Insights Towards Developing Regenerative Therapies: The Lizard, *Anolis carolinensis*, as a Genetic Model for Regeneration in Amniotes

by

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ABSTRACT

Damage to the central nervous system due to spinal cord or traumatic brain injury, as well as degenerative musculoskeletal disorders such as arthritis, drastically impact the quality of life. Regeneration of complex structures is quite limited in mammals, though other vertebrates possess this ability. Lizards are the most closely related organism to humans that can regenerate de novo skeletal muscle, hyaline cartilage, spinal cord, vasculature, and skin. Progress in studying the cellular and molecular mechanisms of lizard regeneration has previously been limited by a lack of genomic resources. Building on the release of the genome of the green anole, *Anolis carolinensis*, we developed a second generation, robust RNA-Seq-based genome annotation, and performed the first transcriptomic analysis of tail regeneration in this species. In order to investigate gene expression in regenerating tissue, we performed whole transcriptome and microRNA transcriptome analysis of regenerating tail tip and base and associated tissues, identifying key genetic targets in the regenerative process. These studies have identified components of a genetic program for regeneration in the lizard that includes both developmental and adult repair mechanisms shared with mammals, indicating value in the translation of these findings to future regenerative therapies.

DEDICATION

I would like to dedicate this manuscript to my parents, who have always encouraged my education and other pursuits: my mom Denise who never put any limits on what I can achieve, and my dad Terry who inspired me to grow up to be a scientist one day. I would also like to dedicate this manuscript to my loving husband Joe who has been by my side every step of the way.

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CHAPTER 1

INTRODUCTION

Many vertebrates are capable of appendage regeneration, though the extent of regenerative ability varies throughout taxa (Agata & Inoue, 2012; Bely & Nyberg, 2010). Urodele amphibians, teleost fish, and anuran tadpoles are capable of regenerating their tails and fins following amputation (Gemberling, Bailey, Hyde, & Poss, 2013; Stocum & Cameron, 2011). In contrast, following metamorphosis, *Xenopus* frogs regenerate a spike-like, cartilaginous structure as a replacement limb (T. Endo, Tamura, & Ide, 2000). Many lizards are capable of tail regeneration following amputation and/or autotomy, and though the replacement tail is a different structure than the original tail, it contains muscle, cartilage, neuroependyma, skin, and vasculature (Fisher et al., 2012; R. W. D. Gilbert, Vickaryous, & Viloria-Petit, 2013b; McLean & Vickaryous, 2011; Ritzman et al., 2012). Additionally in the reptiles, tail regeneration in alligators has been reported in the field, though the structure and regenerative mechanism are unknown (Bellairs & Bryant, 1985; Han et al., 2005). Birds and mammals have limited regenerative capacity in comparison, though some neonatal and juvenile mammals regenerate digit tips (Borgens, 1982; Han, Yang, Lee, Allan, & Muneoka, 2008; Illingworth, 1974; M. Singer, Weckesser, Géraudie, Maier, & Singer, 1987), and African spiny mice autotomize and regenerate skin (Seifert et al., 2012). As amniotes, lizards are the most closely related organisms to mammals that possess the ability to regenerate whole structures. The green anole lizard, *Anolis carolinensis*, which possesses the ability to regenerate its tail following autotomy and has a near-complete reference genome and robust gene annotation (Alföldi et al., 2011; Eckalbar et al., 2013; Ritzman et al., 2012), provides an ideal model for transcriptome-wide studies of molecular pathways and mechanisms involved in lizard tail regeneration.

Mechanisms of Appendage Regeneration in Vertebrate Developmental Model Systems

Teleost fish: Zebrafish. In addition to regenerating amputated fins, zebrafish can regenerate retina, spinal cord, brain, and heart (T. Becker, Wullimann, Becker, Bernhardt, & Schachner, 1997; D. A. Cameron, 2000; Kizil, Kaslin, Kroehne, & Brand, 2012; Otteson & Hitchcock, 2003; Poss, Wilson, & Keating, 2002; Reimer et al., 2008). Zebrafish fins are complex, segmented appendages containing dermal bone and surrounding vascularized, innervated mesenchymal tissue. Following amputation, the fin ray is (1) covered by an epidermis within a few hours, followed by (2) formation of a mass of proliferative cells underlying the epidermis, called a blastema, in 1-2 days, and finally (3) differentiation and regenerative outgrowth (Haas, 1962; J & M, 1992; Nabrit, 1929; Santamaría & Becerra, 1991). This blastema consists of proliferative, morphologically similar cells that originated from the amputation stump via disorganization of fibroblasts and osteoblasts. These proliferative cells are lineage restricted; there is no transdifferentation. Spinal cord proliferation and outgrowth is mediated by Sox2 expression in the ependymal cells (Hui et al., 2014; Ogai et al., 2014). Cre recombinase-based lineage analysis has revealed that differentiated osteoblasts dedifferentiate, migrate distally and contribute to the proliferating blastema, and then contribute to osteoblasts in the regenerated fin (Knopf et al., 2011; Singh, Holdway, & Poss, 2012; Sousa et al., 2011; S. Stewart & Stankunas, 2012; Tu & Johnson, 2011). Similarly, the endothelium, epidermis, and fibroblasts are also lineage restricted (Tu & Johnson, 2011). Curiously, however, the zebrafish fin can regenerate following osteoblast ablation, suggesting that there may be an additional source of cells (Singh et al., 2012). During outgrowth, FGF signaling interactions between the epidermis and underlying

mesenchyme maintain this mass of proliferative cells at the distal portion of the regenerating fin and promote outgrowth (Y. Lee et al., 2009).

Urodele amphibians: Newt and axolotl. Urodele amphibians are widely studied for their superb regenerative abilities; juvenile and adult animals are capable of regenerating replicas of many tissues that are nearly identical to the original, including limbs, jaw, tail, retina, spinal cord, heart, and brain (B. M. Carlson, 2011; Maden, Manwell, & Ormerod, 2013). The limb is a complex appendage, and regeneration involves formation of many tissue types including dermis, epidermis, muscle, cartilage, nerve, vasculature, and other skeletal elements. Limb regeneration in the salamander occurs through three phases: (1) wound healing/formation of a wound epithelium, (2) activation of progenitor cells and (3) "re-development" of the limb (Bryant, Endo, & Gardiner, 2002; Knapp et al., 2013). During the wound healing phase, which begins immediately following amputation, epidermal cells migrate over the wound stump and form a new layer of epidermis. These epidermal cells do not contribute to the blastema (M. R. Carlson, Bryant, & Gardiner, 1998; Hay & Fischman, 1961; Namenwirth, 1974; Satoh, Graham, Bryant, & Gardiner, 2008). Following wound healing, progenitor cells are activated and form a blastema underneath the epidermis. Regenerative success is dependent upon signaling from the damaged nerve during this phase (A. Kumar & Brockes, 2012; Satoh, James, & Gardiner, 2009; Stocum & Cameron, 2011). Historically, the term blastema describes a proliferative mass of homogenous multipotent or pluripotent stem cells at the tip of the regenerating salamander limb that are generated via dedifferentiation (Butler & O'Brien, 1942; Echeverri & Tanaka, 2002; Hay & Fischman, 1961; Namenwirth, 1974; Thornton, 1938; B. M. Wallace & Wallace, 1973). Recent studies, however, have indicated that both dedifferentiation of tissues adjacent to the stump and activation of tissue resident stem cells contribute to the blastemal pool of

progenitor cells present in the regenerating limb (Tanaka & Reddien, 2011) and that these progenitor cells retain a memory of their tissue or embryonic origin (Kragl et al., 2009; Morrison, Borg, & Simon, 2010; Sandoval-Guzmán et al., 2014). Dermis is the most flexible tissue; it is capable of forming cartilage and tendons as well as dermis, all of which originate from the lateral plate mesoderm. Schwann cells only give rise to Schwann cells, even when rescuing irradiated limbs that otherwise wouldn't regenerate. Muscle only makes muscle and is not derived from other tissues (Kragl et al., 2009). Lineage tracing of muscle cells in the newt (*Notophthalmus viridescens)* and axolotl (*Ambystoma mexicanum*) has shown differing results between species regarding dedifferentiation and stem cell-mediated regeneration. In the newt, muscle progenitor cells in the blastema arise from both dedifferentiation of local skeletal muscle fibers and migration of Pax7-positive satellite cells from nearby tissue. In the axolotl, all of the muscle progenitor cells in the blastema arise from Pax7-positive satellite cells; there is no dedifferentiation (Morrison et al., 2010; Sandoval-Guzmán et al., 2014). Though the definition of the term "blastema" may differ and is changing as we know more about progenitor cell origins, we know that this mass of progenitor cells in amphibians arises from either dedifferentiation of mature tissues or activated stem cells residing in these tissues, is in a proliferative state, expresses developmental genes, and is induced by signaling interactions with the overlaying wound epithelium (Brockes & Kumar, 2008; Han et al., 2005; Morrison, Lööf, He, & Simon, 2006; Satoh et al., 2008; Satoh, Bryant, & Gardiner, 2012). The last phase of limb regeneration is termed "re-development" because it is thought to be mainly a recapitulation in the adult of embryonic limb development, including the involvement of FGF, sonic hedgehog, and Hox signaling in limb patterning (Hutchison, Pilote, & Roy, 2007; Muneoka & Bryant, 1982; Nacu & Tanaka, 2011; Roy & Gardiner, 2002; Roy, Gardiner, & Bryant, 2000; Torok, Gardiner,

Shubin, & Bryant, 1998). The distal wound epithelium at this stage is frequently referred to as the apical epithelial cap (AEC) because of its genetic similarity, including FGF signaling interactions with the underlying mesenchyme, to the apical ectodermal ridge (AER) formed during avian and mammalian development (R. N. Christensen & Tassava, 2000; R. N. Christensen, Weinstein, & Tassava, 2002; Han et al., 2005).

Anurans: Xenopus frogs and tadpoles. The regenerative capacity of anuran amphibians, which is represented by *Xenopus laevis* and *Xenopus tropicalis*, is more temporally limited in comparison to the urodeles thus far studied; tail regeneration in tadpoles and limb regeneration in adult frogs produce different structures from the original. The embryonic limb bud regenerates, with a gradual ontogenic decline approaching metamorphosis (Figure 1; Dent, 1962); however, the embryonic tail bud does not (Tucker & Slack, 1995). During the larval stage, tadpoles regenerate their tails from stage 40 until metamorphosis with the exception of a transient refractory period around stage 46-47 associated with feeding behavior (Beck, Christen, & Slack, 2003; Bosco, 1979). After metamorphosis, *X. laevis* froglets regenerate a spike-like, cartilaginous structure following limb amputation (T. Endo et al., 2000). Though the regenerative capacity is not that of the urodele amphibians thus far studied, anurans are an informative regenerative model for two reasons. First, the ontogenic decline in regenerative capability seen in anurans is more similar to regeneration in mammals than that of urodele amphibians, which display superb regenerative capabilities well into adulthood. Second, it is possible to compare naturally occurring limb regeneration in larval stage with incomplete regeneration in the juvenile stage in the same organism.

Tadpole tail regeneration, which has been investigated in *X. laevis* and *X. tropicalis*, results in a fully functional replacement, with regained coordinated swimming, 7 weeks post-amputation (Gaete et al., 2012). This replacement contains spinal cord,

notochord, smooth and skeletal muscle, vasculature, and skin (N. R. Love et al., 2011; J. M. W. Slack, Lin, & Chen, 2007). Though the regenerated tail is functional, it lacks somitic muscle segmentation and intersomitic axons, and the chevron muscle patterning seen in the original tail is also absent (N. R. Love et al., 2011; J. M. W. Slack et al., 2007). The process of tadpole tail regeneration can be characterized by three phases: 1) acute inflammatory response, 2) cell proliferation, and 3) regrowth of differentiating tissues, including neurons, notochord, muscle, and vasculature (N. R. Love et al., 2011). Unlike the urodele amphibians, there is no transdifferentiation; cell lineage tracing has shown that the spinal cord regenerates from adjacent spinal cord cells in the stump and, similarly, the notochord regenerates from adjacent notochord cells in the stump (Gargioli & Slack, 2004). Rather than regenerating from dedifferentiation of pre-existing myofibers, the regenerating myofibers originate from Pax7-positive satellite cells present in adjacent muscle tissue that migrate into the regenerating tail and differentiate (Gargioli & Slack, 2004; Ryffel, 2003). *X. laevis* froglets regenerate a symmetrical cartilage spike following limb amputation (Dent, 1962; Korneluk & Liversage, 1984). Following amputation, (1) a wound epithelium is established, (2) proliferative mesenchymal cells accumulate at the distal tip, (3) cells differentiate and the limb grows outward (T. Endo et al., 2000). The formation of proliferative cells at the tip of the regenerating limb and its regenerative outgrowth is dependent on nerve innervation (T. Endo et al., 2000; Korneluk & Anderson, 1982; Suzuki, Satoh, Ide, & Tamura, 2005), though tadpoles can regenerate denervated developing limb buds (Filoni & Paglialunga, 1990). Myofibers are absent in the regenerated appendage spike, though there are Pax7+ positive myofibers in the adjacent stump (Satoh, Ide, & Tamura, 2005). The symmetrical shape of the regenerated cartilage spike is due to a lack of patterning in both the

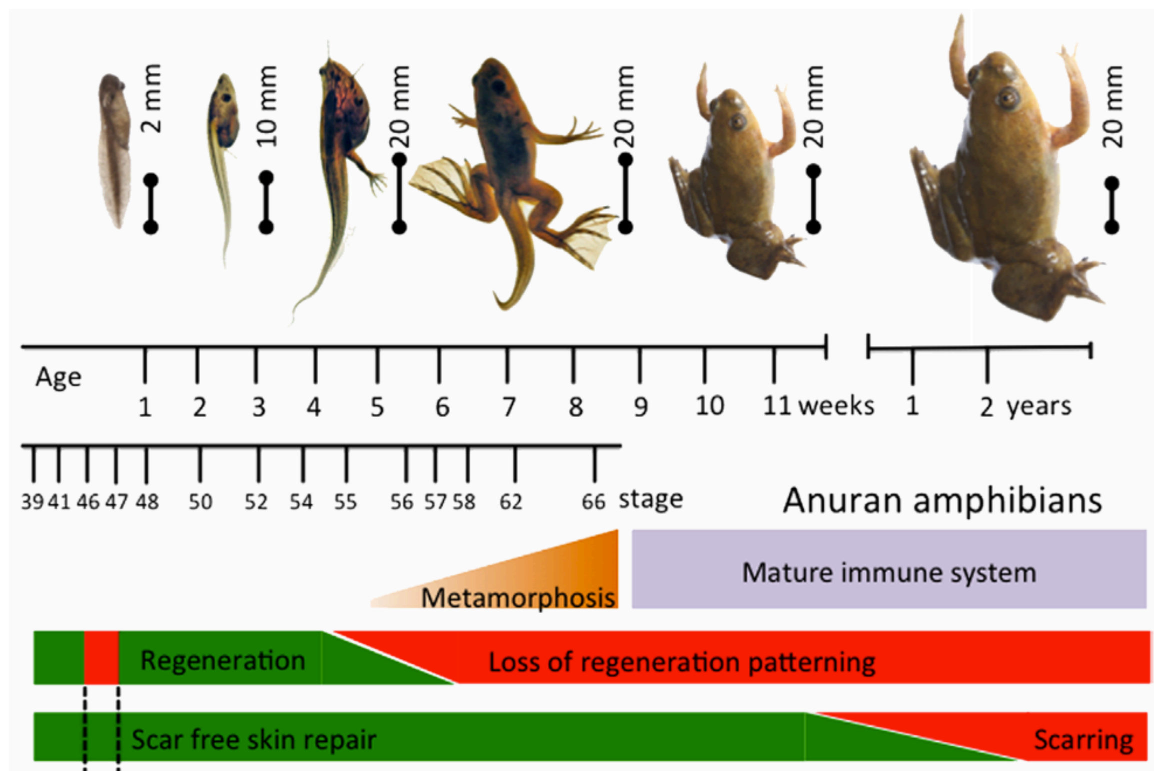


Figure 1. Regeneration in anuran amphibians is temporally limited. With the exception of a transient refractory period (stage 45-47, marked by a dotted line) tadpoles are capable of regeneration prior to metamorphosis. At the onset of metamorphosis, regenerative ability gradually declines from stage 55 to stage 60. This is demonstrated by a loss of digit patterning. While froglets regrow a patterned limb following amputation at stage 53, tadpoles regenerate a spike-like appendage that lacks patterning by stage 60. Adult anuran amphibians do not demonstrate scar-free wound healing (figure from Godwin & Rosenthal, 2014).

anterior-posterior and proximal-distal axes (T. Endo et al., 2000; Ohgo et al., 2010; Satoh et al., 2005; Yakushiji et al., 2007).

Mammals: Mouse and Human. Mammals have highly limited regenerative capacity, with regeneration mostly limited to the neonatal period. Neonatal mice (*Mus musculus*), human children, and juvenile Rhesus monkeys (*Macaca mulatta*) can regenerate amputated digit tips (Borgens, 1982; Douglas, 1972; Illingworth, 1974; M. Singer et al., 1987; Vidal & Dickson, 1993), and mice younger than 7 days are capable of heart regeneration (Porrello et al., 2011). There have also been some case studies of digit tip regeneration in adults, with either the placement of a Hyphecan, a membrane-like cap made from chitin, over the wound (Halim, Stone, & Devaraj, 1998; Lee, Lau, & Chan, 1995) or application of a powder derived from pig's bladder extracellular matrix (Rosenwald 2007; Cohen 2010) following wound debridement. In both mouse and human, regeneration occurs following amputation of the terminal phalanx only and is dependent on whether or not the amputation level includes the nail organ (Borgens, 1982; Mohammad, Day, & Neufeld, 1999; Neufeld & Zhao, 1995). Specifically, the extent of regenerated bone in the regenerate and therefore the length of the regenerated fingertip are dependent upon level of amputation (Han et al., 2008; Neufeld & Zhao, 1995). One main difference from regeneration in other vertebrates is that innervation is not necessary for digit tip regeneration (Mohammad & Neufeld, 2000).

Regenerated digit tips contain bone, connective tissue and tendons, the nail organ, hair follicles, and skin, and are innervated and highly vascularized (Rinkevich, Lindau, Ueno, Longaker, & Weissman, 2011; Said, Parke, & Neufeld, 2004). Following amputation of the digit tip, (1) wound closure occurs in a few days and osteoclasts erode the bone in the stump, (2) a mass of proliferative cells, which originate from connective tissue cells migrating across the wound and marrow in the skeletal stump, is formed, and

(3) cells differentiate into mature tissues in the regenerate (Fernando et al., 2011; Han et al., 2008; Lehoczky, Robert, & Tabin, 2011; Muneoka, Allan, Yang, Lee, & Han, 2008; Neufeld & Zhao, 1995; Revardel & Chebouki, 1987). As in zebrafish and *Xenopus* frogs, there is no transdifferentiation and stem/progenitor cells are lineage-specific; epidermal stem cells and mature keratinocytes originate from the ectodermal tissue overlying the stump, and bone is derived from pre-amputation, mesodermal, osteoblasts (Lehoczky et al., 2011; Rinkevich et al., 2011).

