75	2.28	1.96
SRV_02687_s_at	M6PRBP1	1.69	1.54	1.69
SRV_04819_a_at	TMEM49	1.66	2.11	3.98
SRV_09231_at	ADFP	1.55	1.99	3.43
SRV_03108_a_at	SPLICING FACTOR 2	1.54	2.31	1.56
SRV_00744_a_at	ADFP	1.53	1.94	3.85
SRV_01442_at	C-MYC	1.51	1.86	1.54

Table 5.4. Changed genes in three nervous system injury studies.

Figure 5.1 Immunohistochemical staining of neurites in a blastema at 14 dpa. RT-97 antibody (Red) was used to stain axons in a regenerating limb and Hoescht 33258 was used to stain nuclei. The WE is seen on the right overlying a small blastema.



Figure 5.2. Real-time PCR of genes that change in DRGs following limb amputation. Grey bars represent log2 transformed fold changes according to real-time PCR. White bars represent log2 transformed fold changes according to microarray analysis. Error bars $= \pm$ standard error of the mean. GGF and Transferrin were not represented on the microarray platform.



Figure 5.3 *In situ* hybridization of changed genes in DRGs during limb regeneration. DRGs collected at 14 dpa are represented in A,C,D,F,G,I,J, and K. Sense probes are represented in C,F,I, and K. DRGs collected at 0 dpa are represented in B,E, and H. A-C) ANKRD1, D-F) MMP9, G-I) HRAS, J-K) MBP. Scale bar = 100µm.



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CHAPTER 6

IDENTIFICATION AND CHARACTERIZATION OF MATRIX METALLOPROTEINASES DURING TAIL REGENERATION IN THE MEXICAN AXOLOTL

Monaghan, J.R.,¹ DeMeio, A.L.,¹ Walker, J.A.,¹ Voss, S.R.¹* ¹ Department of Biology, University of Kentucky, Lexington, KY 40506

*Correspondence to: Randal Voss, University of Kentucky, Lexington, KY 40506 Email: srvoss@email.uky.edu

Tel: 859-260-9476

Fax: 859-257-1717

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Introduction

Salamanders have an unusual ability to repair injuries and regenerate missing appendages. This ability traces, in part, to cellular responses that modify the extracellular environment in a way that promotes cell survival, proliferation, differentiation, and patterning. Matrix metalloproteteinases (MMPs) are a family of proteolytic enzymes that regulate cell-cell and cell-extracellular matrix (ECM) interactions by cleaving extracellular substrates. These proteolytic events alter the structural composition of the ECM and regulate the availability of growth factors and cytokines (Page-McCaw et al., 2007; McCawley and Matrisian, 2001). Studies using rodent models have implicated MMP function in development, tissue remodeling, immunity, and disease (Nagase et al., 2006). MMPs also participate in regenerative responses across a wide range of animals including hydra foot and head regeneration (Leontovich et al., 2000; Shimizu et al., 2002), drosophila imaginal disc regeneration (McClure et al., 2008), sea cucumber intestinal regeneration (Quiñones et al., 2002), zebrafish fin regeneration (Bai et al., 2005), amphibian limb regeneration (Vinarsky et al., 2005), and mouse liver regeneration (Alwayn et al., 2008). However, it is unclear exactly when MMPs participate, which MMPs participate, or how MMPs are regulated in these regeneration paradigms. It will be important to address these issues in order to assess the therapeutic potential of MMPs in regenerative medicine.

MMPs are regulated at multiple cellular levels (reviewed by Yan and Boyd, 2007). Following transcription and translation into an inactive enzyme, MMPs are held inactive through binding of the pro-peptide domain to the catalytic site via a zinc ion (Figure 6.2; 6.3). MMPs are then activated by proteolytic cleavage of the pro-peptide domain, freeing the catalytic domain (HEXXHXXGXXH) to cleave substrates. Some MMPs also contain domains thought to evoke substrate specificity such as fibronectin-like and hemopexin-like domains (Nagase et al., 2006; Clark and Cawston, 1989, Chung et al., 2004). Lastly, endogenous inhibitors of MMPs termed tissue inhibitors of MMPs (TIMPs) bind to MMP proteins and regulate their local activity (Nagase et al., 2006). The multi-level nature of MMP regulation ensures that they are only activated when and where they are needed. The importance of MMP regulation is evident in diseases associated with unchecked

MMP activity such as cancer, rheumatoid arthritis, cardiovascular disease, vanishing bone syndrome, atherosclerosis, fibrosis, and ulcers (reviewed by Visse and Nagase, 2003).

MMP activity was first detected in regenerating salamander tissues over 40 years ago (Grillo et al., 1968). Since then, a handful of studies have characterized MMP mRNA levels or protein activity during salamander regeneration (Dresden and Gross, 1970; Yang and Bryant, 1994; Vinarsky et al., 2005; Kato et al., 2003; Chernoff et al., 2000; Yang et al., 1999; Miyazaki et al., 1996; Park and Kim, 1999; Kato et al., 2003). Vinarky et al. (2005) also showed that newt limb regeneration can be partially inhibited if treated with a wide-spectrum MMP inhibitor for 60 days. These data suggest that MMPs are necessary for appendage regeneration, but it is unkown which MMPs are necessary for regeneration or when they may participate in the process. In order to address these questions, we collected MMP sequences from newly-developed salamander genomic databases and characterized their expression throughout regeneration (Putta et al., 2004; Monaghan et al., 2009).