Tail Regeneration in Lizards

As amniotes, lizards are the most closely related organisms to humans that can regenerate appendages. Many lizard species are capable of tail autotomy and/or regeneration (Bellairs & Bryant, 1985). Lizards capable of autotomy, a process induced by physiological and/or mechanical stress whereby the lizard sheds its tail as a mechanism of predator evasion, possess fracture planes in the middle of the centrum of the caudal vertebrae (Arnold 1988; Simpson, 1968). Following autotomy, the severed tail continues to move, often distracting the predator while the lizard escapes (Arnold, 1988; Dial & Fitzpatrick, 1983). There have been a number of studies describing tail regeneration in lizards. Recent research has focused on *E. macularius* (Delorme et al., 2012; E. A. B. Gilbert, Payne, & Vickaryous, 2013a; R. W. D. Gilbert et al., 2013b; McLean & Vickaryous, 2011) and the green anole, *Anolis carolinensis* (Eckalbar et al., 2013; Fisher et al., 2012; Ritzman et al., 2012) as model organisms. Studies in the leopard gecko (*Eublepharis macularius*) suggest that tail regeneration is intrinsic; tails can regenerate regardless of proximity to the fracture plane and whether or not the tail is removed naturally via autotomy or mechanically amputated (Delorme, Lungu, & Vickaryous, 2012).

The green anole has been a model for studies of evolution and development (Eckalbar et al., 2012), population genetics (Tollis & Boissinot, 2014; Wordley, Slate, & Stapley, 2011), reproductive physiology (Lovern & Wade, 2003), behavior (Wade, 2012), and functional morphology (Montuelle, Herrel, Libourel, Reveret, & Bels, 2009). As such, it was the first non-avian reptile to have its genome sequenced and presents an opportunity to advance genetic and molecular approaches to tail regeneration in lizards (Alföldi et al., 2011). There have been a number of studies of the histological and cellular responses involved in green anole lizard regeneration (Alibardi, 1995a; 1995b; 2014a; 2014b; Chlebowski, Przbylski, & Cox, 1973; Cox, 1968; Egar, Simpson, & Singer, 1970; Kamrin & Singer, 1955; Maderson & Licht, 1968; S. B. Simpson, 1968; Turner & Singer, 1973; Zika, 1969), and more detailed and comprehensive histological and anatomical studies of the original and regenerated tail were published recently (Fisher et al., 2012; Ritzman et al., 2012). The structure of the regenerated tail is anatomically distinct from the original. The regenerated tail does not contain bone and consists of a central unsegmented cartilaginous tube. The spinal cord runs through the center of this tube, and regenerated myofibers are arranged radially around the outside of the tube. The proximal cartilage tube undergoes endochondral ossification at the boundary with the original tail skeleton (Lozito & Tuan, 2015). The regenerated spinal cord has a different structure as well; it consists of an ependymal tube that is continuous with the original spinal cord, but lacks the surrounding white matter (axons) and gray matter (cell bodies) (Fisher et al., 2012; E. A. B. Gilbert et al., 2013a). Dorsal root ganglia are not regenerated; the regenerating tail is innervated from proximal ganglia in the intact tail stump (Duffy, Simpson, Liebich, & Davis, 1990; Egar et al., 1970). Anatomically, the original tail possesses equally spaced, interdigitated myomeres along the length of the tail, while the regenerated tail consists of longitudinal myomeres of varying size that are

radially organized (Ritzman et al., 2012). Histologically, these regenerated myomeres possess unique tendonous attachments, and the distribution of connective tissue is distinct from the original tail. Additionally, there are irregularly spaced foramina in the cartilage tube that transmit vasculature but not nerves (Fisher et al., 2012). This structure is more rigid than the original tail, suggesting that the regenerated tail is less capable of controlled, fine-scale movements.

Though the regenerated tail has a different structure than the original tail, it is an impressive example of regeneration of cartilage, *de novo* muscle groups, skin, vasculature, and neural ependymal cells (Fisher et al., 2012; E. A. B. Gilbert et al., 2013a; McLean & Vickaryous, 2011; Ritzman et al., 2012). Following autotomy, where the tail vertebra breaks about halfway through at the fracture plane, there is (1) formation of a blood clot and ECM remodeling associated with contraction of tissues in the stump, (2) formation of the wound epithelium and ablation of the scab, (3) formation of proliferating cells, angiogenesis, and thickening of the wound epithelium and (4) outgrowth and differentiation of mature tissues, including myofibers, cartilage, and the neuroependyma (Lozito & Tuan, 2015; McLean & Vickaryous, 2011; Nambiar, Bhatt, Deshmukh, & Jape, 2008). Similarly to *Xenopus* frog tadpoles and salamanders, regeneration is nerve-dependent; damage to the spinal cord proximal to the regenerating tail, namely the ependymal tube, inhibits regeneration (Kamrin & Singer, 1955; S. B. Simpson, 1964; Whimster, 1978). The ependymal tube provides positional identity to the regenerating appendage (Y. Wang et al., 2011). While blastema formation is fairly well characterized during limb and fin regeneration in amphibians and teleost fish, lizards follow a different mechanism of regeneration, though the source of cells is still relatively unknown. Blastema formation is traditionally characterized by dedifferentiation of tissue, a mass pluripotent proliferating cells focused at the tip of the regenerating appendage,

and the absence of a vascular bed at the distal tip (Butler & O'Brien, 1942; Echeverri & Tanaka, 2002; Hay & Fischman, 1961; Iten & Bryant, 1973; Mescher, 1996; Namenwirth, 1974; Peadon & Singer, 1966; M. Singer, 1974; Smith & Wolpert, 1975; Thornton, 1938; B. M. Wallace & Wallace, 1973). Unlike amphibians, there is no evidence of dedifferentiation in the lizard (Cox, 1969; Hughes & New, 1959; Kahn & Simpson, 1974; S. B. Simpson, 1965). Additionally, in the leopard gecko proliferating cells are present throughout the regenerating tail, rather than localized at the distal tip, and the distal tip is highly vascular (McLean & Vickaryous, 2011). Continuing genetic, molecular, and cellular studies in the lizard should provide more information about the source of cells in the regenerating tail.

Comparative Genetic Studies of Regeneration

A key question for comparative approaches is whether regeneration is an ancestral, monophyletic trait or regenerative capabilities arose multiple times in vertebrates (Bely & Nyberg, 2010). This is especially pertinent for the development of regenerative therapies in humans; mammals possess some regenerative capabilities that are mostly limited to the neonatal period, and the focus of regenerative therapies is to activate shared mechanisms. There are many similarities across regenerating taxa, namely in vertebrates, and recent genetic studies have identified many of these mechanisms in a more detailed manner. Common processes in the initial phases of scar-free wound healing and regeneration in vertebrates include the formation of a wound epithelium, establishment of positional identity, ECM remodeling and degradation facilitated by matrix metalloproteinases and other enzymes following wounding, upregulation of immune response genes, and involvement of developmental signaling pathways, namely Wnt/β-catenin and FGF.

Wound epithelium formation. Before regenerative outgrowth can occur, a wound epithelium must form over the damaged tissue, which is part of the scar-free wound healing process (Campbell & Crews, 2008; Murawala, Tanaka, & Currie, 2012; Takeo, Lee, & Ito, 2015). Successful case studies of regeneration in adult digit tips involve proper debridement and placement of a substitute wound epithelium made of chitin over the wound, underlying the overall significance of the presence of a wound epithelium (Halim et al., 1998; LEE et al., 1995). In the lizard and salamander, the wound epithelium thickens to twice the size of the original epidermis (Delorme et al., 2012; Lozito & Tuan, 2015; McLean & Vickaryous, 2011). This structure is called the apical epithelial cap (AEC) in the salamander to mimic the apical ectodermal ridge (AER) formed during limb development (R. N. Christensen et al., 2002; R. N. Christensen & Tassava, 2000; Han, An, & Kim, 2001). In addition to providing a covering for the wound, signaling between the epithelium and underlying mesenchyme promotes proliferation and outgrowth during regeneration in the frog, salamander, and zebrafish (R. N. Christensen et al., 2002; Ghosh, Roy, Séguin, Bryant, & Gardiner, 2008; Han et al., 2001; Kawakami et al., 2006; Y. Lee et al., 2009; Poss et al., 2000; Yokoyama et al., 2011).

Positional identity. Similarly to the initial development of an appendage, patterning is crucial to successful regeneration. Some positional cues in vertebrate regeneration are known, however many remain elusive. Genetic screening of non-regenerating mutants in the zebrafish identified a number of factors involved in patterning (Makino et al., 2005; Nechiporuk, Poss, Johnson, & Keating, 2003; Poss et al., 2002; Whitehead, Makino, Lien, & Keating, 2005), and microarray analysis of spinal cord regeneration identified a number of genes responsible for dorsoventral patterning, including those involved in the establishment of *shh* gradients, and anterior-posterior

patterning, including members of the Hox cluster (Hui et al., 2014). The symmetrical shape of the regenerated cartilage spike in the *X. laevis* froglet is due to a lack of patterning in both the anterior-posterior and proximal-distal axes. Epigenetic modification of the limb-specific enhancer MFCS1 inhibits *shh* activation, disrupting patterning of the anterior-posterior axis (T. Endo et al., 2000; Satoh et al., 2006; Yakushiji et al., 2007). Inhibition of *shh* signaling by cyclopamine in the salamander produces a similarly shaped digitless phenotype (Roy & Gardiner, 2002). Hox genes *hoxa11* and *hoxa13* specify the autopodial and zeudopodial regions during tadpole limb bud regeneration but are misexpressed during froglet limb regeneration and fail to separate, disrupting proximal-distal patterning (T. Endo et al., 2000; Ohgo et al., 2010). Hox genes also regulate proximal-distal patterning in the salamander, though gene expression in the regenerating limb differs from expression during development (Torok et al., 1998). In lizards, positional identity of the proximal-distal axis in the regenerating tail is regulated by retinoic acid (RA) regulation of CD59 (Y. Wang et al., 2011). Cd59 contains conserved motifs with Prod1, which determines proximal-distal positional identity in limb regeneration in the newt (da Silva, Gates, & Brockes, 2002). Further genetic analysis of regenerating appendages in vertebrates should help to identify genes involved in patterning during vertebrate regeneration.

ECM remodeling. Before regeneration can occur, there must be remodeling of the severed tissues. This process destabilizes the extracellular matrix that provided scaffolding for differentiated tissues in the original appendage, allowing for new matrix to be created for differentiating cells in the regenerated appendage (Stocum & Cameron, 2011; Yokoyama, 2008). Additionally, it is theorized that this remodeling response is partially responsible for upregulation of scar-free wound healing in regenerative organisms as opposed to a fibrotic response (Godwin, Kuraitis, & Rosenthal, 2014;

Vinarsky, Atkinson, Stevenson, Keating, & Odelberg, 2005). Matrix metalloproteases (MMPs) were initially discovered for their degradation of collagen during tadpole metamorphosis (Gross & Lapiere, 1962) and have since been studied for the degradation of ECM components in developmental and disease systems (Galliera, Tacchini, & Corsi Romanelli, 2015; Paiva & Granjeiro, 2014). MMPs have been identified in a number of regenerative organisms, including the salamander, frog, and lizard. Following autotomy in the lizard, the tissues in the stump regress and osteoclasts degrade the distal half of the exposed vertebra (Lozito & Tuan, 2015). The protease MMP9 is present in regenerating lizard tail and most likely contributes to this remodeling phase (Delorme et al., 2012). In the frog, *mmp7* is expressed within 6 hours of tadpole tail amputation (N. R. Love et al., 2011). MMPs are highly expressed during salamander limb regeneration within hours of limb amputation, and are required for regeneration. Tissue remodeling genes continue to be expressed prior to outgrowth of the limb (Kato et al., 2003; Knapp et al., 2013; Monaghan et al., 2009; R. Stewart et al., 2013; Vinarsky et al., 2005; E. V. Yang & Bryant, 1994; E. V. Yang, Gardiner, Carlson, Nugas, & Bryant, 1999). Microarray analysis of multiple regenerative tissues, including spinal cord, heart, tail, forelimb, and hindlimb, in the newt identified multiple MMPs induced early in the regenerative response. Additionally, *mmp-13* was upregulated in all regenerative tissues examined (Mercer et al., 2012).

Immune response. Although the specific role of immune response during regeneration is still being elucidated, there have been a number of studies suggesting that the initiation and completion of wound healing and regeneration in regenerative vertebrates may be controlled by inflammation (Fahmy & Sicard, 2002; Godwin & Brockes, 2006; Godwin & Rosenthal, 2014; Godwin, Pinto, & Rosenthal, 2013; Harty, Neff, King, & Mescher, 2003; Mescher & Neff, 2006). Gene expression studies of the

regenerating axolotl limb (Knapp et al., 2013; Monaghan et al., 2012; R. Stewart et al., 2013), regenerating newt limb and other tissues (Mercola, 2012), *X. tropicalis* frog tadpole tail (N. R. Love et al., 2011), have identified immune response genes that are activated during regeneration, as early as 6 hours following amputation (N. R. Love et al., 2011). Following tail autotomy in lizards, granulocytes and monocytes/macrophages are observed at the site of tail loss and participate in wound healing (Alibardi, 2010b; Alibardi, Celeghin, & Valle, 2012). Macrophages in particular are of interest, as they are a source of inflammatory and anti-inflammatory signals. In mammalian tissue repair, macrophages arrive in wounds 24-48 hours following injury and are responsible for clearing dead cells and releasing proinflammatory cytokines. Finally, macrophages reduce inflammation and stimulate angiogenesis, fibroblast migration, and replication (Park & Barbul, 2004). Macrophage depletion or transcriptional repression during muscle repair in mammals results in a fibrotic response (Ruffell et al., 2009; Tidball & Wehling-Henricks, 2007). In axolotl limb regeneration, macrophage depletion in the first 24 hours following limb amputation results in wound closure with regenerative failure associated with fibrosis and dysregulation genes regulating extracellular matrix formation. When endogenous macrophages have been restored, regeneration is restored as well following reamputation, indicating particular importance of the macrophage response in regeneration (Godwin et al., 2013).

Wnt/β-catenin and FGF signaling pathways. Gene expression and functional genetic studies have revealed a crucial role for Wnt/β-catenin signaling during vertebrate appendage regeneration. Gene expression studies of axolotl limb regeneration have identified Wnt/β-catenin, BMP, and FGF signaling as playing key roles in wound healing, cell proliferation/blastema formation, and "re-development" of the limb (Kawakami et al., 2006; Knapp et al., 2013; Makanae, Mitogawa, & Satoh, 2014; Satoh,

Makanae, Hirata, & Satou, 2011; C.-H. Wu, Tsai, Ho, Chen, & Lee, 2013a; Yokoyama, Ogino, Stoick-Cooper, Grainger, & Moon, 2007). Additionally, the Wnt pathway is differentially expressed during digit tip regeneration in mice (Chadwick et al., 2007). Wnt/β-catenin signaling is necessary for limb regeneration in the axolotl, developing limb and tail regeneration in *Xenopus* tadpoles, and fin regeneration in zebrafish (Ghosh et al., 2008; Kawakami et al., 2006; Sugiura, Tazaki, Ueno, Watanabe, & Mochii, 2009; Yokoyama et al., 2007). Functional studies in zebrafish implicate Wnt/β-catenin signaling as a sort of control center in the regenerating fin that regulates proliferation and pattering through regulation of other pathways, including FGF, BMP, and Hedgehog signaling (Wehner et al., 2014). While Wnt/β-catenin and FGF signaling promote blastemal proliferation and outgrowth, non-canonical Wnt signaling inhibits these processes (Y. Lee, Grill, Sanchez, Murphy-Ryan, & Poss, 2005; Stoick-Cooper et al., 2006). Promotion of Wnt/β-catenin and FGF signaling can also enhance regenerative capabilities; *Xenopus* froglets grew limbs with multiple digits after implantation of larval limb progenitor cells with activated Wnt/β-catenin signaling and the addition of sonic hedgehog, FGF10, and thymosin β4 (Lin, Chen, & Slack, 2013). Additionally, modulation of Wnt/β-catenin signaling can induce limb regeneration of the apical ectodermal ridge (AER) and limb bud in chick embryos, which normally do not regenerate (Kawakami et al., 2006; Kostakopoulou, Vogel, Brickell, & Tickle, 1996; Summerbell & Tickle, 1977; Yokoyama et al., 2011). Extracellular factors such as Wnt5 in the salamander and frog (Ghosh et al., 2008; Kawakami et al., 2006; Sugiura et al., 2009; Yokoyama et al., 2011) and several FGFs in the salamander and zebrafish (R. N. Christensen et al., 2002; Han et al., 2001; Y. Lee et al., 2009; Poss et al., 2000) produce a positive feedback loop between the epithelium and underlying mesenchyme to promote proliferation and regulate differentiation. In mammalian digit tip regeneration, Wnt signaling in the nail

epithelium confers regenerative ability (Takeo et al., 2013). These studies demonstrate the importance of the Wnt/β-catenin pathway across vertebrate model systems, suggesting that there is a conserved genetic program for regeneration.

Conclusion. While there are many similarities in vertebrate regeneration, the extent of conserved, convergent, or divergent genetic mechanisms across vertebrates during the regenerative process remains unclear. Microarray studies are somewhat limited in their scope and can have high up-front costs. Genome sequencing of tetrapod vertebrate model systems, including the chicken, the lizard *A. carolinensis*, and the frog *X. tropicalis* has highlighted the degree to which homologous genes are conserved across evolution (Wallis et al., 2004; Alföldi et al., 2011; Hellsten et al., 2010; Kusumi et al., 2011). Advances in sequencing technologies have facilitated sequencing and assembly of large genomes (20 – 40 Gbp) that were previously thought to be too difficult to assemble (Neale et al., 2014). There is a current effort to sequence the Mexican axolotl (*Ambystoma mexicanum*) genome, which has yet to be sequenced, mainly due to its large size (>20 Gbp; Salamander Genome Project). The continuing emergence and decreasing cost of RNA-Seq-based gene expression studies in regenerative model organisms should aid in identification of conserved genetic mechanisms in vertebrates. Lizards, as amniotes, are particularly of interest in this regard as they are the most closely related organism to mammals capable of appendage regeneration in the adult and are more likely to share a conserved genetic program for the regenerative process.