Materials and Methods

Sequence identification and alignments

Matrix metalloproteinase sequences were identified for the axolotl by searching the *Ambystoma* EST database (www.ambysoma.org) and NCBI databases. These sequences were assembled with annotated vertebrate MMPs to develop gene models. Sequence assembly was accomplished using Seqman (DNASTAR). As many of these gene models were partial sequences, it was necessary to extend sequences by PCR and 5' or 3' RACE (Invitrogen). All gene models have been deposited in the NCBI nonredundant nucleotide database.

Animal model, RNA extraction, and real-time RT-PCR

All surgical manipulations were carried out on *Ambystoma mexicanum* according to the University of Kentucky Animal Care and Use guidelines (IACUC #01088L2006). Tail amputations were made at the bottom 1/3 of the tail. Total RNA was isolated from three tails collected 1 mm proximal to the injury plane at 0, 3, 6, 12, 24 hpa, 7, and 14 dpa (mass = 10.34 ± 1.97 standard deviation [SD]; snout-vent length [SVL] = 6.42 ± 1.40)

using TRIzol Reagent (Invitrogen) followed by Qiagen Mini columns (Qiagen). cDNA was produced using poly-T primers provided in the iScript Select cDNA synthesis Kit (Bio-Rad). Real-time PCR was performed in 10 μ l reactions including 300 pM forward and reverse primers, 10 ng cDNA, and FastStart Universal SYBR Green Master Mix (Roche). Primers used are shown in Table 6.1. PCR reactions and melt curve analysis were run on a StepOne Real-time PCR System (Applied Biosystems). Gene expression values (Ct) were produced for three biological replicates by averaging Ct values for two technical replicates. The $\Delta\Delta$ Ct method was chosen to calculate ratios (Livak and Schmittgen, 2001). Ct values were normalized to a control gene (GAPDH) and then a ratio (R) was calculated using the mean of the 0 hpa samples. R-values were log2 transformed for statistical analysis and graphical presentation (Fig. 4,5,7). One-factor analysis of variance was performed followed with Fisher's PLSD tests to test for significance between time points (Stat-View).

In situ hybridization and histology

Tail tissues were sectioned in the transverse and coronal planes for histology and *in situ* hybridization. Figures 6.4L, 6.5M, 6.6H, and 6.7G show cartoons of a typical transverse section through an axolotl tail. In the middle of the axolotl vertebral column is the centrum, containing remnants of the notochord. The spinal cord lies dorsal to the centrum within the neural arch. The hemal arch lies ventral to the centrum, containing the caudal artery and vein. Processes (apophyses) are represented as circles at the dorsal or ventral end of the arches. It was difficult to distinguish between cartilagenous and ossified vertebral tissues. For this reason, cells lying within matrix lacunae of processes were referred to as osteocytes, multinucleated cells surrounding processes were referred to as chondrocytes, and cells on the external surface of vertebrae were referred to as perichondrial fibroblasts.

Probe templates were created from PCR products produced by either M13 primers (cloned MMP fragments into a pGEM-T vector) or MMP-specific primers appended with SP6 and T3 promoters (cDNA produced from regenerating tail RNA). PCR products were cleaned up using Qiagen PCR purification kits (Qiagen). 200-500 ng of PCR product was incubated at 37 °C for 2 hrs in a 10 µl digoxygenin-labeling *in vitro* transcription reaction

(Roche DIG-RNA labeling kit). DNA was degraded for 20 min. with 10 U DNase I, stopped with 2 µl 200 mM EDTA, and cleaned up on Sigmaspin Sequencing Reaction Clean-up columns (Sigma). RNA was quantified on a nanodrop spectrophotometer, diluted to 50 ng/µl in THE RNA storage solution (Ambion), and stored at -80°C. Tissue samples were fixed in freshly made 4% paraformaldehyde / 1XPBS at 4 °C overnight, cryoprotected at 4 °C by immersion in 10% sucrose / 1xPBS for 1 hr, 20% sucrose / 1xPBS for 1hr, and 30% sucrose / 1xPBS overnight. Tissues were mounted in OCT medium, sectioned at 16 µm, and used after 2 hr of drying at room temperature. Hybridization mix included 50% formamide, 10 mM Tris-Cl (pH 8.0), 200 µg/ml yeast tRNA, 10% dextran sulfate, 1x Denhardts's solution, 0.25% SDS, 1 mM EDTA, and 1 µg/ml of DIG-labelled RNA probe. Incubations were performed at 64 °C overnight in a humidified chamber and detected with an overnight incubation in an NBT/BCIP solution (Roche). Sections were mounted with Permount, imaged using an AX-80 Olympus microscope, and photographed using an Olympus DP-70 camera. Figures were assembled using Photoshop 7.0 and brightness and contrast were changed to whole figures. Tissues were treated identically up to sectioning for histology except tissues were decalcified with 500 mM EDTA for 2 days. Tissues were then sectioned at 16 µm and stained with Eosin Y and Gill's hematoxylin #2 (Sigma-Aldrich).