CHAPTER 2

TRANSCRIPTOMIC ANALYSIS OF TAIL REGENERATION IN THE LIZARD ANOLIS CAROLINENSIS REVEALS ACTIVATION OF CONSERVED VERTEBRATE DEVELOPMENTAL AND REPAIR MECHANISMS

Abstract

Lizards, which are amniote vertebrates like humans, are able to lose and regrow a functional tail. Understanding the molecular basis of this process would advance regenerative approaches in amniotes, including humans. We have carried out the first transcriptomic analysis of tail regeneration in a lizard, the green anole *Anolis carolinensis*, which revealed 326 differentially expressed genes activating multiple developmental and repair mechanisms. Specifically, genes involved in wound response, hormonal response, musculoskeletal development, and the Wnt and MAPK/FGF pathways were differentially expressed along the regenerating tail axis. Furthermore, we identified 2 microRNA precursor families, 22 unclassified non-coding RNAs, and 5 novel protein-coding genes significantly enriched in the regenerating tail. However, high levels of progenitor/stem cell markers were not observed in any region of the regenerating tail. Furthermore, we observed multiple tissue-type specific clusters of proliferating cells along the regenerating tail, not localized to the tail tip. These findings predict a different mechanism of regeneration in the lizard than the blastema model described in the salamander and the zebrafish, which are anamniote vertebrates. Thus, lizard tail regrowth involves the activation of conserved developmental and wound response pathways, which are potential targets for regenerative medical therapies.

Background

Regeneration of appendages in the adult is observed in a number of vertebrates, including in the lizard tail, the salamander limb and tail (McCusker & Gardiner, 2011), and the zebrafish caudal fin (Gemberling et al., 2013). Molecular and cellular analyses in these model organisms are beginning to reveal conserved versus divergent mechanisms for tissue regeneration (Hui et al., 2014; Knapp et al., 2013; Looso et al., 2013; Nacu et al., 2013; Q. Wu, Wang, Guo, Ge, & Lu, 2013b), which impacts the translation of these findings to human therapies. Regeneration in newts is associated with proteins specific to urodele amphibians, casting doubt on the conservation of these regenerative pathways with other vertebrates (Looso et al., 2013). In addition, muscle formation during limb regeneration differs between newts and the axolotl (Sandoval-Guzmán et al., 2014). Mammals possess some neonatal regenerative capabilities, including mouse and human digit tip regeneration (Rinkevich et al., 2011; Takeo et al., 2013) and heart regeneration in the mouse (Porrello et al., 2011), but these processes are limited in the adult organism (Fernando et al., 2011). Lizards, which are amniote vertebrates, are evolutionarily the closest regeneration model organism to humans capable of appendage regeneration in the adult. An examination of the genetic regulation of regeneration in an amniote model will advance our understanding of the conserved processes of regeneration in vertebrates, which is relevant to develop strategies of tissue regeneration in humans.

In response to threats, lizards have evolved the ability to autotomize, or self-amputate, their tails and regenerate a replacement (Figure 2A) (Alibardi, 2010a; Cox, 1969). The patterning and final structure of the lizard tail is quite distinct between initial development and the process of regeneration (Fisher et al., 2012; Ritzman et al., 2012). Whereas the original tail skeleton and muscular groups are segmentally organized, reflecting embryonic patterning, the regenerated tail consists of a single unsegmented

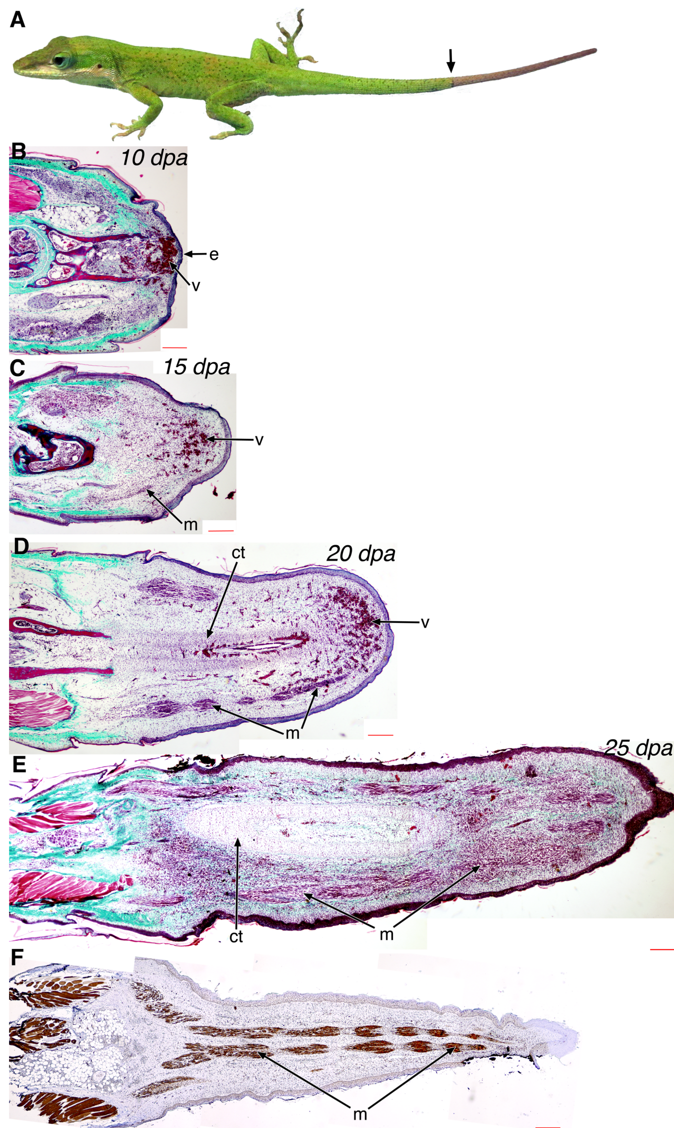


Figure 2. Overview of the stages of lizard tail regeneration. A: *Anolis carolinensis* lizard with a regenerating tail (distal to arrow). **B-E**: Histology of the 10dpa (B), 15 dpa (C), 20 dpa (D), and 25 dpa (E) regenerating tail by Gomori's trichrome stain, with which connective tissues and collagen stain green-blue, muscle, keratin, and cytoplasm stain red, and nuclei are black. **F**: Immunohistochemistry of myosin heavy chain in a 25 dpa regenerating tail using the MY-32 antibody. e, wound epithelium; v, blood vessels; m, muscle; ct, cartilaginous tissue. Composites: B-F. Scale bars in red: 200 μm.

cartilaginous tube surrounded by unsegmented muscular bundles (Fisher et al., 2012; Ritzman et al., 2012). In addition, the segmental organization of the spinal cord and dorsal root ganglia in the original tail are absent in the replacement, with regenerated axons extending along the length of the endoskeleton (Duffy et al., 1990; S. B. Simpson & Duffy, 1994). While the regenerative process in lizards has been described previously (Cox, 1969; Fisher et al., 2012; Hughes & New, 1959; Ritzman et al., 2012; S. B. Simpson, 1965), both the source of regenerating tissue and the cellular and molecular mechanisms that are activated during the regenerative process remain unclear. Dedifferentiation has been proposed to be a major source of proliferating cells in the anamniote salamander blastema model (Kintner & Brockes, 1984). However, no clear evidence of dedifferentiation has been identified in tail regeneration in the lizard, an amniote vertebrate (Cox, 1969; Fisher et al., 2012; Hughes & New, 1959; S. B. Simpson, 1965). A temporal-spatial gradient of tissue patterning and differentiation along the regenerating tail axis has been described (Cox, 1969; Hughes & New, 1959; S. B. Simpson, 1965).

The green anole lizard, *Anolis carolinensis*, is an emerging model organism, and has provided insights in the fields of evolution and development (Eckalbar et al., 2012; Koshiba-Takeuchi et al., 2009), population genetics (Tollis & Boissinot, 2014; Wordley et al., 2011), reproductive physiology (Lovern & Wade, 2003), behavior (Wade, 2012), and functional morphology (Montuelle, Daghfous, & Bels, 2008). Large-scale gene expression analyses of biological processes such as tail regeneration in the green anole have previously been limited by a lack of genomic resources. However, the *A. carolinensis* genome was recently made available (Alföldi et al., 2011). In addition, our group has generated a robust genome annotation based on 14 deep transcriptomes using both directional and non-directional RNA-Seq data from a diverse number of tissues (Eckalbar et al., 2013). These genomic resources provide a platform for transcriptome-

wide analysis of the genes involved in regeneration in the green anole. Here we describe, to our knowledge, the first transcriptomic analysis of lizard tail regeneration.

Materials and Methods

Animals and collection of regenerating tail samples. Animals were collected and maintained in strict accordance with Protocol Number 12-1247R approved by the Institutional Animal Care and Use Committee at Arizona State University. Adult *Anolis carolinensis* lizards were purchased from Marcus Cantos Reptiles (Fort Myers, FL) or Charles D. Sullivan Co., Inc. (Nashville, TN). Animals were housed as previously described (Fisher et al., 2012; Ritzman et al., 2012). Autotomy was induced by applying slight pressure to the tail until the tail was released. Animal health was monitored following autotomy. We collected 5 biological replicates of regenerating tail sections at 25 days post autotomy (dpa). Regenerating tails (n=5) at 25 dpa were divided into five sections (approximately 1 mm each) for RNA-Seq analysis.

RNA-Seq. RNA-Seq of the lizard embryos has been described previously (Eckalbar et al., 2012). Total RNA was isolated from tissue samples, including 25 dpa regenerating tail (n=5) and satellite cells (n=3; mirVana miRNA Isolation Kit total RNA protocol only, Ambion). The Ovation RNA-Seq kit (NuGEN) was used to synthesize double stranded cDNA. Paired-end sequencing libraries were then generated using Illumina manufacturer protocols and sequenced on an Illumina HiSeq 2000. For our analysis, 4 of the 5 regenerating tail replicates were multiplexed together and 2 of the 3 satellite cell replicates were multiplexed together.

Bioinformatic analysis. RNA-Seq reads were trimmed to eliminate nucleotide bias where necessary. Trimmed reads were then mapped to the *A. carolinensis* genome (Alföldi et al., 2011) using Bowtie2.1.0 and TopHat2.0.8 with the

ASU_Acar_v2.2.1 annotation revised from Eckalbar et al., 2013. For Cuffdiff analysis, TopHat aligned reads were assembled using Cufflinks2.1.1 and genes with differential expression were identified using Cuffdiff2.1.1 with the following options: `--upper-quartile-norm --multi-read-correct`. Cuffdiff data were then imported into CummeRbund (Trapnell et al., 2012; 2010). For DESeq2 analysis, raw counts were generated from TopHat aligned reads using HTSeq and normalized for library size in DESeq2 (Anders & Huber, 2010; Anders et al., 2013; Anders, Pyl, & Huber, 2014). In order to identify variant genes using DESeq2, normalized data were fitted to a negative binomial general linear model and adjusted for multiple testing using the Benjamini-Hochberg method, and a likelihood ratio test was performed. CummeRbund and DESeq2 are part of the Bioconductor set of software packages (Gentleman et al., 2004), which use the R statistical programming environment (http://www.R-project.org). P-values for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes were generated using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional analysis tool (D. W. Huang, Sherman, & Lempicki, 2009a; 2009b). Significant GO terms ($p<0.05$) were mapped with the REViGO online tool (http://revigo.irb.hr), which removes redundant GO terms and visualizes the semantic similarity of remaining terms (Supek, Bošnjak, Škunca, & Šmuc, 2011). For all heatmaps, genes were clustered by Jensen-Shannon divergence of the log10(FPKM+1) value.

***A. carolinensis* genome annotation revision.** A revised annotation of the *A. carolinensis* genome was reported using fourteen deep transcriptomes (ASU Acar v2.1; Eckalbar et al., 2013). RNA-Seq data was assembled using the ABySS and Trans-ABySS pipeline (Birol et al., 2009; Robertson et al., 2010; J. T. Simpson et al., 2009). Each of the 25 dpa regenerating tail sections was assembled individually in ABySS using

every 5th kmer ranging from 26bp to 96bp. These assemblies were then combined using trans-ABySS to create a merged assembly with reduced redundancy. This merged assembly was then mapped to the genome using BLAT inside trans-ABySS. De novo assembled contigs were then filtered to require at least 90% coverage of the contig to the genome and to require at least one 25 bp gap. Seqclean was first used to remove Illumina adapters and any contaminants from the UniVec databases from the de novo assembled transcripts and the EST libraries. The cleaned de novo assembled transcripts from ABySS/Trans-ABySS were then assembled using the PASA reference genome guided assembly, and PASA alignment and assembly was executed using default parameters (Haas, 2003; Loke et al., 2005; Rhind et al., 2011; Shen et al., 2008). The PASA assemblies were then used to update the ASU Acar v2.1 annotations inside PASA to v2.2. The annotation was further updated to v2.2.1 with a subset of manual annotations.

Isolation of satellite cells from A. carolinensis. Lizard satellite cell isolation was adapted from mammalian (Allen, Temm-Grove, Sheehan, & Rice, 1997; Lees, Rathbone, & Booth, 2006; Tatsumi et al., 2006) and avian (Feldman & Stockdale, 1991; Yablonka-Reuveni, Seger, & Rivera, 1999) methods. Following euthanasia, large limb muscle groups were dissected in PBS and minced. Cells were separated by protease treatment and suspensions were initially plated to remove adherent fibroblasts and other debris. Satellite cells remaining in suspension were then collected and plated onto Matrigel-coated tissue culture plates in growth medium (Ham's F-10, 20% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 40 μg/mL gentamicin, 20 ng/mL bFGF) at 30°C in a 5% CO2 humidified chamber. While a number of conditions were tested, 30°C was the optimal temperature identified.

Histological analysis. For paraffin sectioning, regenerated tails were fixed and embedded as described previously (Fisher et al., 2012). Embedded tails were

sectioned into 20 μm sections using a CM1950UV Leica Cryostat and placed on HistoBond slides. Paraffin-embedded tissue sections were stained according to hematoxylin-eosin or Gomori's trichrome and mounted in Permount as described previously (Fisher et al., 2012). Hematoxylin stains nuclei and nucleoli blue and eosin stains cytoplasmic and extracellular matrix proteins pink/red, while hydrophobic cells such as adipocytes and myelin will remain clear. With Gomori's trichrome stain, connective tissues and collagen appear green-blue; muscle, keratin, and cytoplasm are red; and nuclei are black.

Immunohistochemistry. Paraffin-embedded tissue sections were deparaffinized, rehydrated, and bathed in sodium citrate buffer (pH 6.0). Cells were fixed in 100% methanol. Tissue sections and cells were stained using the Histostain-SP Broad Spectrum kit (Invitrogen) as follows: Tissue sections and cells were blocked in serum, incubated with primary antibody (MY-32, Sigma Aldrich, MFCD00145920; PCNA, Santa Cruz Biotechnology, sc-7907; MCM2, Abcam, ab4461) incubated with secondary antibody, and incubated with HRP-strepavidin complex, with blocking and antibody incubations at 37°C. Tissue sections and cells were counterstained with hematoxylin and mounted in Permount (Fisher Scientific).

Immunofluorescence. Cells were fixed in 100% methanol, blocked in serum, incubated with PAX7 antibody (Developmental Studies Hybridoma Bank), and incubated with secondary antibody, with blocking and antibody incubations at 37°C. Slides were then counterstained with DAPI.

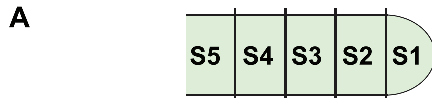
Data Access. RNA-Seq data for the lizard embryo samples, which have been previously reported (Eckalbar et al., 2012), are deposited in at the National Center for Biotechnology Information (NCBI) BioProject, under BioProject PRJNA149661. RNA-

Seq data for the lizard tail regeneration and satellite cell samples are deposited under BioProject PRJNA236326.

Results

Histology of early regenerative stages. Progressively increasing tissue patterning and differentiation are evident in the early regenerative stages of the lizard tail. The first 10 days are characterized by wound healing (0 - 10 days post autotomy (dpa); Figure 2B). By 10 dpa, a wound epithelium has formed over the autotomized stump and blood vessels have formed immediately below. There was no appreciable outgrowth at this stage. Outgrowth begins after the wound epithelium forms and is characterized by early outgrowth of the ependyma from the spinal cord into the surrounding mesenchymal tissue (10 - 15 dpa). By 15 dpa, there was noticeable outgrowth of highly vascularized tissue and myofibers began to form (Figure 2C). With continued tail outgrowth, the central cartilage tube and surrounding skeletal muscle began to differentiate (15 - 20 dpa; Figure 2D). Note that the tip of the tail remains vascular (10 - 20 dpa, Figure 2B-D). By 25 dpa, further lengthening of the regenerating tail was observed, along with formation of muscle and cartilage surrounding the ependymal core (Figure 2E). Further outgrowth with continued tissue differentiation is evident post-25 dpa, and there is no significant outgrowth after 60 dpa (Fisher et al., 2012). In fact, by 25 dpa, myosin heavy chain (MHC) positive skeletal muscle was present along the length of the developing tail, except at the very distal tip (Figure 2F). Spatially, there is an increase in patterning and differentiation along the regenerating tail was observed at early outgrowth stages (e.g., 15-25 dpa, Figure 2C-E), with differences in tissue organization particularly evident along the proximal-distal axis.

A



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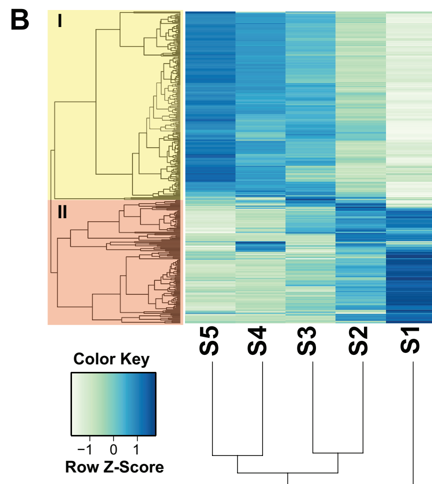

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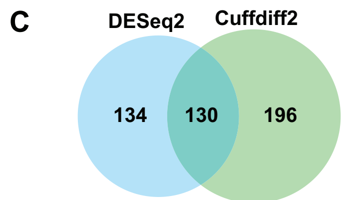

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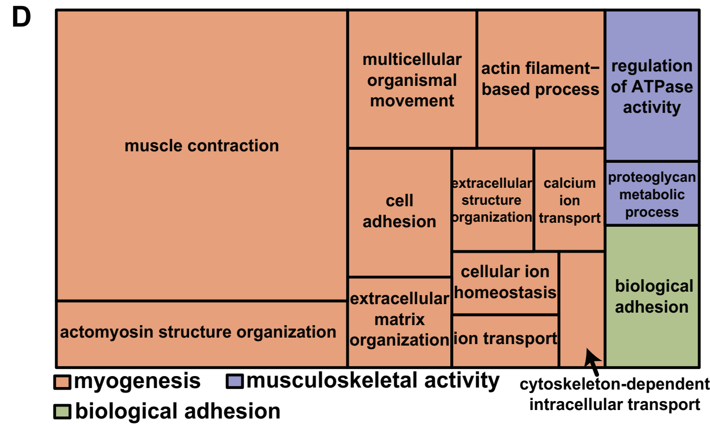

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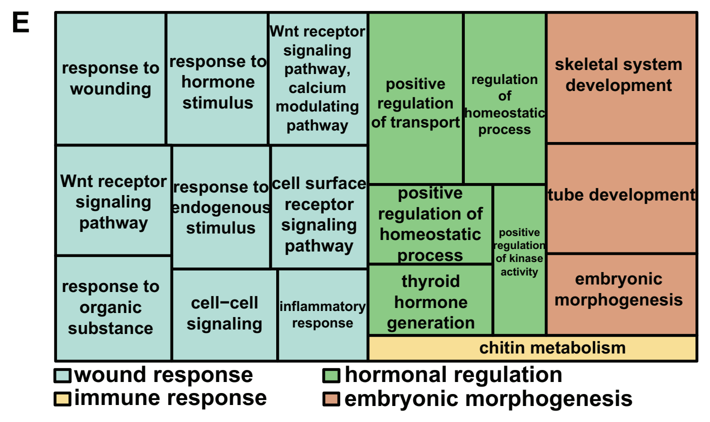

Figure 3. Transcriptomic analysis of gene expression in the 25 dpa regenerating lizard tail. **A**: 25 dpa regenerated tail tissue was divided into five equal sized segments (S1-S5) with S1 representing the most distal regenerating tip, and total RNA was extracted for RNA-Seq analysis. **B**: A heatmap showing 326 genes that were differentially expressed, i.e., displayed significant differences between any two segments in the regenerating tail as determined by Cuffdiff ($p<0.05$). Genes were clustered by Jensen-Shannon divergence of the log10(FPKM+1) value into two major groups, as shown in the dendrogram on the left. 129 genes displayed increased expression distally towards the tail tip (Cluster II) while 197 displayed increased expression proximally (Cluster I). This clustering also demonstrated that the distal-most regenerating tail tip (S1) was the outlier among these samples. **C**: Venn diagram of differentially expressed genes identified by DESeq2 and Cuffdiff2. **D-E**: A treemap overview of differentially expressed genes in (D) Cluster I and (E) Cluster II based on representative Gene Ontology Biological Processes. The relative sizes of the treemap boxes are based on the |log10(p-value)| of the respective GO term. Related terms are visualized with the same color, with the representative category for each color group denoted in the legend.