Results and Discussion

Identification of MMPs in the salamander

To identify MMPs in the Mexican axolotl, the *Ambystoma* EST database (www.ambysoma.org) and NCBI databases were searched for presumptive salamander MMPs. These sequences were assembled into 16 contiguous sequences (contigs) and extended using PCR, 5' RACE, and 3' RACE. Contigs then were used as query sequences to search the NCBI human RefSeq protein database (BLASTX; Table 6.2). Eight of the 16 contigs yielded full-length protein coding sequences (relative to human proteins). The other contigs ranged from 96.7% (MMP2) to 15.5% (MMP16) coverage of human MMP protein sequences. Table 6.2 shows the best human hit and best non-redundant hit for each of the 16 presumptive MMP sequences. Sequence similarity alone was insufficient to determine the orthology of many MMP sequences so contigs were named according to

their best human hit or their presumptive orthologs previously identified in the Japanese or American newts. Overall, sequences similar to four collagenases (*aCola*, *aColb*, *aColc*, *aCol3*), two gelatinases (*aMMP2*, *aMMP9*), four stromelysins (*aMMP3/10a*, *aMMP3/10b*, *aMMP3/10c*, *aMMP11*), three membrane-type MMPs (*aMMP14*, *aMMP17*, *aMMP23*), three other MMPs (*aMMP7*, *amphMMP*, *aMMP19*), and a tissue inhibitor of MMP (*atimp1*) were identified. Approximate coverage of each predicted protein sequence to its most similar human MMP is shown in Figure 6.3. Alignment of predicted protein sequences for MMPs with > 50% coding sequence coverage shows the high conservation among the MMPs in the propeptide domain, catalytic domain, and hemopexin-like domain (Figure 6.2). Notice the fibronectin-like repeats in aMMP2 and aMMP9 that define vertebrate gelatinases. Also notice the highly variable hinge domain located between the catalytic and hemopexin-like domains. Below, we show the association of the identified MMPs with aspects of tail regeneration. Each MMP family is treated separately and a summary of expression patterns is provided in Table 6.3.

Wound epithelium formation and function

The wound epithelium (WE) is a critical element of salamander appendage regeneration because preventing WE formation inhibits regeneration (Goss, 1956; Mescher, 1976; Tassava and Garling, 1979; Thornton, 1957; Lheureux, 1983). It is possible that MMP activity in the WE is necessary for regeneration. We found that four MMPs were strongly expressed in the WE basal keratinocytes during the first 24 hpa including *aMMP9* (Fig. 6.5A,C), *aCola* (Fig. 6.4A,D,J), *aColb* (Fig. 6.4B,E), and *aMMP3/10a* (Fig. 6.6A,C,G). *aMMP9* expression was seen only at 12 hpa in basal keratinocytes of the epidermis and was lost by 24 hpa (Figure 6.5C,E,G). It is possible that *aMMP9* expression in the epidermis is associated with keratinocyte migration and is not needed for downstream WE function. Similar observations have been made by others (Yang et al., 1999; Satoh et al., 2007, 2008). Indeed, MMP9 is up-regulated in migrating keratinocytes in mammals (Mohan et al., 1999) and it can cleave collagen type IV, the major constituent of basement membranes. Collagen IV proteolysis has been shown to expose cryptic sites that promote cell migration (Giannelli et al., 1997; Xu et al., 2001).

Thus, it is possible that the role of MMP9 is to cleave collagen IV located in the basement membrane, leading to epithelial cell migration.

aCola, aColb, and aMMP3/10a expression were all observed at 24 hpa. The strongest expression was always observed in basal keratinocytes, but some expression was observed where migrating epithelial sheets overlay the wound surface (Figure 6.4D,E, 6.6C,G). Six hypotheses have been proposed for the function of MMP expression in the WE: 1) MMPs may initiate and support keratinocyte migration and wound closure. This hypothesis is supported by mammalian studies showing defects in wound closure after disrupting MMP function (Bullard et al., 1999; Agren et al., 1999, 2001; Lund et al., 1999; Mirastschijski et al., 2002, 2004; Pilcher et al., 997; Dumin et al., 2001) 2) MMPs may participate in phagocytosis of wound debris from the wound epidermis (Singer and Salpeter, 1961; Reviewed by Carlson, 2007). 3) MMPs may prevent basement membrane formation under the WE, promoting epithelial/mesenchymal interactions (Yang et al., 1999). Epithelial/mesenchymal interactions are thought to be necessary to support blastemal growth (Tsonis, 1996). 4) MMPs may initiate blastema formation by promoting histolysis of underlying tissue. This is supported by experimental evidence showing that histolysis does not take place if the WE is not formed (reviewed by Singer and Salpeter, 1961). 5) MMPs may release growth factors such as FGFs from the ECM to make them available to blastemal cells (Hondermarck and Boilly, 1992; Boilly et al., 1991). Mammalian studies have shown that MMPs 1 and 3 can cleave ECM components to make FGF bioavailable to cells (Whitelock et al., 1996). 6) MMP activity may promote regeneration by preventing scar formation. This is supported by the association of scar formation and regenerative ability in fetal mammalian skin (Bullard et al., 1997; Ferguson and O'Kane, 2004) and the observation that a scar-like epidermis formed when newt limbs were treated with GM6001 (Vinarsky et al., 2005). It is likely that these MMPs may have redundant roles and work synergistically in WE formation and function. The exact function of the WE is just starting to be appreciated during appendage regeneration, but it is clear that MMPs will be intimately involved in these functions (Campbell and Crews, 2008).