Sequencing and differential expression testing of regenerating tail transcripts. To identify differentially expressed genes along the proximal-distal axis of regenerating tails, we carried out RNA-Seq analysis on five tails at 25 dpa. Tails were sectioned into five segments of equal length (Figure 3A). RNA-Seq analysis identified 326 differentially expressed genes with $p<0.05$ after correcting for multiple testing using Cuffdiff2 (Trapnell et al., 2012; 2013), 302 of which have mammalian orthologs (Figure 3B). Data were also analyzed by DESeq2 (Anders et al., 2013; Anders & Huber, 2010), which yielded 264 differentially expressed genes, 252 of which have mammalian orthologs (Figure 3C). These Cuffdiff2 differentially expressed genes clustered into two major groups, representing genes elevated towards the proximal base (Cluster I, Figure 3D) or the distal tip (Cluster II, Figure 3E).

Differential expression of genes involved in developmental and repair mechanisms in the regenerating tail. Our RNA-Seq analysis identified Gene Ontology (GO) groups associated with the differentiation of tissues present in the proximal regenerating tail, predominantly those that are specific to skeletal muscle (Figure 3D; Table 1). Sarcomeric proteins, including myosin heavy chains and actinins, were elevated in the proximal tail. This pattern of expression was validated by the presence of myosin heavy chain positive muscle fibers (Figure 2F). Myogenic regulatory factors associated with muscle growth and repair were also elevated in the proximal tail. These include the transcription factors *pax7*, mohawk (*mkx*), and *tcf15*, which are expressed in myogenic stem/progenitor cells (D. M. Anderson, Beres, Wilson-Rawls, & Rawls, 2009; Fan, Li, Rozo, & Lepper, 2012; Takahashi et al., 2007), NFATc1, which regulates muscle hypertrophy (Sakuma et al., 2003), and the TGFβ family member myostatin (*mstn*), which modulates muscle mass (Manceau et al., 2008); *Anolis* Gene Nomenclature Committee standards used for gene symbols; (Kusumi et al., 2011). Also,

Table 1

Selected Cluster I Gene Ontology categories represented along the regenerating tail axis.

Category	GO Term	Description	Count	P-value	Genes
myogenesis	GO:0006936	muscle contraction	30	6.63E-29	*mybpc2, tnnc2, tnnc1, myl3, mybpc1, mybpc3, myl1, pgam2, myot, des, myom2, myl6b, myom1, chrna1, scn5a, dtna, kcnma1, actc1, acta1, actn2, myh6, tnni2, trdn, tnnt3, tnnt1, ryr1, stbd1, chrne, casq2, chrng*
	GO:0007517	muscle organ development	28	3.44E-22	*mef2c, myod1, myl2, tnnc1, myl3, mybpc3, myl1, trim72, speg, myl6b, pax7, obsl1, mkx, mkl2, chrna1, actc1, acta1, mstn, mylpf, myh6, csrp3, flnb, murc, neb, xirp1, itga7, vgll2, tcf15*
	GO:0007519	skeletal muscle tissue development	9	3.73E-07	*myod1, acta1, myl3, myl6b, pax7, mylpf, vgll2, chrna1, csrp3*
	GO:0042692	muscle cell differentiation	11	4.86E-07	*myod1, actc1, acta1, xirp1, myl2, speg, lgals1, obsl1, myh6, mkl2, chrna1*
	GO:0050881	musculoskeletal movement	6	1.14E-06	*tnnt3, tnnt1, tnnc2, tnnc1, chrna1, tnni2*
	GO:0030029	actin filament-based process	14	1.28E-06	*actc1, tnxb, myl2, acta1, myl1, pdlim3, myh6, gas7, flnb, xirp1, xirp2, myl6b, limch1, obsl1*
	GO:0007155	cell adhesion	21	3.41E-05	*hapln1, tnxb, mybpc2, clstn2, egfl6, lpp, mybpc1, col22a1, mybpc3, col28a1, mgp, actn2, col2a1, actn3, ecm2, col9a1, itga7, acan, susd5, col11a2, thbs4*
	GO:0001501	skeletal system development	12	4.79E-04	*bmp3, col9a1, col9a2, tbx15, lect1, clec3a, pax7, acan, mgp, col2a1, col11a2, tcf15*
	GO:0030198	extracellular matrix organization	7	7.29E-04	*csgalnact1, tnxb, adamts20, acan, col2a1, col11a2, ecm2*
	GO:0030705	cytoskeleton-dependent intracellular transport	4	0.0166	*actc1, myl6b, myl1, myh6*
	GO:0006873	cellular ion homeostasis	11	0.0055	*kcnma1, jph2, xirp1, pygm, atp2a1, ryr1, chrna1, chrne, csrp3, sypl2, chrng*
chondrogenesis	GO:0051216	cartilage development	8	1.10E-05	*bmp3, col9a1, lect1, pax7, acan, mgp, col2a1, col11a2*
	GO:0002062	chondrocyte differentiation	4	7.90E-04	*col9a1, acan, col2a1, col11a2*
	GO:0001502	cartilage condensation	3	0.0162	*acan, mgp, col2a1*
musculoskeletal activity	GO:0043462	regulation of ATPase activity	5	1.82E-05	*tnnt3, myl3, tnnc1, mybpc3, myh6*
	GO:0006029	proteoglycan metabolic process	4	0.0099	*csgalnact1, lect1, acan, col2a1*
biological adhesion	GO:0022610	biological adhesion	21	3.48E-05	*hapln1, tnxb, mybpc2, clstn2, egfl6, lpp, mybpc1, col22a1, mybpc3, col28a1, mgp, actn2, col2a1, actn3, ecm2, col9a1, itga7, acan, susd5, col11a2, thbs4*

the MADS box factor *mef2c*, and the myogenic regulatory factor *myod1*, which synergize to activate muscle specific gene transcription, were elevated (Wilson-Rawls, Molkentin, Black, & Olson, 1999). As growth and repair of skeletal muscle in vertebrates normally relies on the expansion and differentiation of muscle-specific progenitor cells, the enrichment for genes associated with the regulation of this population predicts a similar mechanism of muscle growth and repair occurring in a zone of active regeneration. Furthermore, the increase in *mkx* transcription raises the possibility of a coordinated growth between tendons and muscle in the regenerating tail, given that the orthologous gene is required for growth and repair in mammals (D. M. Anderson et al., 2012).

Our transcriptome analysis identified multiple genetic pathways activated towards the tip of the regenerating tail. Genes differentially elevated at the tip were enriched for GO categories related to i.) wound response, ii.) hormonal regulation, and iii.) embryonic morphogenesis (Figure 3E; Table 2). Wound and inflammatory response genes elevated in the distal regenerating tail include *igfbp4*, *mdk*, *ptx3*, and *pdgfra*. Mouse *Ptx3* is required for fungal resistance (Garlanda et al., 2002), and *Mdk* plays a role in angiogenesis(Reynolds et al., 2004). Hormonal and homeostatic regulation genes included those involved in thyroid hormone generation, such as *cga* and *dio2*. Thyroid hormone plays a critical role in neuromuscular growth, both during normal development and in repair after injury. *Dio2* has been shown to co-regulate myogenesis and muscle regeneration in the mouse (Dentice et al., 2010). In the rat model, triiodothyronine (T3) treatment after sciatic nerve injury has been shown to enhance reinnvervation of muscles (Panaite & Barakat-Walter, 2010). In the tadpole, thyroid hormone is critical for limb development during metamorphosis, where limb muscle growth, innervation of the limb, cartilage growth, and skin development are all thyroid hormone-dependent (Brown et al., 2005). Genes involved in homeostatic regulation and vascular development include

Table 2

Selected Cluster II Gene Ontology categories represented along the regenerating tail axis

Category	GO Term	Description	Count	P-value	Genes
wound response	GO:0009611	response to wounding	10	0.0040	*pcsk1, scube1, pdgfra, pla2g7, entpd1, ptx3, mdk, igfbp4, f2r, spp1*
	GO:0009725	response to hormone stimulus	8	0.0059	*cga, pcsk1, krt19, tnfrsf11b, bsg, th, pdgfra, spp1*
	GO:0007223	Wnt receptor signaling pathway, calcium modulating pathway	3	0.0067	*wnt5a, wnt16, ror2*
	GO:0016055	Wnt receptor signaling pathway	5	0.0079	*dkk2, wnt5a, wnt16, ror2, wif1*
	GO:0007166	cell surface receptor signaling pathway	20	0.0106	*wnt5a, cga, edn3, fgfr4, il1r1, wnt16, gpr158, bsg, maml2, ptpn22, thy1, dkk2, ednra, or5v1, pdgfra, ror2, wif1, pdgfc, entpd1, f2r*
	GO:0010033	response to organic substance	11	0.0098	*ednra, cga, pcsk1, krt19, il1r1, tnfrsf11b, bsg, th, pdgfra, f2r, spp1*
	GO:0006954	inflammatory response	6	0.0433	*scube1, pla2g7, ptx3, igfbp4, f2r, spp1*
hormonal regulation	GO:0051050	positive regulation of transport	7	0.0020	*ednra, edn3, pcsk1, rab8b, ptx3, f2r, thy1*
	GO:0032844	regulation of homeostatic process	5	0.0046	*ednra, tnfrsf11b, f2r, spp1, thy1*
	GO:0006590	thyroid hormone generation	2	0.0350	*cga, dio2*
embryonic morphogenesis	GO:0001501	skeletal system development	9	5.81E-04	*wnt5a, tnfrsf11b, pdgfra, ror2, mepe, cbfb, igfbp4, spp1, twist1*
	GO:0035295	tube development	7	0.0019	*wnt5a, ednra, fgfr4, sall1, pdgfra, ptk7, twist1*
	GO:0048598	embryonic morphogenesis	7	0.0096	*wnt5a, sall4, th, ptk7, ror2, twist1, ptprq*
immune response	GO:0006030	chitin metabolic process	2	0.0407	*chi3l1, chit1*

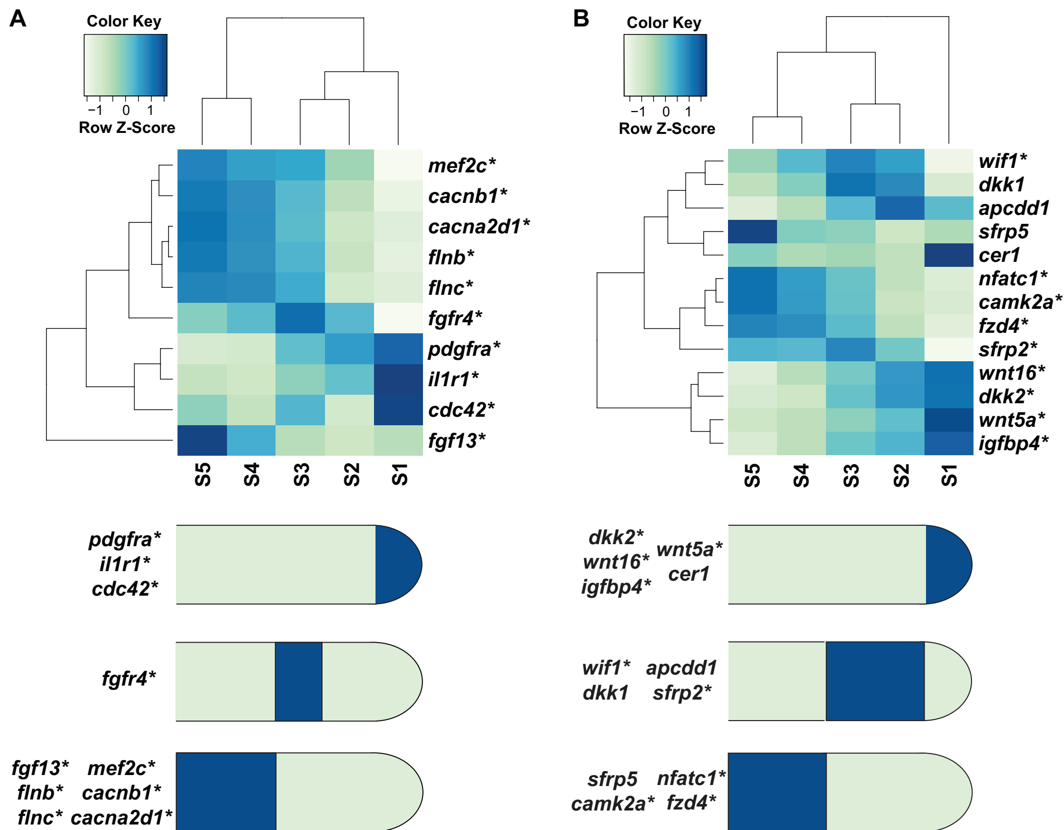

Figure 4. MAPK/FGF and Wnt pathway genes differentially expressed in the 25 dpa regenerating lizard tail. **A-B**: Based on RNA-Seq analysis described in Fig. 3, the heatmaps show the 10 MAPK/FGF pathway genes (A), or 9 Wnt pathway genes (B) defined by KEGG, that were differentially expressed (DE), i.e., displayed significant differences between any two segments in the regenerating tail as determined by Cuffdiff2 ($p<0.05$), along with previously identified Wnt inhibitors. A diagram summarizing the tail segment(s) with highest expression level for each MAPK/FGF (A) or Wnt (B) pathway gene is also shown. DE genes are denoted with an asterisk.

ednra and *edn3*, which are members of the endothelin family and regulate vasoconstriction and cell proliferation (Goldie, 1999), the thrombin receptor *f2r*, which promotes vascular development by negatively regulating hematopoietic differentiation of mouse embryonic stem cells (Yue et al., 2012), and *thy1*, which is a marker of angiogenesis (W. S. Lee et al., 1998). The *wnt5a* ligand and its receptor, *ror2*, were both significantly expressed at the tip, indicating non-canonical Wnt signaling, which can promote chondrogenesis (T. F. Day, Guo, Garrett-Beal, & Yang, 2005; DeChiara et al., 2000). Skeletal system development genes elevated in the regenerating tail include the basic helix-loop-helix transcription factor *twist1*, which regulates a number of pathways, including FGF, by chromatin modification via histone acetyltransferases (Hamamori et al., 1999).

Differentially expressed genes analyzed for Kyoto Encyclopedia of Genes and Genomes (KEGG) categories ($p < 0.05$) identified axon guidance and neural development genes, including slit homolog 2 (*slit2*), actin binding LIM protein family member 2 (*ablim2*), and netrin receptor unc-5 homolog C (*unc5c*) (Table 2). KEGG groups enriched in the regenerating tail also include the Wnt and MAPK/FGF signaling pathways. FGF signaling plays a key role in developmental patterning, proliferation, and differentiation (Pownall & Isaacs, 2010). Differentially expressed MAPK/FGF pathway genes at the tail tip include *pdgfra*, *il1r1*, and *cdc42* while *mef2c*, *cacnb1*, *cacna2d1*, *flnb*, *flnc*, and *fgfr13* are elevated at the proximal regenerating tail (Figure 4A). A number of recent reports from mouse digit tip and salamander limb regeneration identified Wnt pathway involvement (Knapp et al., 2013; Takeo et al., 2013; Q. Wu et al., 2013b). Wnt signaling promotes the differentiation of embryonic stem cells as well as cells from skeletal muscle, osteogenic, and cardiogenic lineages (Cruciat & Niehrs, 2013). The tip to the middle of the regenerating tail are enriched with Wnt inhibitors, including *dkk2*,

igfbp4, *wif1*, and *sgfrp2* (Figure 4B). The expression of soluble Wnt inhibitors from this region could create a proximal-distal gradient of Wnt signaling that is necessary to maintain the actively growing zone of the regenerating tail in a proliferative, undifferentiated state.

Novel and uncharacterized transcripts in the regenerating tail. We sought to characterize the 22 differentially expressed genes, representing 29 transcript isoforms, without clear orthology, i.e., BLAST alignment scores against the nonredundant protein database were either E ≥ 1.0, identity was ≤ 50%, or no match was identified. These transcripts could potentially be protein-coding genes specific to squamate reptiles, either novel or highly divergent within the squamate lineage, or could represent noncoding RNA species. Transcripts were queried against the protein family (Pfam; Punta et al., 2012) and RNA family (Rfam; Burge et al., 2013) databases, and coding potential was evaluated using the Coding-Non-Coding Index (CNCI; L. Sun et al., 2013), which evaluates coding potential by profiling adjoining trinucleotide sequences (Table 3). Four transcripts were identified as retrotransposons, including the gag-pol polyprotein and RNA-directed DNA polymerase from mobile element jockey-like, which are enriched in the proximal regenerating tail. Of the remaining transcripts, 3 were predicted as protein-coding and 22 were characterized as non-coding by the CNCI. The protein-coding gene ASU_Acar_G.15880, which is differentially expressed in the proximal regenerating tail, has a DUF4585 (domain of unknown function) domain, and orthologous genes found in the king cobra (*Ophiophagus hannah*; GenBank: ETE69491.1) and green sea turtle (*Chelonia mydas*; GenBank: EMP32806.1; NCBI: XP_007063098.1) genomes and the axolotl (*Ambyostoma mexicanum*) transcriptome. The 2 remaining protein-coding transcripts were not matched to any known domains in the Pfam database. Of the 22 non-coding transcripts, we identified 2 differentially

Table 3

Novel and uncharacterized transcripts in the regenerating tail

	Transcript ID	CNCI score	CNCI classification	Length (bp)	Longest ORF (bp)	Domain/ Homology	Highest Section
Predicted RNA only	ASU_Acar_T.1063.1	0.0	non-coding	216	213	lncRNA	S1
	ASU_Acar_T.14483.1	-0.0029	non-coding	698	153	lncRNA	S4
	ASU_Acar_T.14483.2	-0.0784	non-coding	1256	195	lncRNA	S4
	ASU_Acar_T.14483.5	-0.0029	non-coding	712	153	lncRNA	S2
	ASU_Acar_T.14483.7	-0.0029	non-coding	1430	195	lncRNA	-
	ASU_Acar_T.17546.1	-0.0390	non-coding	225	222	lncRNA	S1
	ASU_Acar_T.17964.1	-0.1550	non-coding	219	123	lncRNA	S4
	ASU_Acar_T.18922.3	-0.0664	non-coding	1627	291	lncRNA	S3
	ASU_Acar_T.5235.1	0.0	non-coding	216	213	lncRNA	S3
	ASU_Acar_T.7180.1	-0.0038	non-coding	243	240	lncRNA	S5
	ASU_Acar_T.8849.1	-0.0532	non-coding	291	288	lncRNA	S4
	ASU_Acar_T.8944.1	-0.2007	non-coding	279	276	lncRNA	S1
	ASU_Acar_T.20175.1	-0.0204	non-coding	261	258	lncRNA	S1
	ASU_Acar_T.1922.1	-0.0114	non-coding	2286	213	miR-133	S5
	ASU_Acar_T.19355.1	-0.0064	non-coding	2549	219	miR-324	S5
	ASU_Acar_T.10886.1	-0.1770	non-coding	637	384	ncRNA	S1
	ASU_Acar_T.13829.1	-0.0563	non-coding	189	186	ncRNA	S4
	ASU_Acar_T.14483.4	0.0	non-coding	183	180	ncRNA	S3
	ASU_Acar_T.14483.6	-0.0073	non-coding	459	456	ncRNA	S4
	ASU_Acar_T.14791.1	0.0	non-coding	199	114	ncRNA	S1
	ASU_Acar_T.1721.1	-0.0170	non-coding	192	189	ncRNA	S2
	ASU_Acar_T.18922.5	-0.0947	non-coding	192	189	ncRNA	S4
	ASU_Acar_T.2935.1	0.0000	non-coding	195	192	ncRNA	S1
	ASU_Acar_T.3586.1	0.0000	non-coding	195	192	ncRNA	S1
Protein Coding - Not Described	ASU_Acar_T.15880.1	0.1481	coding	14705	4992	DUF4585	S2
	ASU_Acar_T.14483.3	0.0510	coding	3395	2766	unknown	S5
	ASU_Acar_T.18922.1	0.2979	coding	1833	561	unknown	S4
	ASU_Acar_T.18922.2	0.1975	coding	1212	1041	unknown	-
	ASU_Acar_T.18922.4	0.2288	coding	1746	474	unknown	S4
	ASU_Acar_T.18922.6	0.2764	coding	1714	336	unknown	S5
	ASU_Acar_T.18922.7	0.1938	coding	1383	1296	unknown	-
	ASU_Acar_T.19198.1	0.0293	coding	264	261	unknown	S1
	ASU_Acar_T.21065.1	0.3766	coding	2064	1053	unknown	S1
Retro-transposons	ASU_Acar_T.14133.1	0.2166	coding	3618	3615	gag-pol polyprotein	S5
	ASU_Acar_T.591.1	0.1336	coding	762	759	gag-pol polyprotein	S3
	ASU_Acar_T.591.2	-0.0102	non-coding	198	195	gag-pol polyprotein	S5
	ASU_Acar_T.4168.1	0.0918	coding	2010	1863	rna-directed dna polymerase	S5

expressed genes in the proximal tail categorized within the miRNA precursor families miR-133 and miR-324. miR-133 acts in a negative feedback loop with serum response factor (SRF) to promote myoblast differentiation in vitro, and suppresses BMP2-induced osteogenesis by targeting Runx2 (Y. Chen, 2006; Z. Li et al., 2008). The remaining 20 non-coding transcripts represent potential modulators of genes down-regulated in regeneration. In summary, these unidentified transcripts represent novel protein-coding genes, long non-coding RNAs, and microRNAs that may regulate the regenerative process in concert with identified genes and signaling pathways.

Comparison of regenerating tail with stem/progenitor cells and developing embryo. Tissue regeneration in the lizard tail requires a source of cells; these could be tissue-specific oligopotent or progenitor stem cells, as in mammalian tissue repair, since there is no evidence of dedifferentiation in the lizard as observed in the salamander (Cox, 1969; Hughes & New, 1959; Kusumi & Fisher, 2012; S. B. Simpson, 1965). We analyzed the regenerated tail in comparison with lizard embryos and satellite cells; both are highly enriched for stem cell populations. We profiled the transcriptome of lizard embryos at the 28-38 somite pair stages (Eckalbar et al., 2010). At this stage, the embryo contains paraxial mesoderm, a multipotent cell source for skeletal muscle, cartilage, bone, and tendon. Satellite cells capable of differentiating into skeletal muscle in response to injury serve as progenitor/stem cells for adult muscle repair in mammals (Asakura, Komaki, & Rudnicki, 2001). We isolated a PAX7 positive cell population from adult lizard skeletal muscle that was morphologically comparable to mouse satellite cells. These cells differentiated into multinucleated, MHC positive myotubes, and express many of the same lineage-specific genes (Figure 5A). The lizard embryos and satellite cells each possess distinct gene expression signatures based on gene markers for mouse and human embryonic, hematopoietic, and mesenchymal stem cells and satellite cells. In

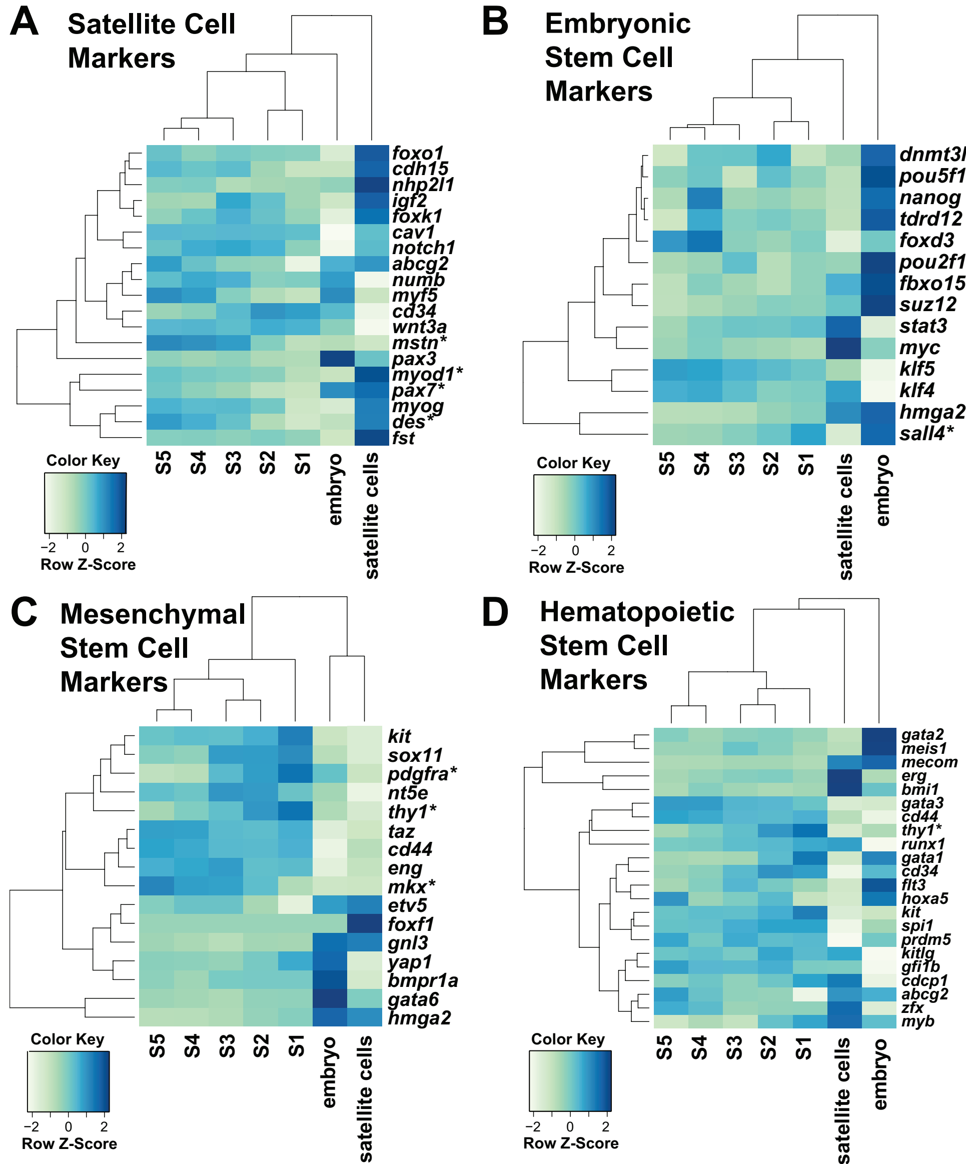

Figure 5. The 25 dpa regenerating tail has limited relative expression of stem cell markers. **A-D**: Heatmap showing gene expression of satellite cell (A) and embryonic (B), mesenchymal (C), and hematopoietic stem cell markers in lizard embryos (n=2), satellite cells (n=3), and 25dpa regenerating tail sections (n=5). DE genes along the regenerating tail axis are denoted with an asterisk.

contrast, these genes are expressed at low levels without a distinct proximal-distal pattern in the regenerating tail (Figure 5A-D). These data predict a role for stem cells distributed throughout the regenerating tail, instead of being localized to the distal tip with a distal-to-proximal gradient of differentiation within the tail. While there are genes elevated in the regenerating tail relative to the embryo and satellite cells, genes elevated in the regenerating tail tip are primarily involved in the formation of tissues specific to the tail such as keratin-associated beta protein, and genes elevated in the proximal regenerating tail are primarily involved in tissue differentiation. The lack of intensity in the signal compared to the embryo and satellite cells could be due to stem cells comprising only a minority population in the regenerating tail.

Distributed pattern of cell proliferation in the regenerating tail. Proliferation and specification of progenitor cells is required for growth of the regenerating tail. While the regenerating tail did not express high levels of stem cell factors, selected progenitor/stem cell markers still displayed differential expression along the proximal-distal axis (Figure 5A-D). These genes included platelet-derived growth factor receptor *pdgfra*, which is expressed in subtypes of mesenchymal progenitor cells involved in muscle repair (Cairns et al., 2012). In addition, genes elevated in the tail tip include the *kit* ligand and *sox11* transcription factor, and genes elevated towards the proximal tail included the previously discussed transcription factor *mkx*. To visualize the pattern of proliferating cells within the regenerating tail, we analyzed the distribution of minichromosome maintenance complex component 3 (MCM2) in the regenerating tail (Figure 6A-E). MCM2 positive cells are observed in distributed, discrete regions in the regenerating tail, including the condensing cartilage tube and ependymal core (Figure 6B-C) and in developing muscle (Figure 6D-E). A second marker of proliferation, proliferating cell nuclear antigen (PCNA), showed a

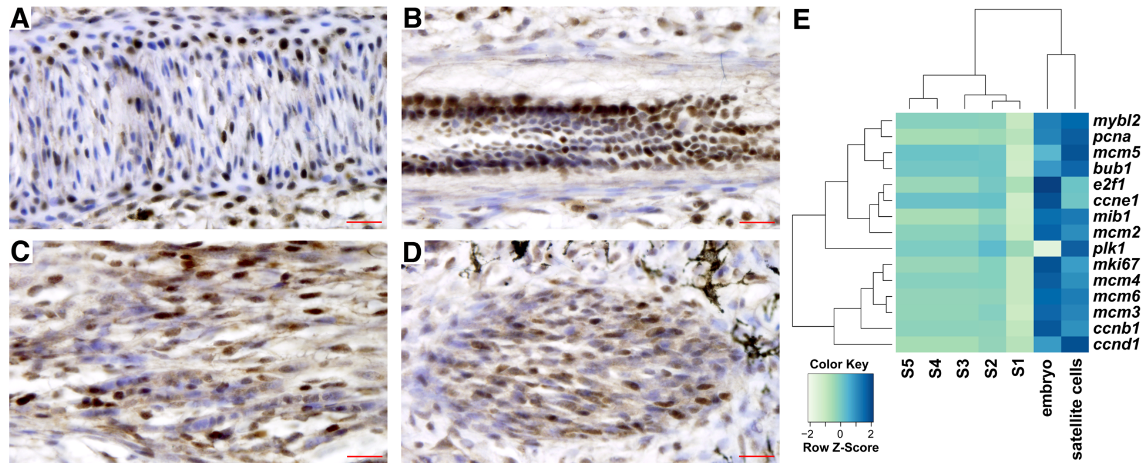

Figure 6. Histological and RNA-Seq analysis of proliferation in the 25 dpa regenerating tail. **A-D**: MCM2 immunohistochemistry of the 25dpa regenerating tail (brown nuclei), counterstained with hematoxylin (blue nuclei). The condensing cartilage tube (A), ependymal core (B), developing muscles near the proximal base (C) and tip (D) of the regenerating tail are shown. **E**: A heatmap showing gene expression of proliferative markers in the regenerating tail, the embryos, and satellite cells. DE genes along the regenerating tail axis are denoted with an asterisk. Scale bars in red: 20 μm (A-D).

similar pattern of expression, confirming that proliferating cells are distributed throughout the regenerating tail in comparison to low levels of proliferating cells in the original tail. This pattern of proliferation is corroborated by RNA-Seq analysis of proliferation markers along the regenerating tail (Figure 6F). No segment along the proximal-distal axis of the regenerating tail demonstrated elevated expression of these markers, indicating that there is no single growth zone.

Discussion

While transcriptomic analysis has been carried out in anamniote regenerative models, including the zebrafish tail, the newt limb, and the axolotl limb (Hui et al., 2014; Knapp et al., 2013; Looso et al., 2013; Q. Wu et al., 2013b), the genetic profile of pathways activated in regeneration of amniote appendages has not been described. Through transcriptomic analysis of lizard tail regeneration, we have identified that genes in pathways involved in developmental processes, including myogenesis, chondrogenesis, and neurogenesis, as well as adult processes, such as wound and immune responses, and are differentially expressed along the regenerating tail axis. The Wnt pathway was significantly enriched along the regenerating lizard tail axis, and activation of this pathway has also been noted in salamander tail tip and mouse digit tip regeneration (Knapp et al., 2013; Takeo et al., 2013; Q. Wu et al., 2013b). Specifically, the Wnt pathway members *wnt5a* and *wif1* are differentially expressed in lizard as well as the salamander (Knapp et al., 2013; Q. Wu et al., 2013b). The activation of Wnt signaling in two amniote lineages, mammals and squamate reptiles, and in urodele amphibians supports a role for this pathway in regeneration that is conserved among tetrapod vertebrates. Transcriptomic analysis also revealed that genes involved in thyroid hormone generation (GO category GO:0006590; Table 2) were differentially expressed,

suggesting a regulatory connection between regeneration of the lizard tail and musculoskeletal transformations during amphibian metamorphosis. The lizard *dio2* gene is the ortholog of deiodinase, iodothyronine, type I, which in mammals converts thyroxine prohormone (T4) to bioactive 3,3',5-triiodothyronine (T3; Croteau, Davey, Galton, & St Germain, 1996). In *Xenopus laevis*, T3 is the key signal for the process of metamorphosis from tadpole to adult frog (Furlow & Neff, 2006) (Furlow & Neff, 2006). Many of the changes associated with metamorphosis are also observed in the remodeling of the tail stump and outgrowth of the lizard tail. The lizard *cga* gene is the ortholog of chorionic gonadotropin, alpha chain, which encodes the alpha chain of thyroid-stimulating hormone and other key hormones (Boothby, Ruddon, Anderson, McWilliams, & Boime, 1981). During tadpole metamorphosis, both thyroid hormone (TH) and thyroid-stimulating hormone (TSH) rise, despite the normal expectation that TH would down-regulate TSH (Buckbinder & Brown, 1993). Changes in TH regulation of TSH may also be altered in regeneration, which has not been studied in the lizard. It is possible that among the amniotes, the lizard retains genetic pathways associated with thyroid hormone regulation of metamorphosis in amphibian vertebrates. Similarly, we previously identified conserved features in Notch pathway regulation of lizard and amphibian development, specifically a gradient of hes6 expression in the presomitic mesoderm that was not observed in other amniote vertebrates and presumably lost (Eckalbar et al., 2010). Our transcriptomic analysis has highlighted the activation of multiple genetic pathways, sharing genes that have been identified as regulating development or wound response processes in other vertebrate model systems.

Developmental systems display different patterns of tissue outgrowth. For example, some tissues are formed from tissue patterning from a localized region of a single multipotent cell type, such as the axial elongation of the trunk through production

of somites from the presomitic mesoderm (Kusumi, May, & Eckalbar, 2013). Other tissues are formed from the distributed growth of distinct cell types, such as the development of the eye from neural crest, mesenchymal, and placodal ectodermal tissue (Graw, 2010). The regeneration of the amphibian limb involves a region of highly proliferative cells adjacent to the wound epithelium, the blastema, with tissues differentiating as they grow more distant from the blastema. However, regeneration of the lizard tail appears to follow a more distributed model. Stem cell markers and PCNA and MCM2 positive cells are not highly elevated in any particular region of the regenerating tail, suggesting multiple foci of regenerative growth. This contrasts with PNCA and MCM2 immunostaining of developmental and regenerative growth zone models such as skin appendage formation (Chodankar et al., 2003), liver development (Suksaweang et al., 2004), neuronal regeneration in the newt (Berg et al., 2010), and the regenerative blastema (Santos-Ruiz, Santamaría, Ruiz-Sánchez, & Becerra, 2002), which all contain localized regions of proliferative growth. Skeletal muscle and cartilage differentiation occurs along the length of the regenerating tail during outgrowth; it is not limited to the most proximal regions. Furthermore, the distal tip region of the regenerating tail is highly vascular, unlike a blastema, which is avascular (Mescher, 1996). These data suggest that the blastema model of anamniote limb regeneration does not accurately reflect the regenerative process in tail regeneration of the lizard, an amniote vertebrate.

Regeneration requires a cellular source for tissue growth. Satellite cells, which reside along mature myofibers in adult skeletal muscle, have been studied extensively for their involvement in muscle growth and regeneration in mammals and other vertebrates (D. M. Anderson et al., 2012; Asakura et al., 2001; Fan et al., 2012; R. M. George et al., 2013; Takahashi et al., 2007). For example, regeneration of skeletal muscle in the axolotl

limb involves recruitment of satellite cells from muscle (Sandoval-Guzmán et al., 2014). Satellite cells could contribute to the regeneration of skeletal muscle, and potentially other tissues, in the lizard tail. Mammalian satellite cells in vivo are limited to muscle, but in vitro with the addition of exogenous BMPs, they can be induced to differentiate into cartilage as well (Asakura et al., 2001; Cairns et al., 2012). High expression levels of BMP genes in lizard satellite cells could be associated with greater differentiation potential, and further studies will help to uncover the plasticity of this progenitor cell type.