Histolysis and blastema formation

One surprising observation in this study was the low MMP expression in muscle cells undergoing histolysis versus strong staining in cartilage, bone, and epidermis. Similar observations were made during limb regeneration for MMP3/10b and MMP9 in the Japanese newt (Kato et al., 2003) and MMP9 in larval axolotls (Yang et al., 1999), but strong expression was observed in blastema cells for MMP9 and collagenase in the American newt (Vinarsky et al., 2005). We found some expression of *aCola* (Figure 6.4D,J), *aColb* (Figure 6.4E), *aMMP9* (Figure 6.5G), and *TIMP1* (Figure 6.7D) transcripts in areas of tissue histolysis. In particular, *aColb* was expressed in cells resembling neutrophils and leukocytes throughout the early wound site (Fig. 4B,K). Human MMP8, or neutrophil collagenase, is expressed in neutrophils following injury, suggesting that *aColb* and human MMP8 play similar functions (Van Lint and Libert, 2006). Regardless, muscle fibers seemed to be stained the least of all tissues. It is possible that the WE, bone, and inflammatory cells are secreting the necessary MMPs to promote muscle histolysis and blastema formation. Further investigation is needed to see if MMP activity is needed for muscle histolysis.

Bone and cartilage remodeling

One of the most striking expression patterns observed was broad MMP expression in bone and cartilage found throughout the vertebrae. We observed expression in cells resembling osteocytes, osteoclasts, perichondrial fibroblasts, periosteal fibroblasts, and chondrocytes. By 24 hpa, strong expression was observed by *aMMP9* (Figure 6.5E), *aCola* (Figure 6.4D,J), and *aCol3* (Figure 6.4F) in vertebrae. Expression of these genes extended out to 14 dpa, suggesting that they play roles in bone and cartilage remodeling. Indeed, knockout mice of *MMP9* and *Col3* show growth-plate defects (Vu et al., 1998; Stickens et al., 2004; Inada et al., 2004), suggesting that the function of these genes may be conserved between development in mammals and regeneration in salamanders.

Two MMPs were found to be up-regulated only during later stages of regeneration including *aMMP2* (Figure 6.5B) and *aMMP14* (Figure 6.7A). *aColc* showed a similar gene expression pattern, but was also expressed earlier (Figure 6.4C). Each of these genes was also expressed in similar cell types including chondrocytes and perichondrial fibroblasts of the centrum (Figure 6.4O,6.5K,6.7C). Interestingly, aMMP3/10b was also

found to be expressed in osteocytes at 14 dpa. Given the short time frame studied here, these mRNAs likely continue to increase beyond 14 dpa. Mouse knockouts of *MMP2* and *MMP14* have defects in bone and/or connective tissue formation and remodeling (Stickens et al., 2004; Inada et al., 2004; Holmbeck et al., 1999; Zhou et al., 2000; Inoue et al., 2006; Page-McCaw et al., for Review). Also, two out of three human skeletal diseases caused by loss-of-function MMP mutations are associated with *coll3* and MMP2 (Martignetti et al., 2001; Kennedy et al., 2005). MMP14 is also known to activate MMP2 *in vivo* and function redundantly (Nagase and Woessner, 2000; Oh et al., 2004). This evidence suggests that *aCol3*, *aMMP2*, and *aMMP14* may participate similarily during the reformation of vertebrae, especially the cartilage tube, during tail regeneration. Other studies are needed to test whether these MMPs are critical at the cartilage-forming stages of salamander appendage regeneration.

Nervous system and vascular MMP9 expression

aMMP9 transcripts were localized to multiple tissues including the nervous system and the vasculature (Fig. 5H,J). *aMMP9* has previously been localized to spinal cord tissue during the early stages of spinal cord regeneration in the axolotl (Monaghan et al., 2007) as well as dorsal root ganglia neurons during limb regeneration (personal observations). MMP9 has been shown to be involved in neurite elongation in mammals (Shubayev and Myers, 2004). aMMP9 expression in multiple CNS injury paradigms suggest that it may have a role in nervous system injury or regeneration. Functional analyses are necessary to test this hypothesis.

aMMP9 was also expressed in areas resembling vascular formation at 21 dpa. It was surprising to find that aMMP9 was not expressed elsewhere in the regenerating tail at this time point. Support for the role of *aMMP9* in vascular remodeling comes from mouse studies showing that MMP9 mutant mice have defects in angiogenesis as well as MMP9 mutants causing defects in smooth muscle cell blood vessel ensheathment (Vu et al., 1998; Chantrain et al., 2004). Overall, *aMMP9* was expressed in a wide array of cell types in this study and likely is associated with multiple functions during tail regeneration.