Conclusion

In summary, we have identified a coordinated program of regeneration in the green anole lizard that involves both recapitulation of multiple developmental processes and activation of latent wound repair mechanisms conserved among vertebrates. However, the process of tail regeneration in the lizard does not match the dedifferentiation and blastema-based model as described in the salamander and zebrafish, and instead matches a model involving tissue-specific regeneration through stem/progenitor populations. The pattern of cell proliferation and tissue formation in the lizard identifies a uniquely amniote vertebrate combination of multiple developmental and repair mechanisms. We anticipate that the conserved genetic mechanisms observed in regeneration of the lizard tail may have particular relevance for development of regenerative medical approaches.

CHAPTER 3

DIFFERENTIAL EXPRESSION OF CONSERVED AND NOVEL MICRORNAS DURING TAIL REGENERATION IN THE GREEN ANOLE LIZARD, ANOLIS CAROLINENSIS

Abstract

The green anole lizard (*Anolis carolinensis*) is evolutionarily the most closely related organism to humans that can lose and regrow an appendage. Regeneration in lizards involves differential expression of hundreds of genes that regulate wound healing, musculoskeletal development, hormonal response, and embryonic morphogenesis. While microRNAs are able to regulated large groups of genes, their role in lizard regeneration has not been investigated. MicroRNA sequencing of lizard regenerating tail and associated tissues revealed 350 novel and 196 known microRNA precursor families. Eleven microRNAs were differentially expressed between the regenerating tail tip and base during maximum outgrowth (25 days post autotomy), including miR-133a, miR-133b, and miR-206, which have been reported to regulate regeneration and stem cell proliferation in model systems. Three novel differentially expressed microRNAs were identified in the regenerating tail tip. Differentially expressed microRNAs were identified in the regenerating lizard tail, including known regulators of stem cell proliferation. The identification of 3 novel microRNAs suggests that regulatory networks, either conserved in vertebrates and previously uncharacterized or specific to lizards, are involved in regeneration. These findings suggest that differential regulation of microRNAs may play a role in coordinating the timing and expression of hundreds of genes involved in regeneration.

Background

Among amniotes, while mammals and birds display only limited capacity for regeneration in the adult, lizards retain the ability to regrow their tails, including the formation of multiple tissues such as spinal cord, skeletal muscle, vasculature, cartilage, and skin, throughout their lives (Chapter 2; Fernando et al., 2011; Han et al., 2008; Kusumi & Fisher, 2012; Porrello et al., 2011; Stocum & Cameron, 2011). Transcriptomic analysis of the green anole lizard, *A. carolinensis*, revealed the differential expression of genes involved in wound response, hormonal response, and musculoskeletal development as well as the Wnt and MAPK/FGF pathways (Chapter 2). While many orthologous genes can be identified between the genomes of the green anole versus mouse and human (Kusumi et al., 2011), a key question about the evolution of regeneration in vertebrates focuses on what genetic changes are responsible for lizards retaining their regenerative capacity and mammals and birds losing this ability.

Changes in the coding or cis-regulatory sequences of multiple individual genes could account for the differential capacity for regeneration within vertebrates. However, given the large number of genes regulating this process, regulators of multiple genes may be involved. MicroRNAs can modulate the expression levels of large numbers of genes, and divergent microRNA regulation could contribute to differences in regeneration between reptilian and mammalian vertebrates. MicroRNAs are highly conserved across metazoa (Wheeler et al., 2009) and play critical roles in regulating a variety of biological processes, including proliferation and differentiation of neurons and cardiac and skeletal muscle tissue during development (Stefani & Slack, 2008), hematopoietic and embryonic stem cell differentiation (Pourrajab, Babaei Zarch, BaghiYazdi, Hekmatimoghaddam, & Zare-Khormizi, 2014; Undi, Kandi, & Gutti, 2013), and T-cell development, maturation, differentiation, and activation (Kroesen, Teteloshvili, &

Czepiel, 2015). MicroRNAs also play a key role in regulating muscle development and repair, which has been extensively studied in mouse and other model systems (Williams, Liu, van Rooij, & Olson, 2009). The role of microRNA regulation in adult regeneration is an active area of research in vertebrate models.

The expression of microRNAs during development and regeneration has been investigated in amphibians (including the axolotl, the newt, *Xenopus* adult and tadpoles) and in teleosts such as the zebrafish. In the axolotl, microRNAs regulate limb and tail regeneration (Holman, Campbell, Hines, & Crews, 2012; Sehm, Sachse, Frenzel, & Echeverri, 2009). In the newt, distinct sets of microRNAs, specifically the let-7 family, are expressed during lens and inner ear hair cell regeneration (Nakamura et al., 2010; Tsonis et al., 2007). In zebrafish, microRNAs play an important role in heart, spinal cord, and caudal tail fin regeneration (Thatcher, Paydar, Anderson, & Patton, 2008; Yin et al., 2008; Yu et al., 2011).

MicroRNAs from whole animal for the green anole lizard have been reported (Lyson et al., 2012), but no studies have been carried out to identify microRNAs in tail regeneration of any lizard species. To investigate the role of microRNAs in lizard regeneration, we performed deep sequencing of RNA smaller than 100 bp. We targeted our analysis on microRNAs from two distinct regeneration tail tissues, the growing tip and base, which yielded differentially expressed transcripts on total RNA transcriptomic analysis (Chapter 2). MicroRNA profiles from adult brain and skeletal muscle were assayed to help in annotation of small RNAs. From this sequencing data and subsequent microRNA annotation, we identified differentially expressed microRNAs between the tip and base of the regenerating tail that may play important roles in regulating stem cell proliferation and differentiation during regeneration. Furthermore, we predicted the mRNA targets of lizard microRNAs and correlated their expression with mRNA

expression identified in a previous study (Chapter 2). This study advances our understanding of which post-transcriptional regulators may regulate regenerative capacity in the lizard.

Materials and Methods

Animal care and tissue collection. All animals were collected and maintained according to Institutional Animal Care and Use Committee guidelines at Arizona State University (Protocol Number 12-1247R). Adult *A. carolinensis* lizards were purchased from Charles D. Sullivan, Inc. (Nashville, TN) or Marcus Cantos Reptiles (Fort Myers, FL) and housed as described previously (Eckalbar et al., 2013). Autotomy was induced by firmly holding a point on the tail 5 cm from the base, while the lizard was otherwise allowed to move on a flat surface. Regenerated tails were then collected 25 days post autotomy (dpa). 25 dpa regenerating tails were cut into three sections each, representing the base, middle, and tip of the regenerating tail. For microRNA isolation, three tip and base sections were respectively pooled, leading to three replicates each containing three pooled tail samples for each tip and base tissue sample. Brain and muscle tissues were collected from lizards immediately following euthanasia.

microRNA sequencing and annotation. Small RNAs were extracted from lizard tissues, including 25 dpa regenerating tail base (n=3) and tip (n=3), brain (n=1), and skeletal muscle (n=1), following the miRVana kit protocol (Ambion). Small RNAs were then barcoded for multiplexed sequencing on two Illumina GAIIx lanes, generating single end 40 base pair reads, and raw sequencing reads from the resulting small RNA libraries were demultiplexed through services provided by LC Sciences. Using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), the adapters used for sequencing (TGGAATTCTCGGGTGCCAAGG) were trimmed from the demultiplexed

reads while keeping only reads 18bp or greater, and trimmed reads were quality filtered by removing all sequencing reads with less than 80% of the bases with at least a Q20 Illumina quality score. The resulting adapter trimmed and quality filtered reads for each for the samples were then mapped to the AnoCar2.0 repeat masked genome available from Ensembl (Ensembl Build 67; Alföldi et al., 2011) using the miRDeep2 package (Friedländer et al., 2008; Friedländer, Mackowiak, Li, Chen, & Rajewsky, 2012) mapper.pl script with the following options: d, e, h, i, j, m. This generated a collapsed set of non-redundant reads while retaining read counts along with the genomic location of the mapped reads. miRDeep2 was then used to annotate novel microRNAs in *A. carolinensis*, as well as validate predicted microRNAs from miRBase. Specifically, (1) mapped reads generated by the mapper.pl script, (2) miRBase predicted microRNAs for *A. carolinensis* (Kozomara & Griffiths-Jones, 2010; 2013), and (3) the miRBase microRNA sequence datasets for human, mouse, chicken, frog, and zebrafish were all passed through the miRDeep2.pl script (Friedländer et al., 2008; 2012). Novel microRNA genes predicted by miRDeep2 are assigned a score based on read support and secondary structures consistent with the biogenesis of microRNAs. Novel microRNAs predicted by miRDeep2 were retained for further analysis if they had a miRDeep2 score of 5 or above, corresponding to an estimated false discovery rate of 6%.

Statistical analysis of microRNA expression. To determine miRNA expression levels, the set of collapsed, non-redundant reads from the mapper.pl miRDeep2 script were first aligned to the miRBase microRNAs and novel microRNAs predicted by miRDeep2 using the quantifier.pl script as part of the miRDeep2 package. This step produced a raw counts file that was then used as input into the DESeq R/Bioconductor package for further statistical analysis (Anders et al., 2013; Anders & Huber, 2010). Differential expression tests in DESeq (adjusted $p < 0.05$) were conducted

only for miRNA genes with at least 10 reads of support in each of the samples being tested, using the following parameters: `fitType="local"` and `sharingMode="fit-only"`.

MicroRNA target prediction. The mRNA targets of the known miRBase and novel microRNAs were predicted using RNAhybrid and miRanda against 3' UTR sequences extracted from the ASU_Acar_v2.2.0 gene annotation (Betel, Koppal, Agius, Sander, & Leslie, 2010; Eckalbar et al., 2013; Enright et al., 2003; John et al., 2004; Krüger & Rehmsmeier, 2006; Landgraf et al., 2007; Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004). The RNAhybrid prediction first calibrates the location and scale parameters of the extreme value distribution for each microRNA by using the RNAcalibrate tool against the 3' UTR sequences in order to improve the p-value calculations for each target prediction for each specific microRNA. These calibrated parameters were then used as input for the d-option for the final RNAhybrid prediction step. Additionally, the minimum free energy parameter was set to -20 kcal/mol with a p-value ≤ 0.01. The set of miRanda microRNA target predictions was generated by setting the minimum free energy to -20 kcal/mol and requiring no mismatch in the seed region. Only overlapping miRNA target predictions from both RNAhybrid and miRanda were retained. Additionally, microRNA targets were filtered for transcripts that were the target of two or more microRNAs.

Comparison of microRNA expression and mRNA target expression. Expression of microRNAs in the regenerating tail was compared to the expression of their mRNA targets, with a cut-off of 2-fold change between the tip and base of the regenerating tail. DESeq was used to determine the expression levels of the known and novel microRNAs as outlined above, while corresponding transcript expression levels were determined previously (Chapter 2). Transcript-microRNAs interactions were then

filtered for co-expression of both the microRNA and mRNA in either the tip or base of the regenerating tail. All one or greater DESeq normalized values for expression of microRNAs were retained. Similarly, transcripts were required to have at least a Cufflinks estimated FPKM of 1 or greater in at least one section of the regenerating tail to be retained for further analysis.

Availability of supporting data. All microRNA raw sequencing data is available from the NCBI Short Read Archive/NIH Bioproject accession number PRJNA278692.

Results

Identification of microRNAs in the regenerating lizard tail. In tail regeneration in the green anole lizard, there is rapid outgrowth at 25 days post autotomy (dpa). We collected nine regenerating tails at this 25 dpa stage and dissected and pooled tissue from the tip and base to obtain sufficient RNA for sequencing (n=3 per pool; 3 pools as biological replicates) (Fig. 7A-B; Table 4). These regenerating tail tissues and stages corresponded to our previous RNA-Seq gene expression analysis, permitting comparison of microRNA and mRNA levels (Chapter 2). The 326 differentially expressed genes identified in our previous study clustered into two groups characterized by elevated gene expression in the regenerating tail tip or base (Chapter 2). Therefore, we sought to identify microRNAs in these tissues that could regulate the regenerative process. To aid in annotating novel microRNAs and confirm the presence of previously identified microRNAs in the green anole, we sequenced microRNAs in adult skeletal muscle and brain, which represent component tissues of the regenerating tail (muscle and central nervous system). Annotation was carried out using mirDeep2 (Friedländer et al., 2008; 2012), a tool designed to identify known and novel microRNAs from small-

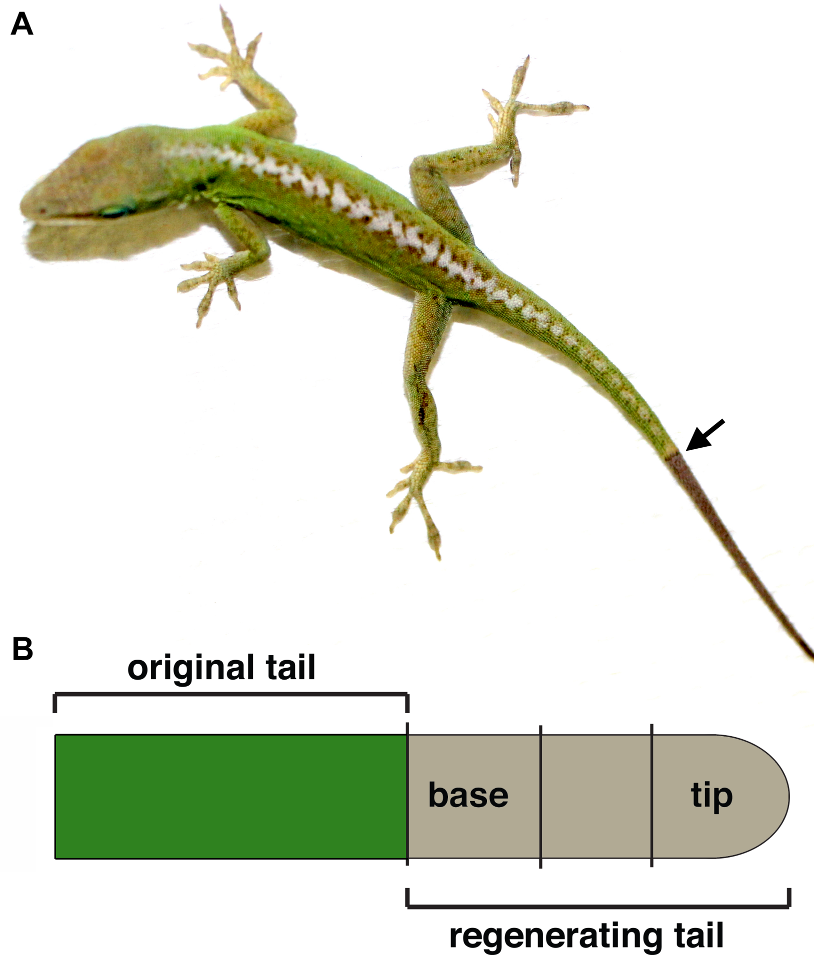

Figure 7. Experimental design of microRNA analysis of lizard tail regeneration. **A**: Image of a green anole lizard with a fully regenerated tail (arrow at break point). **B**: A 25 dpa regenerating tail was divided into three equal sized segments, with the distal regenerating tip and proximal regenerating base collected for microRNA analysis (3 pools, n=3 per pool).

Table 4

microRNA sequencing read summary

Sample	Number of Reads				
	Sequenced	Adapter Trimmed	Quality Filtered	Unique	Mapped to Anocar2.0
ALL TISSUES	58,931,365	51,635,802	48,210,322	1,704,571	642,584
Regenerating Tail Tip (replicate 1)	6,896,312	4,911,787	4,638,573	267,572	80,047
Regenerating Tail Tip (replicate 2)	8,771,826	7,690,607	7,073,991	213,808	67,955
Regenerating Tail Tip (replicate 3)	8,738,345	8,054,177	7,339,012	205,521	84,089
Regenerating Tail Base (replicate 1)	6,905,196	6,084,203	5,763,610	317,605	134,040
Regenerating Tail Base (replicate 2)	9,398,842	8,815,680	8,181,644	245,564	87,691
Regenerating Tail Base (replicate 3)	5,898,914	5,514,428	5,107,890	157,094	62,711
Adult Skeletal Muscle	3,510,208	2,890,930	2,744,587	124,822	48,387
Adult Whole Brain	8,811,722	7,673,990	7,361,015	172,585	77,664

RNA sequencing together with the miRBase database of published microRNAs (Kozomara & Griffiths-Jones, 2010; 2013). Our mirDeep2 analysis identified a total of 546 precursor microRNA families using a miRDeep2 score of 5 (corresponding to a true positive rate of 94 ± 1%) for novel microRNAs (Figure 8). This compares to 282 microRNA anole precursor families already identified in miRBase (Lyson et al., 2012). Of the 546 precursor microRNA families that we identified from regenerating tail, brain, and skeletal muscle, 196 of these precursors were also present in miRBase. The remaining 350 microRNA precursor families were identified by miRDeep2 as potentially novel. Of these, 215 are most likely orthologs of microRNAs found in other systems, displaying either 100% seed identity or a reciprocal BLAST hit to vertebrate microRNA precursors found in miRBase. This left 135 microRNA precursor families with no currently known ortholog based on sequence alone (Kozomara & Griffiths-Jones, 2010; 2013). Analysis of synteny conservation of these microRNA precursors did not identify any clear orthologues in the mouse or human based on genomic location.

Tissue specific patterns of microRNA gene expression. Altogether, 12 microRNAs are uniquely expressed in the regenerating tail base compared to only three anole microRNAs identified in the regenerating tail tip (Figure 8). Mostly highly expressed microRNAs in regenerating tissue are expressed in both the tip and the base of the regenerating tail (Table 5). While most microRNAs are shared amongst tissues, the brain displayed the largest number of unique microRNAs (Figure 8). 489 microRNAs were expressed in brain, 340 are expressed in skeletal muscle, and 473 were expressed in regenerating tail tissue. Highly expressed microRNAs in the brain include a number of regulators of neuronal development and differentiation such as miR-124a, miR-124b, miR-9, and miR-26 (Table 6) (Cao, Pfaff, & Gage, 2007; Caputo et al., 2011; Dill, Linder, Fehr, & Fischer, 2012; Maiorano & Mallamaci, 2009; Makeyev, Zhang, Carrasco, &

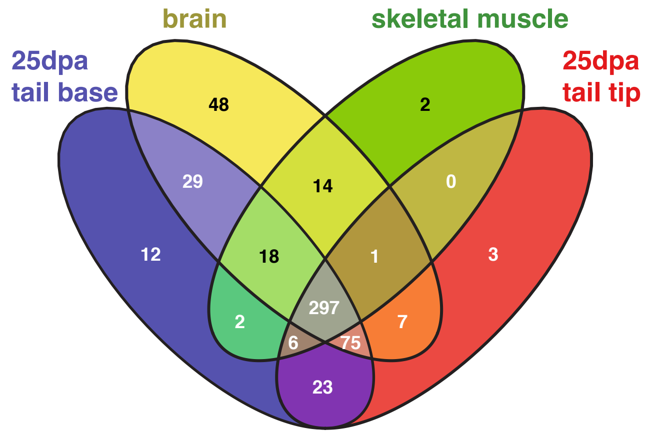

Figure 8. Distribution of microRNAs expressed in the brain, skeletal muscle, and 25 dpa regenerating tail tip and base (minimum count of 1).