Other MMPs and TIMP1 expression during tail regeneration

Several other MMPs were identified that may further studies of axolotl development and regeneration. A novel sequence was identified that contains all predicted MMP protein domains (Fig. 2; *amphMMP*); this sequence shows poor alignment to previously characterized mammalian and salamander MMPs. This sequence was named amphMMP (amphibian-specific MMP) because it had homology to a hypothetical Xenopus tropicalis protein (60% similarity; AAI55488), a translated EST sequences from Xenopus laevis (BJ032306), and an MMP protein found in the domesticated silkworm (Bombyx mori; NP 01116499; 37% similar). amphMMP may be the Xenopus genes XMMP (NP 001079285; 26% similarity) or collagenase 4 (AAH84654; 38% similar), but their low homology to these MMPs suggests that a yet unidentified MMP is present in the amphibian genome. Another sequence named aMMP7 showed similarity to H. sapiens collagenase 3 (57/% similar) but contained a stop codon sequence at predicted amino acid 291. An identical transcript has been previously characterized and named collagenase 3 (Yang et al., 1999). aMMP7 is 59% similar to aMMP3, suggesting that it is transcribed from a different genomic loci. Multispecies alignments of the predicted *aMMP7* protein sequence shows that it truncates at a similar region in the H. sapiens and X. laevis matrilysin proteins (Fig. 2; Harrison et al., 2004; NP 002414; NP 001079682). Further work is needed to confirm orthology, including possibly genome mapping. Contigs similar to several other MMPs including membrane type MMPs 17 and 23, stromelysin MMP11, and MMP19 were identified, but were either not expressed during tail regeneration or did not change expression (data not shown). It will be necessary in the future to address whether these genes are pseudogenes and transcribe functional MMPs. These sequences will be valuable for comparisons to MMPs in other vertebrates.

Conclusion

In summary, this study identified several previously unidentified MMPs in the Mexican axolotl and showed when and where these genes are expressed during regeneration. Our results suggest that most MMPs are highly orchestrated - they are expressed during regeneration in specific tissue types and only at certain times. The overlaping expression patterns of MMPs also suggest that MMPs have redundant functions during regeneration. The results presented here suggest hypotheses for how
MMPs participate in regeneration, when they participate, and upon which tissues they may target. These insights bring us closer to understanding the roles of MMPs in complex tissue regeneration.

Table 6.1. Primers used for real-time PCR and in situ hybridization. RT-PCR stands for real-time PCR. ISH stands for *in situ* hybridization. Each ISH primer contained either a T3 or SP6 promoter sequence appended to the 5' end of the primer.

Gene	RT-PCR	ISH
aCola_F	CACCCGAAATCTGTGACAA	AGTTCAGTCTTCCCCAGGATG
aCola_R	CGGCGCCAGAAGAATC	AAGAACGTTTTCCCTGTTGC
aColb_F	TGGACCAAGTTCGCTTGT	ACAGGAGCTCAGGGAAGAATG
aColb_R	GCTTGCGGATGTTCTTTG	AAGTGGACGTCTCCACCAAC
aCol3_F	GTGTGGAGCGAGGTTACG	CTGCATCGATGGATAGCTCA
aCol3_R	TCGGGTTCCAAAGGAGAT	GGCAAACTCGACGAAGAGAC
aMMP9_F	GAATGCACATGGACCAGAA	AGCGCACGATGTCTTTCTGTA
aMMP9_R	TTGGCACAGAACATTGGAG	GCGGTGGTACTCCAACTCAT
aMMP2_F	ACCCGTTGCAAGAAAGAAG	CGTTATGTTCGACGTTCCCT
aMMP2_R	GTGTTGGAGTCCAGCTTCC	CTTGGACTGGTGTCCCTGAT
aMMP3/10a_F	ACCCGTTGCAAGAAAGAAG	clone
aMMP3/10a_R	GTGTTGGAGTCCAGCTTCC	clone
aMMP3/10b_F	TGGAAGAAGACTGCGTTGA	ACCTTCCACTCCTGCTGCTAC
aMMP3/10b_R	ACACTTTGAAGGCCCTTTG	GCCCGTCAAATGGATAGAAA
aMMP14_F	AGGACTTCCCAGCGACAT	ACACGCGGTTATCTCGTACC
aMMP14_R	CGTCAAACACCCAATGCT	TCCGTGATGGACATGAGAAA
aTimp1_F	CGAAGAGCCAAGCTGGA	AAGACGGCCTTCTGCTATTCA
aTimp1_R	CATCGGGGGTGTAGAGGAAC	TAGATATGGGCCAGTCCTCG
aMMP16_F	ACTGGCGCTATGACGAGA	
aMMP16_R	GGCTAGGGAGGTGTGTCC	
aMMP23_F	AGGACCGTGTTCTCACCA	
aMMP23_R	GCACAAGGAGGGGAAAAG	
aMMP11_F	TCAGCCAGACAGCCAGAT	
aMMP11 R	GGTCAGGTGGGTCCTCTC	

Table 6.2. Best human protein hits for 16 axolotl MMPs. % Cov. = Percent coverage of the salamander sequence compared to the human protein. % Id. = Percent identical/similar between axolotl and human protein. Best NR hit = best non-redundant NCBI protein database hit. Nv = *Notophthalamus viridescence*, Cp = *Cynops phyrrhogaster*, Xt = *Xenopus laevis*, Xt = *Xenopus tropicalis*, R = Rabbit, Rr = *Rattus rattus*, Op = Opossum.