Table 5

Highly expressed microRNAs in regenerating tail tip and base (DESeq normalized counts)

microRNA precursor	tip expression	microRNA precursor	base expression
aca-miR-21	212122	aca-miR-21	187018
aca-miR-10b	78808	aca-miR-199b	60669
aca-miR-27b	67317	aca-miR-27b	56981
aca-miR-199b	65229	aca-miR-199a-2	55663
aca-miR-199a-2	29690	aca-miR-199a-1	55631
aca-miR-199a-1	29657	aca-miR-10b	55446
aca-miR-203	29477	aca-miR-99b	34538
aca-miR-26-2	28893	aca-miR-26-2	32110
aca-miR-26-1	28813	aca-miR-26-1	32022
aca-miR-99b	20212	aca-miR-203	25853
aca-miR-10a	18919	aca-let-7a	17446
aca-miR-205a	16783	aca-miR-10a	16705
aca-let-7f-1	16737	aca-let-7f-1	16311
aca-let-7a	16118	aca-miR-1a-1	16175
aca-let-7f-2	15753	aca-miR-1a-2	16150
aca-miR-181a-3	14394	aca-let-7f-2	15625
aca-miR-181a-2	14348	aca-miR-140	14167
aca-miR-181a-1	14347	aca-miR-148a	11433
aca-let-7e	10906	aca-let-7e	11273
aca-miR-148a	10513	aca-let-7c-1	10238

Table 6

Highly expressed microRNAs in brain and skeletal muscle (DESeq normalized counts)

microRNA precursor	brain expression	microRNA precursor	muscle expression
aca-miR-124b	96714	aca-miR-1a-1	144296
aca-miR-125b-1	75541	aca-miR-1a-2	144242
aca-miR-125b-2	73617	aca-miR-133a-1	55682
aca-miR-99b	64863	aca-miR-133a-2	55682
aca-miR-26-2	43364	aca-miR-26-2	43039
aca-miR-26-1	43234	aca-miR-26-1	42941
aca-miR-125a	41711	aca-miR-21	33124
aca-miR-124a-2	39123	aca-miR-99b	28191
aca-miR-124a-1	39122	aca-miR-124b	26041
aca-miR-124a-3	39122	aca-miR-125b-1	23844
aca-miR-100	30873	aca-miR-27b	23384
aca-miR-9-3	22674	aca-miR-125b-2	23191
aca-miR-9-1	22665	aca-miR-143	16657
aca-miR-9-2	22665	aca-miR-99a	12634
aca-let-7c-1	21340	aca-miR-125a	12331
aca-let-7c-2	21340	aca-miR-124a-2	11107
aca-miR-99a	20749	aca-miR-124a-1	11107
aca-let-7a	19748	aca-miR-124a-3	11107
aca-miR-27b	16237	aca-miR-100	10673
aca-miR-181a-3	12498	aca-miR-451	10081

Maniatis, 2007; Yoo, Staahl, Chen, & Crabtree, 2009). miR-124a, miR-9, and miR-181a specifically are some of the most abundant microRNAs expressed in the vertebrate central nervous system (Coolen, Katz, & Bally-Cuif, 2013; Miska et al., 2004; Sanuki et al., 2011)(Sanuki et al., 2011; Coolen et al., 2013; Miska et al., 2004). Highly expressed microRNAs in the skeletal muscle include the muscle specific microRNAs, or myomiRs, miR-1 and miR-133a (Y. Chen, 2006; Mccarthy & Esser, 2006), along with miR-26, miR-125b, and miR-27 all of which are involved in myogenesis and skeletal muscle repair (Table 6) (Dey, Gagan, Yan, & Dutta, 2012; Y. Ge, Sun, & Chen, 2011; Lozano-Velasco & Galiano-Torres, 2014; Sjogren, Egan, Katayama, Zierath, & Krook, 2014). Having identified the tissue specificity of the identified microRNAs, we focused on differential expression within the regenerating tail.

Differential expression analysis of regenerating tail microRNAs and coordinated expression with mRNAs. Small-RNA sequencing of the 25 dpa regenerating lizard tail tip and base identified the expression of 546 microRNAs. In general, most of the microRNAs were highly correlated between these two tissues, with only 11 differentially expressed microRNAs (Figure 9A; adjusted $p < 0.05$). The impact of differential expression of 11 microRNAs is of course amplified by a larger number of predicted target genes (Table 7) (Bartel, 2009).

The differentially expressed microRNAs could be clustered into four groups, where many microRNAs up-regulated in the base share high levels of expression with skeletal muscle (Figure 9B). Nine of these microRNAs have elevated expression in the tail base, including miR-1, miR-133a, miR-133b, and miR-206, which have been shown to play key roles in regulating skeletal muscle differentiation and function (D. M. Anderson et al., 2006; Y. Chen, 2006; H. K. Kim, 2006; M. Koning, Werker, van der Schaft, Bank, & Harmsen, 2012; Koutsoulidou, Mastroyiannopoulos, Furling, Uney, &

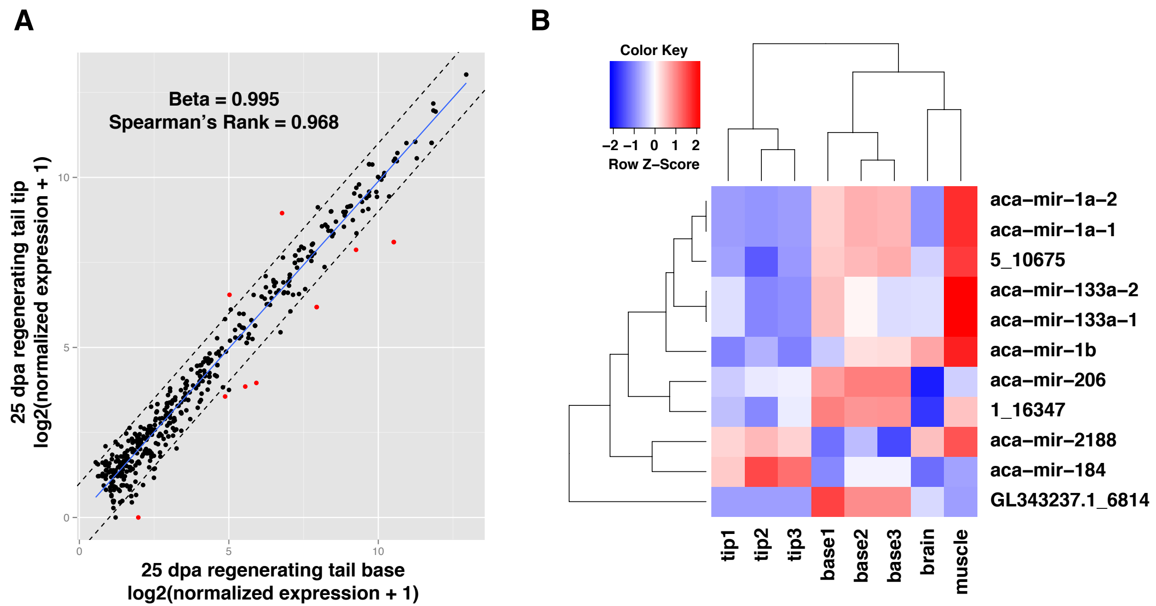

Figure 9. Differential expression of microRNAs in the 25 dpa regenerating lizard tail. **A:** Regression of normalized microRNA expression in the regenerating tail tip and base (Beta-value = 0.995, Spearman's Rank = 0.968). Each point on the graph represents a microRNA. Dashed lines represent the cutoff for two-fold change. Differentially expressed microRNAs, i.e., displayed significant expression differences as determined by DESeq ($p < 0.05$) are represented in red. **B:** Heatmap of differentially expressed microRNAs. Expression in each replicate is shown. MicroRNAs were clustered by Jensen-Shannon divergence of DESeq variance stabilization transformed expression data.

Table 7

Predicted mRNA targets of up-regulated, differentially expressed microRNAs (orthologous and novel)

Regenerating tissue source	Up-regulated, differentially expressed microRNA	Predicted gene targets
tail tip	aca-miR-184	*adprhl2*, *anpep*, *ar*, *b3gnt1*, *bai2*, *ccar1*, *ccdc50*, *cdkn2c*, *cox4i2*, *depdc5*, *fam160a2*, *gpi*, *h6pd*, *kars*, *limk1*, *me1*, *ncan*, *nek6*, *oxnad1*, *pcsk4*, *pdia3*, *pdpk1*, *phlda1*, *ring1*, *rtn2*, *slc30a2*, *slc43a1*, *suclg1*, *sugp2*, *tgfb1*, *tmem214*, *usp21*, *xxylt1*, G.11044, G.14682, G.15668, G.19327, G.19921, G.20484, G.21669, G.21917, G.21923, G.22240, G.22349, G.22365, G.22632
tail tip	aca-miR-2188	*aaas*, *b3gnt7*, *crtac1*, *grm7*, *hsf1*, *hsp90b1*, *itpkb*, *itsn1*, *mta1*, *mylk2*, *nmnat3*, *pou2f2*, *tpd52*, *zbtb45*, G.1698, G.6382
tail base	1_16347	*abcf3*, *adhfe1*, *amn1*, *c11orf35*, *cep76*, *chrna4*, *ddit4*, *dmtn*, *dpy30*, *dpysl3*, *fam57a*, *ikzf3*, *jarid2*, *lrrc4b*, *moxd1*, *mtpap*, *nmnat2*, *pfdn4*, *rps6kl1*, *scarf2*, *smpd2*, *sox13*, *sws2*, *tlk2*, *tpt1*, *tpx2*, *trmt1*, G.11229, G.11992, G.14528, G.16037, G.19728, G.19926, G.4056
tail base	5_10675	*adam33*, *ap1b1*, *arhgef33*, *ccdc104*, *efcab4a*, *fermt2*, *ggt1*, *klhl38*, *ncoa4*, *nkd1*, *pipox*, *plxna4*, *ppfia4*, *psmc6*, *psmf1*, *rgs18*, *sall1*, *sdf2l1*, *traf3ip3*, *trim65*, *txlna*, *zfyve1*, G.11978, G.20962, G.21441, G.4400
tail base	aca-miR-1b	*efhd1*, *irak4*, *sema4c*, *slain2*, *snai2*, *tktl1*, G.22875, G.9382
tail base	aca-miR-206	*ankrd17*, *c5orf30*, *cd44*, *cep192*, *chrac1*, *gbe1*, *notch3*, *poldip3*, G.14293, G.4173, G.9382
tail base	GL343237.1_6814	*ddb2*, *elmsan1*, *irf7*, *kank4*, *kifap3*, *klhdc3*, *ldb2*, *map1lc3b*, *nfia*, *orc4*, *ppp1r9b*, *ptprh*, *secisbp2*, *swap70*, *vash2*, *znf385c*, G.12700, G.2381, G.3078, G.4859
tail base	aca-miR-1a-1; aca-miR-1a-2	*ankrd17*, *efhd1*, *gbe1*, *ikbkap*, *irak4*, *pdgfa*, *sema4c*, *slain2*, *snai2*, *tktl1*, G.14293, G.9382
tail base	aca-miR-133a-1; aca-miR-133b	*abcf3*, *adhfe1*, *amn1*, *arhgdia*, *c10orf12*, *c11orf35*, *cacna1b*, *cep76*, *cfdp1*, *chrna4*, *col1a1*, *creld1*, *ddit4*, *dmtn*, *dpy30*, *dpysl3*, *fam57a*, *gria1*, *gtpbp1*, *ikzf3*, *lrrc4b*, *moxd1*, *mtpap*, *nmnat2*, *pfdn4*, *ppapdc2*, *rps6kl1*, *scarf2*, *smpd2*, *sox13*, *tm2d3*, *tpt1*, *tpx2*, *trmt1*, *vcp*, G.10949, G.11229, G.11992, G.14528, G.16037, G.19284, G.19728, G.19926, G.3656, G.4056, G.5104

Phylactou, 2011; van Rooij, Liu, & Olson, 2008). In zebrafish, the miR-133 precursor family regulates regeneration in the tail fin (Yin et al., 2008), the heart (Yin, Lepilina, Smith, & Poss, 2012), and spinal cord (Yu et al., 2011). In mice, miR-1 and miR-206 regulate satellite cell proliferation via repression of *Pax7* translation, thereby promoting myotube formation (J. F. Chen et al., 2010; R. Koning et al., 2008). miR-184, which is differentially expressed in the regenerating tail tip, regulates proliferation and differentiation of neural stem cells (C. Liu et al., 2010). Of the 11 differentially expressed microRNAs, three were predicted by miRdeep2 analysis as novel, indicating that an ortholog could not be identified based on sequence.

Predicted targets of these novel microRNAs are listed in Table 7. A number of genes predicted to be targeted by the three novel microRNAs are involved in mitosis and cell cycle control, including antagonist of mitotic exit network 1 homolog (*amn1*), centrosomal protein 76kDa (*cep76*), jumonji, AT rich interactive domain 2 (*jarid2*), leucine rich repeat containing 4B (*lrrc4b*), origin recognition complex, subunit 4 (*orc4*), protein phosphatase 1, regulatory subunit 9B (*ppp1r9b*), proteasome macropain 26S subunit ATPase 6 (*psmc6*), proteasome macropain inhibitor subunit 1 (*psmf1*), tousled-like kinase 2 (*tlk2*), tumor protein translationally-controlled 1 (*tpt1*), and the microtubule-associated gene *tpx2*. In addition, a number of genes involved in neurogenesis or synapse formation were targets, including cholinergic receptor, nicotinic, alpha 4 (*chrna4*), dihydropyrimidinase-like 3 (*dpysl3*), plexin A4 (*plxna4*), sphingomyelin phosphodiesterase 2 neutral membrane (*smpd2*), and EF-hand domain family member D1 (*sws2/efhd1*). Finally, Wnt pathways members fermitin family member 2 (*fermt2*), *n*aked cuticle homolog 1 (*nkd1*), and spalt-like transcription factor 1 (*sall1*) were among the predicted targets. Given the cell proliferation and tissue

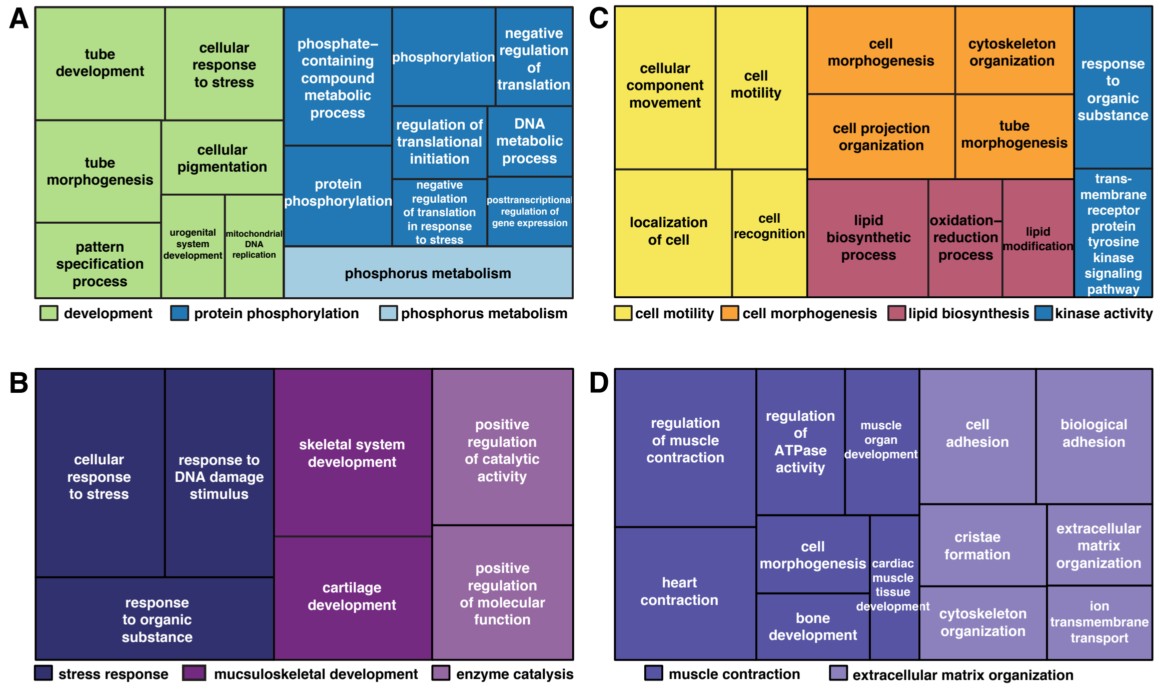

Figure 10. microRNAs and their co-expressed mRNA targets in the 25 dpa regenerating lizard tail. **A–B:** A treemap overview of significant ($p < 0.05$) Gene Ontology Biological Processes for down-regulated microRNAs and their up-regulated mRNA targets in the 25 dpa regenerating tail tip (A) and regenerating tail base (B). **C–D**: A treemap overview of significant ($p < 0.05$) Gene Ontology Biological Processes for up-regulated microRNAs and their up-regulated mRNA targets in the 25 dpa regenerating tail tip (C) and regenerating tail base (D). The relative sizes of the treemap boxes are based on the |log10(p-value)| of the respective GO term. Related terms are visualized with the same color, with the representative category for each color group denoted in the legend.

formation taking place within the regenerating tail base, these novel microRNAs may play a key role in regulating the regenerative process.

We have previously shown that there are at least 326 differentially expressed genes in the regenerating lizard tail, including genes in the Wnt and FGF/MAPK pathways as well as those involved in wound repair, hormonal regulation, and musculoskeletal development. We identified co-expressed microRNA/target mRNA pairs that both have at least 2-fold change in expression between the regenerating tail tip and base. DAVID analysis using Gene Ontology Biological Processes was used to categorize the microRNAs and their co-expressed target transcripts (D. W. Huang, Sherman, & Lempicki, 2009a; 2009b). Of particular interest are coordinated profiles of expression where the microRNA changes reinforce the mRNA gene expression, i.e., microRNAs levels are decreased where the expression of their mRNA targets are increased, as these could represent post-transcriptional microRNA repression (Figure 10A-B). Additionally, the group of highly expressed mRNA genes whose regulatory microRNAs are also up-regulated in the corresponding tissue are of interest as they could represent translational microRNA repression (Figure 10C-D).