Name	Human hit	% Cov.	E-value	Best NR hit	% Id.	E-value
aCola	MMP1	100	5.00E-156	Nv coll	83/91	0
aColb	MMP1	100	4.00E-143	Nv coll	63/78	8.00E-175
aColc	MMP10	96.6	9.00E-132	Nv coll	61/75	3.00E-165
aCol3	MMP13	100	2.00E-176	Cp col3	81/89	0
aMMP2	MMP2	96.7	0	X1 MMP2	82/90	0
aMMP9	MMP9	88	0	Nv MMP9	90/95	0
aMMP3/10a	MMP3	100	3.00E-147	Nv 3/10a	80/89	0
aMMP3/10b	MMP3	100	1.00E-141	Cp 3/10b	72/83	0
aMMP3/10c	MMP3	100	3.00E-148	Nv 3/10a	75/85	0
amphMMP	MMP19	100	3.00E-53	Xt hyp.	60/74	0
aMMP7	MMP13	100	2.00E-83	R MMP13	59/74	1.00E-86
aMMP11	MMP11	23.6	1.00E-46	X1 MMP11	86/92	3.00E-56
aMMP14	MMP14	72.6	5.00E-172	Xt MMP14	78/86	2.00E-178
aMMP17	MMP17	15.5	8.00E-19	Rr MMP17	42/60	1.00E-19
aMMP19	MMP19	27.4	8.00E-50	Xt hyp.	81/92	7.00E-64
aMMP23	MMP23B	51.8	5.00E-80	Op MMP23	78/89	5.00E-90

Table 6.3. Summary of MMP and Timp1 gene expression patterns. This table summarizes *in situ* hybridization results for MMPs and *timp1*. Figures 6.4-6.7 show how strong each gene is expressed in particular tissue types. E, Epidermis; F, perichondrial fibroblasts; MH, area of muscle histolysis; M/N, macrophage/neutrophils; Ch, centrum chondrocytes; Oc, osteoclast; N, neuron; V, vasculature; O, osteocytes; Na, was not assayed; --, no expression observed.

Name	12 hpa	24 hpa 7 dpa		14 dpa	21 dpa
aCola	E,F	E,F,MH	Oc	Oc	Na
aColb	E,M/N	E,F,M/N	Ch	Ch	Na
aCol3	Na	Ob,F	Ob,F	Ob,F	Na
aMMP2			Ch	Ch	Na
aMMP9	E,Ob,F,	F,M/N,MH,Ob,Ch	O,Oc,F,Ch	O,Oc,F,Ch	V,N,F
aMMP3/10a	E	E			Na
aMMP3/10b			Ob	Ob	Na
aMMP14			Ch,F	Ch,F	Na
atimp1	Na	E,MH,F	Ch,F	Ch,F	Na

Figure 6.1. Hematoxylin-Eosin staining was performed on cryosections at 3 hpa (A), 6 hpa (B), 12 hpa (C), 24 hpa (D), and 21 dpa (E). Scale bar 200 μm



Figure 6.2. Predicted protein sequence alignment of axolotl MMPs. Sequences covering >50% of the presumptive protein coding sequence were used for the multiple sequence alignment. The top box shows the conserved cysteine switch region in the propeptide domain. The second box highlights the putative zinc-binding domain necessary for catalytic activity. The top sequence underlined highlights the catalytic domain and the bottom sequenced underlined represents the hemopexin domain. Notice the large insert within the catalytic region of aMMP2 and aMMP9 that represents the fibronectin-like repeats.



Figure 6.3. Cartoon showing all 16 MMPs identified in this study. Black lines represent approximate coverage of the predicted coding sequence and amino acid (aa) lengths are presented to the right. The top cartoon represents the prototypical MMP protein. Other cartoons represent domains found in each family of MMPs including collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others.



Figure 6.4. Temporal (A,B,C) and spatial (D-K,M-O) expression of *aCola* (A,D,G,J,M), *aColb* (B,E,H,K,N), and *aCol3* (C,F,I,O) during tail regeneration. Temporal gene expression patterns assayed by real-time quantitative PCR are shown in A, B, and C. All y-axes are log-2 fold changes from the average day 0 samples. Spatial expression is shown at 24 hpa (D-K) and 14 dpa (M-O). Note the specific staining when antisense probes were used (D,E,F,J,K,M,N,O) and the lack of staining when sense probes were used (G,H,I). J and K are close-up photos of D and E. L represents a tail cross section showing approximate locations of images. Dorsal is upward. Horizontal lines represent coronal sections (D-K) and boxes represent cross sections (M-O). SC, spinal cord. Error bars = \pm standard error of the mean (SEM). Scale bar = 100 µm.



Figure 6.5. Temporal (A,B) and spatial (C-L) expression patterns of *aMMP9* (A,C-J) and *aMMP2* (B,K,L) during tail regeneration. Temporal gene expression patterns assayed by real-time quantitative PCR are shown in A and B. All y-axes are log-2 fold changes from the average day 0 samples. Spatial expression is shown at 6 hpa (C,D), 24 hpa (E-H), 14 dpa (I,K,L), and 21 dpa (J). Sense probes were used on sections shown in D, F, and L. G is a close-up of E. Arrows indicated neuron-like cells and arrowheads indicate vasculature. M represents a tail cross section showing approximate locations of images. Horizontal lines represent coronal sections (C-H) and boxes represent transverse sections (I-L). Error bars = \pm SEM. Scale bar = 100 µm.



Figure 6.6. Temporal (A,B) and spatial (C-G) expression patterns of *aMMP3/10a* (A,C,E,G) and *aMMP3/10b* (B,D,F) during tail regeneration. Temporal gene expression patterns assayed by real-time quantitative PCR are shown in A and B. All y-axes are log-2 fold changes from the average day 0 samples. Spatial expression is shown at 24 hpa (C,G) and 14 dpa (D). Sense probes are shown in E and F. G) close-up of C. The horizontal line in H represents a coronal section (C,E,G) and the box represents a transverse section (D,F). SC = spinal cord. NC = notochord. Scale bar = 100 μ m.



Figure 6.7. Temporal (A,B) and spatial (C-F,H) expression patterns of *aMMP14* (A,C,E) and *aTIMP1* (B,D,F,G) during tail regeneration. Temporal gene expression patterns assayed by real-time quantitative PCR are shown in A and B. All y-axes are log-2 fold changes from the average day 0 samples. Spatial expression is shown at 24 hpa (D,F,H) and 14 dpa (C,E). Sense probes were used on sections shown in E and F. H is a close-up of D. The horizontal line in G represents a coronal section (D,F,H) and the box represents a transverse section (C,E). Scale bar = 100 μ m.



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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

The studies presented in this dissertation describe the physiological genomics of spinal cord and limb regeneration in the salamander. These studies are some of the first to use unbiased genomic technologies to identify gene candidates for salamander limb regeneration. Below I highlight some of the interesting gene candidates and possible future directions to address their roles in regeneration.

Spinal cord regeneration is likely the most relevant model of salamander regeneration to the human condition. For this reason, we performed our first microarray analyses on the regenerating spinal cord (Chapter 2). In this study, we found that spinal cord injury changes thousands of genes from baseline suggesting that regeneration is a global process. Among these genes, we were interested in ones that may contribute to the activation or maintenance of ependymoglia. Ependymoglia are thought to act as neural precursors during regeneration and understanding how they are maintained and activated is critical to our understanding of regeneration. Gene pathways known to regulate neural precursors were identified including BMP, FGF, and Wnt signaling pathways. Several genes that code for molecules involved in these pathways were differentially regulated during regeneration included *follistatin, secreted frizzled-related protein 2, wnt5a,* and *fibroblast growth factor-binding protein 1.* Although this work is important for identifying genes candidates that regulate ependymoglia activation, over-expression and knockdown experiments of these genes during spinal cord regeneration will be needed to see if they disrupt ependymoglia function.

One of the most interesting results from this study was the extreme change in mRNA abundance of matrix metalloproteinases (MMPs). The dramatic upregulation of MMPs motivated us to extend these studies to the entire MMP gene family, the results of which are presented in Chapter 6. Overall, we found that MMP9 was expressed in the spinal cord during regeneration. Further analysis showed that it was mostly expressed in cells resembling neurons, but also ependymoglia (Figure 2.4C; Figure 6.5; data not shown). Chapter 5 shows that MMP9 is also expressed in dorsal root ganglia neurons during limb regeneration and in the retina following optic nerve injury (Elizabeth Debski,

personal communication). The similar MMP9 expression patterns in these three injury paradigms suggest that MMP9 is specifically associated with regenerating or injured neurons. In mammals, MMP9 has been associated with increased axon outgrowth in spinal cord injury models (Duchossoy et al., 2001), retinal ganglion cell axon regeneration (Ahmed et al., 2005), and peripheral nerve regeneration (Ferguson and Muir, 2000). MMP9 may facilitate nervous system regeneration by breaking down inhibitory neurite outgrowth molecules including CSPGs (Ferguson and Muir, 2000) and myelin-inhibitory products (Milward et al., 2008), promote remyelination (Larsen et al., 2003), or release growth factors that could then support neuron survival and growth (Pizzi and Crowe, 2007 for review). It will be interesting to find out if disrupting MMP9 activity in neurons in vitro affects neurite outgrowth. The identification and characterization of Nogo-A in the salamander nervous system (Chapter 3) also raises the possibility that MMPs clear the injury site of Nogo-A and other inhibitory molecules after injury. Indeed, the inhibitory molecules tenascin-R and myelin-associated glycoprotein are quickly cleared from the salamander optic nerve after injury, and this is correlated with axon regeneration (Becker et al., 1999). It is possible that neuronal expression of MMP9 mediates this process.

A second gene that showed an interesting expression pattern across multiple nervous system injury paradigms was ankyrin repeat domain-containing 1 (ANKRD1). It was found to be upregulated in all neural injury models and was highly expressed in dorsal root ganglia neurons 14 days after limb regeneration (Figure 5.2 and 5.3). Real-time PCR also found this gene to be upregulated by 3 dpa, suggesting that the expression of this gene is expressed throughout regeneration (data not shown). This transcription factor has traditionally been thought of as muscle specific gene, but recent data in a rat injury model have associated ANKRD1 with dorsal root ganglia neuron outgrowth (Stam et al., 2007). It is possible that the large upregulation of ANKRD1 in regenerating neurons may explain the high axon regenerative capacity of axolotl neurons. Localization of this gene in regenerating spinal cord and retinal ganglion neurons would support this hypothesis. Knockdown of ANKRD1 is also needed *in vitro*, possibly using morpholino technologies, to address whether ANKRD1 mediates axon outgrowth.

The role of nerves in limb regeneration is one of the most perplexing questions in the regeneration field. We attempted to address this question by identifying the downstream targets of nerves during limb regeneration as well as molecules expressed by sensory neurons during limb regeneration (Chapters 4 and 5). Chapter 4 shows that many differences were found between denervated and regenerating limbs. Many of these genes were associated with the presence of a blastema in the innervated limb and absence in the denervated limb. The utility of this experiment is that we now have a gene expression signature of a limb blastema. Understanding what the blastema is at the cellular and molecular level will be necessary in future experiments to address whether we can experimentally perturb this process. The next step is to locate the expression of these genes between denervated and regenerating limbs in order to identify where they are expressed. For example, expression in the WE would suggest that the WE is the primary target of the regenerating nerves, which then creates a regeneration competent environment. An interesting candidate gene pathway identified in Chapter 5 that may fit this profile is the L-serine synthesis pathway. The three enzymes involved in this pathway were differentially regulated between denervated and regenerating limbs at 5 and 14 days after limb amputation. Attempting to rescue regeneration in denervated limbs with local administration of L-serine would functionally link L-serine to limb regeneration. Such a simple model for how nerves support limb regeneration may be directly relevant to wounding in mammalian systems.

Chapter 5 highlighted up-regulated genes in the DRG during limb regeneration. We suggest that these could be involved in axon regeneration, upstream of the NTF, or possibly be the NTF. All of this work is correlative in nature, but sets the groundwork for functional analysis of these genes. It will be important to localize expression of these genes to sensory neurons and show secretion from peripheral nerves. It will also be important to over-express gene candidates in denervated, amputated limbs.

The successes of these early experiments provide optimism that we may soon understand the underlying mechanisms of regeneration. Regardless, some major hurdles need to be met before we can understand how complex regeneration is accomplished. For example, we need to know how important the wounding/immune response is in both spinal cord and limb regeneration. Is there something unique about the salamander immune response that supports regeneration? It will also be necessary to understand the relationship between nerves, the wound epidermis, and their support of blastema formation during limb regeneration. These three elements are essential for creating a regeneration-permissive environment, but little is known about their specific function or relationship with one another. Thirdly, it is necessary for us to know how plastic salamander cells are following injury. Are salamanders able to regenerate spinal cord and limb tissue because they have a large supply of resident stem cells or are they capable of creating a local supply of stem cells by dedifferentiating cells after injury? Once each of these questions is answered in the salamander system, it may be plausible to replicate the salamander "injury" environment at a mammalian injury site. It is also plausible that knowledge gained from the salamander system may be useful for tissue engineering. Tissue engineering is the process of growing tissue or organs *in vitro* and implanting this tissue in the human patient. The salamander regeneration model gives a unique example of the environment necessary to support complex adult tissue growth and may aid in the engineering of these *in vitro* systems. The descriptive work provided in this dissertation is an important step towards these goals. The next challenge will be to identify which of the descriptive observations presented here is necessary for regeneration.

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VITA

James R. Monaghan

Born March 21, 1979; Chicago, IL

EDUCATION

1997-2001	DePauw University, Greencastle, IN Bachelor's of Arts, Biology
2002-2003	Indiana University-Purdue University Indianapolis, Indianapolis, IN Graduate courses in Biochemistry and Molecular Biology
2003-2009	University of Kentucky, Lexington, KY PhD candidate, Biology Dissertation: Physiological genomics of spinal cord and limb regeneration in a salamander, the Mexican axolotl

PROFESSIONAL EXPERIENCE

2000 - 2001	Internship, Medical and Molecular Genetics Department, Advisor: Stephen Dlouhy, PhD
2001 – 2003	Full-time employment, Medical and Molecular Genetics Department, Indiana University School of Medicine
2005 – 2009	Undergraduate student mentor, University of Kentucky, under the direction of S. Randal Voss.
2001 – 2003	Teaching Assistant, Molecular and Biochemical Genetics (Q613), IUPUI
2003 - 2004	Teaching Assistant, Microbiology 209, University of Kentucky
2006-2007	Pre-doctoral Representative: Bluegrass Chapter Society for Neuroscience
2006-2009	Graduate Student Representative: Graduate Affairs Committee, Department of Biology
2006-2007	President: Graduate Student Association, Department of Biology

HONORS AND AWARDS

- 1997 Summer Honors Program at Indiana State University
- 1997 Thomas C. Murray Scholarship
- 1997 DePauw University Merit Award
- 1998 DePauw University Scholar Athlete
- 2003 Gertrude Flora Ribble Graduate Scholarship Award
- 2006 Keystone Symposia Travel Scholarship, Stem Cell Conference
- 2007 Dissertation Year Fellowship, University of Kentucky Graduate School
- 2007 Society for Neuroscience Chapters Graduate Student Travel Award

PUBLICATION HISTORY

Putta S, Smith JJ, Walker JA, Rondet M, Weisrock DW, **Monaghan JR**, Samuels AK, Kump K, King DC, Maness NJ, Habermann B, Tanaka E, Bryant SV, Gardiner DM, Parichy DM, Voss SR. (2004). From biomedicine to natural history research: EST resources for ambystomatid salamanders. *BMC Genomics*. 13(5):54 (17 pages).

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• Co-first authors

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ABSTRACTS or PRESENTATIONS

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James Robert Monaghan

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