In the regenerating tail tip, genes involved in phosphorus metabolism, phosphorylation, development of tubular structures, cell motility, cell morphogenesis, lipid biosynthesis, and kinase activity are highly expressed. This would be expected in the regenerating tail tip, where organization of structures with epithelial cell organization such as the vasculature, ependymal, and cartilage tube would require active signal transduction via phosphorylation (Figure 10A; 10C). MicroRNAs that reinforce this pattern of expression are all novel. In the regenerating tail base, genes involved in musculoskeletal development, enzyme catalysis, response to organic substances, muscle contraction, and extracellular matrix organization display increased expression, as might

be expected in differentiating skeletal muscle and cartilage present in that tissue (Figure 10B; 10D). MicroRNAs that reinforce this pattern of expression included many novel microRNAs, including two of the differentially expressed novel microRNAs (5_10675 and 1_16347), as well as let-7b, which is regulates neural stem cell proliferation and is additionally expressed during lens regeneration in the newt (Nakamura et al., 2010; Tsonis et al., 2007; C. Zhao et al., 2010).

Discussion

This study describes the first microRNA transcriptome analysis of regeneration in the green anole. We identified 546 microRNA precursor families from regenerating tail and adult brain and skeletal muscle, with 411 microRNAs orthologous to families in other vertebrate species. Given previous analysis finding a distributed pattern of cell proliferation throughout the regenerating green anole tail (Chapter 2), we did not expect that collecting the tail tip would enrich for a focal region of stem cell proliferation or reveal a gradient of differentiation. The cellular organization at the tail tip differs from the base in being enriched for forming vasculature, growing ependyma, and coalescing cartilage tube. Differentially expressed mRNAs and microRNAs both reflect this enrichment for those tissues in the tail tip. Conversely, the regenerating tail base is enriched for differentiating skeletal muscle groups, and this pattern was observed in mRNA and microRNA expression. The identification of only 11 differentially expressed microRNAs in the regenerating lizard tail reinforces the finding that progenitor/stem cell proliferation and differentiation are taking place in regions across the regenerating tail (Chapter 2). The finding that there are 3 novel microRNAs are differentially expressed in the regenerating tail is intriguing. However, these novel microRNAs likely have

homologues in other vertebrates, but the lack of reptilian genomes and microRNAs sequenced to date limits our ability to clearly identify orthology.

Though microRNA target prediction is a useful tool, prediction algorithms often have varying target lists, and their false positive and false negative rates are difficult to assess (Min & Yoon, 2010; Pasquinelli, 2012). While comparing microRNA expression with the expression of its mRNA target helps resolve and identify microRNA/mRNA target pairs for further analysis, it would be beneficial to further verify these genes for downstream analyses. Since microRNAs are an example of post-transcriptional regulation, the addition of proteomic data would provide a unique insight into verification of microRNA targets. Specifically, proteomic data would help assess whether certain microRNAs act at the post-transcriptional a translational level, namely aiding in differentiation of translational regulators versus false positives in cases where a microRNA target is upregulated.

Given that most microRNA precursor families expressed in lizard tail regeneration have identified orthologues in other vertebrates, comparison with microRNAs identified in other regenerative models could be instructive. For example, the small RNA miR-133 is downregulated during heart regeneration and in tip of the regenerating tail in zebrafish (Yin et al., 2012). In the anole, we identified high levels of miR-133a in the regenerating tail base compared to the tail tip. The small RNA miR-184, which is differentially expressed in the tip of anole regenerating tail, has also been identified in zebrafish tail fin regeneration (Thatcher et al., 2008). In addition to regulating neural stem cell proliferation and differentiation, miR-184 targets the RNA-induced silencing complex (RISC) member argonaute2 (C. Liu et al., 2010; Roberts, Warren, Griffiths, & Ross, 2013; Tattikota et al., 2014). During newt lens regeneration, miR-1 and miR-206 regulate cell proliferation (Nakamura et al., 2010). Orthologues of

these two microRNAs are both differentially expressed in the regenerating anole tail base. While previous studies did not identify novel microRNA precursor families specific to regeneration, we identified 3 previously unknown differentially expressed microRNAs in the regenerating tail base. This may reflect the ability of RNA-Seq to identify novel sequences, while microarray analysis that is limited by probe sets included in the arrays. Comparative analysis of the role of microRNAs in vertebrate regeneration would be advanced by further deep sequencing of small RNA populations in other model systems.

Conclusion

Given that microRNAs are able to regulate a large number of genes, it is possible that microRNA regulation the regenerative process can contribute to differences in regenerative capacity among vertebrates. Divergence in vertebrate microRNA regulation could arise by a number of possible models including, i.) the deletion or loss of microRNAs regulating regeneration within the mammalian lineage, ii.) the change in downstream transcripts targeted by microRNAs in the mammalian lineage, and iii.) the emergence of novel reptile-specific microRNAs that promote regeneration. The latter model appears less parsimonious given the conservation of regeneration across vertebrates, including teleosts, amphibians, and amniotes (in lizards). In addition to microRNA-based regulation, genomic changes may of course affect coding genes and non-coding regulatory sites such as enhancers, silencers, and insulators. Further analysis in the lizard and comparison with other regenerative models will allow us to further distinguish between these possibilities.

CHAPTER 4

TRANSCRIPTOMIC ANALYSIS OF EARLY TAIL REGENERATION DEMONSTRATES MECHANISMS REGULATING SCAR-FREE WOUND HEALING

Abstract

Vertebrate appendage regeneration can be described as having two phases. The first phase is characterized by scar-free wound healing and occurs prior to outgrowth. The second phase involves patterning and outgrowth of the regenerating appendage and reactivation of developmental pathways. Common processes in the early stage of regeneration across vertebrates include extracellular matrix remodeling, establishment of positional identity, and wound epithelium formation, though the genetic mechanisms underlying these processes are relatively unknown. Transcriptome sequencing of lizard tails at early, pre-outgrowth stages of regeneration (6 hours, 5 days, and 10 days post autotomy) has identified a number of genes involved in these processes, including matrix metalloproteases, the Wnt/planar cell polarity pathway, pro-inflammatory cytokines, and neurotrophic factors. Additionally, a number of genes, all of which have mammalian orthologs, are significantly expressed immediately preceding outgrowth, as well as at the regenerating tail tip mid-outgrowth (25 dpa). These findings identify genes that may regulate scar-free wound healing and promote regenerative outgrowth during tail regeneration.

Background

The initial stages of scar-free wound healing that precede regenerative outgrowth are an important aspect of regenerative success in vertebrates. Pre-outgrowth stages of appendage regeneration in vertebrates share many common processes. First, there must

be remodeling of the severed tissues before regeneration can occur. The existing extracellular matrix (ECM) at the wound site is destabilized, allowing for a new matrix to be created for newly differentiating cells (Stocum & Cameron, 2011; Yokoyama, 2008). ECM remodeling is theorized to be partially responsible for the upregulation of scar-free wound healing, as opposed to the fibrotic response seen in organisms that do not regenerate (Godwin & Rosenthal, 2014; Vinarsky et al., 2005). Secondly, a wound epithelium must form over the damaged tissue before outgrowth can occur (Campbell & Crews, 2008; Murawala et al., 2012; Takeo et al., 2015), which in addition to covering the wound, acts as a signaling center to promote proliferation and outgrowth during regeneration in the frog, salamander, and zebrafish (R. N. Christensen et al., 2002; Ghosh et al., 2008; Han et al., 2001; Kawakami et al., 2006; Y. Lee et al., 2009; Poss et al., 2000; Yokoyama et al., 2011). Third, though the role of immune response during regeneration is unknown, a number of studies have suggested that inflammation may play a role in the initiation and completion of wound healing and regeneration in vertebrates (Fahmy & Sicard, 2002; Godwin et al., 2013; Godwin & Brockes, 2006; Harty et al., 2003; King, Neff, & Mescher, 2012; Mescher & Neff, 2006). Fourth, positional identity is a crucial process for regenerative success; similarly to development, the new appendage must undergo patterning.

Several additional factors also contribute to vertebrate regeneration. Nerve innervation plays a significant role in regenerative outcomes. In the salamander, lack of signaling from the damaged nerve results in scar-free wound healing without subsequent regeneration (A. Kumar & Brockes, 2012; Satoh et al., 2009; Stocum & Cameron, 2011), and similarly, lack of innervation in *Xenopus* froglet regenerating limbs results in a lack of proliferation and regenerative outgrowth (T. Endo et al., 2000; Korneluk & Anderson, 1982; Suzuki et al., 2005). Also, major developmental pathways are reactivated during

regeneration (Wnt, hedgehog, FGF, IGF, BMP/TGFβ, and MMP), and inhibition of these pathways has shown disruption either during the wound healing, proliferation, or patterning phases of regeneration (Carlson 2011; Stocum 2012). In fact, Wnt signaling can enhance regeneration in *Xenopus* froglets and chick embryos that do not normally regenerate (Kawakami et al., 2006; Yokoyama et al., 2011), indicating the importance of these pathways.

Additionally, macrophages are of interest as they regulate ECM remodeling, fibroblast formation, angiogenesis, and peripheral nerve innervation in wound repair (Barron & Wynn, 2011; Lucas et al., 2010; Martini, Fischer, López-Vales, & David, 2008; Nucera, Biziato, & De Palma, 2011). By controlling the inflammatory response, macrophages directly regulate repair and regeneration (reviewed in Delavary et al., 2011). Cytokines may regulate many genes, including those involved in the cell cycle (Q. Zhang, Sakamoto, & Wagner, 2014), and they regulate proliferation of multiple cell types, namely that of fibroblasts, keratinocytes, and endothelial cells (reviewed in Delavary, van der Veer, & van Egmond, 2011). Additionally, macrophages stimulate the production of soluble effector molecules, including platelet-derived growth factors (PDGFs), insulin-like growth factors (IGFs), hepatocyte growth factors (HGFs), fibroblast growth factors (FGFs), transforming growth factors (TGFs), colony-stimulating factors (CSFs), Wnt ligands, and other molecules related to the immune system (Stefater, Ren, Lang, & Duffield, 2011). In addition to regulating many processes during vertebrate development, such as programmed apoptosis in the mouse brain and chick retina (Frade & Barde, 1998; Marín-Teva et al., 2004), programmed apoptosis and Wnt7b secretion during eye development in the mouse (Lang & Bishop, 1993; Lobov et al., 2005), and neuron survival and neurite process outgrowth in the mouse (Michaelson, Mehler, Xu, Gross, & Kessler, 1996), recent studies have begun to investigate the role of macrophage

regulation in amphibian regeneration. Both pro-inflammatory and anti-inflammatory signals are upregulated simultaneously during regeneration in salamanders and *Xenopus* tadpoles, suggesting that the balance of the inflammatory response is important for successful regenerative outcomes (Godwin et al., 2013; King et al., 2012). Depletion of macrophages in the axolotl prior to limb amputation results in collagen deposition and formation of a thick scar at the limb stump instead of regeneration, while depletion of macrophages during regeneration prior to regenerative outgrowth results in a delay in regeneration (Godwin et al., 2013). This further underlies the importance of elucidating the role of macrophages and the immune/inflammation response in vertebrate regeneration.

Genetic mechanisms for some of these processes in vertebrates are known and others are still being elucidated. Transcriptomic analysis of mid-outgrowth regenerating tail (25 dpa) in the green anole lizard, *Anolis carolinensis*, identified differentially expressed genes related to wound healing, hormonal response, skeletal system development, and the Wnt and MAPK/FGF pathways, however the genetic mechanisms underlying scar-free wound healing in lizards have yet to be identified. Therefore, we sought to characterize the genetic mechanisms involved in lizard tail regeneration in stages preceding outgrowth.

Materials and Methods

Animal care, collection of regenerating tail samples, and RNA Sequencing. All animals were collected and maintained according to Institutional Animal Care and Use Committee guidelines at Arizona State University (Protocol Number 12-1247R). Adult *A. carolinensis* lizards were purchased from Charles D. Sullivan, Inc. (Nashville, TN) or Marcus Cantos Reptiles (Fort Myers, FL) and housed as

described previously (Eckalbar et al., 2013; Chapter 2). Autotomy was induced by firmly holding a point on the tail 5 cm from the base, while the lizard was otherwise allowed to move on a flat surface. Regenerated tails (n=2) were then collected 6 hours, 5 days, and 10 days post autotomy (dpa). Total RNA was isolated from regenerating tail tissue samples, following the miRVana kit protocol (Ambion), and double-stranded cDNA was synthesized using the Ovation RNA-Seq kit (NuGEN). Paired-end sequencing libraries were generated using Illumina manufacturer protocols (TruSeq v3) and sequenced on an Illumina HiSeq 2000.

Bioinformatic analysis. RNA-Seq reads were demultiplexed with the Illumina CASAVA software, followed by adapter trimming and further quality score trimming (QC30) with Trimmomatic (Bolger, Lohse, & Usadel, 2014). Trimmed reads were then aligned and mapped to the *A. carolinensis* genome (Alfoldi et al., 2012) with Bowtie2.1.0 and Tophat2.0.8 (Trapnell et al., 2010; 2012), using the ASU_Acar_v2.2.1 annotation described previously (Eckalbar et al., 2013; Chapter 2). BAM files from Tophat were sorted and converted to SAM format using samtools, and HTSeq 0.6.1 was used to generate count data using coding sequences in the ASU_Acar_v2.2.1 annotation (Anders et al., 2014). Differentially expressed genes were then identified using the default parameters in DESeq2_1.6.3, which includes multiple testing corrections (M. I. Love, Huber, & Anders, 2014), which is part of the Bioconductor (Gentlemen et al., 2004) suite of packages within the R statistical programming environment (http://www.R-project.org). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional analysis tools was used to annotate functional clusters based on Gene Ontology (GO) biological processes and molecular function categories (D. W. Huang, Sherman, & Lempicki, 2009a; P. Huang et al., 2011). For all heatmaps, genes

were clustered by Euclidean distance of mean-centered DESeq2 regularized log2 transformed values.

Results and Discussion

Sequencing and differential expression testing of early regenerative timepoints. In order to identify differentially expressed genes during the early, pre-outgrowth stages of regeneration, we carried out RNA sequencing and analysis on tails at three pre-outgrowth stages: 6 hours post autotomy (hpa), 5 days post autotomy (dpa), and 10 dpa. 91 differentially expressed genes (adjusted $p < 0.05$) were identified between 6 hpa and 5 dpa, 15 of which are highly significant ($p < 0.0001$) (Figure 11A-B; Table 8). There are a number of upregulated genes at 6 hpa involved in an inflammatory immune response, including matrix metalloproteinase 7 (*mmp7*), which establishes a chemokine gradient thereby recruiting neutrophils (Parks, Wilson, & López-Boado, 2004), the proinflammatory cytokine interleukin-8 (*il8*), which mediates neutrophil activation (Baggiolini, Walz, & Kunkel, 1989), chemokine-like receptor 1 (*cmklr1*), which regulates macrophage chemotaxis and promotes phagocytosis of apoptotic cells (Cash, Christian, & Greaves, 2010; Samson et al., 1998; Yoshimura & Oppenheim, 2011), and leukocyte-specific transcript 1 (*lst1*), an immunomodulatory gene that inhibits leukocyte proliferation (Mulcahy, O'Rourke, Adams, Molloy, & O'Gara, 2006; Rollinger-Holzinger et al., 2000). A similar inflammatory immune response is observed in *Xenopus* tadpoles, where *mmp7* is activated as early as 6 hours following amputation (N. R. Love et al., 2011). The significant DAVID functional cluster at this stage contains terms involved in regulation of cell death (Figure 11C; Table 9).

Most of the genes upregulated at 5 dpa have higher expression at 10 dpa (pink bar, Figure 11A), indicating that gene expression may increase over time leading to the

Table 8

Differentially expressed genes at 6 hpa, 5 dpa, and 10 dpa

	0.001 < p < 0.05	0.0001 < p < 0.001	p < 0.0001
6 hpa vs. 5 dpa	**68**	**8**	**15**
upregulated at 6 hpa	*adam28, ca13, cat, cmklr1, cpn2, dennd4a, hbad, hbb-b1, ier3, irg1, lectin, lst1, mmp7, nr4a3, pnp, rfesd, rnase1, sdr16c5, selenbp1, sla, slc25a4, traf1, tnfrsf1b, tubb1, uap1, uox, G.11931, G.16699*	*gpr84, il8, kr13, mcl1, ptgs2*	*bkj, li-ac-x, mmp3, olfm4, selenbp1, serpinb2, G.16704*
upregulated at 5 dpa	*acp5, ccnd1, chit1, ckap2, col6a3, cyp2d20, ddx60, dut, enpp2, fbln1, glis3, gpx7, hist2h2ac, igfbp4, il4i1, knstrn, krt19, li-ac-27, li-ac-30, li-ac-35, li-ac-37, loxl1, lrrc17, lum, mcm6, olfml3, sfrp2, sulf1, tagln3, tgfbi, tmem68, tmem68, top2a, tyms, G.15595, G.20270, G.2702*	*col3a1, mdk,* G.20239	*atp6v0d2, ctsk, cyp2d14, cyp2j2, cyp2j6, li-ac_x, s100a9, tmem68*
5 dpa vs. 10 dpa	**287**	**16**	**30**
upregulated at 5 dpa	*ctsk, dnah2, li-ac-27, li-ac_x, rbp4, tgm3, tmprss_x, traf3ip2, zbed4, zbed4,* G.14133, G.17324, G.21860, G.3533	-	*atp6v0d2*
upregulated at 10 dpa	*actl6a, actr6, acvr1, adam12, adamts2, adk, aebp1, afap1, ak6, akr1d1, alg8, ap2m1, ap3b1, apcdd1, aqpep, arf1, arfgap1, asph, aspn, atp2c1, atp6ap2, bub3, c6orf203, c7orf60, cald1, calm2, calu, cask, cbfb, cbx5, ccnd1, cd34, cdc16, cdh11, cenph, cenpq, chordc1, cisd1, ckap2, cmpk1, col12a1, col5a1, col5a2, col6a1, col6a2, col6a3, col7a1, commd2, crtap, ctps1, cyb5r3, cyp2d14, cyp2j2, cyp2j6, cyth3, dbf4, dcn, dcun1d5, ddx39b, dkc1, dkk3, dlgap5, dnajc2, dsg2, dut, dynlt1, eif4e, emilin2, eml4, entpd5, eny2, epb41l2, epha4, ergic3, eya1, fads2, fam198a, fam198b, farp1, fat1, fbln1, filip1l, fkbp10, flrt2, fnbp1l, frem1, frmd4a, fstl1, galnt5, gid8, gli2, glis3, glo1, golm1, gtf2f1, gulp1, h2afy2, hdac2, hdgfrp3, helb, hells, hist1h1o1, hmgb1, hmgn1, hmgn2, hmgn5, hmmr, hnrnpa2b1, hnrnpk, hspa4l, hspb6, il1r1, il4i1, itm2b, jag2, kdelc1, kiaa1430, kif11, kif15, kif20b, kif26b, knstrn, kpna2, krt14, krt24, l3mbtl3, lamc1, lef1, li-ac-35, lman1, lmnb1, lpar4, lrrc17, lsm3, lsp1, lum, lypd3, maoa, mark1, matr3, mcm6, mettl5, mfge8, mllt3, mmp13, mmp14, mmp2, mnd1, myh10, nasp, nbl1, nbn, ndnf, nfyb, nid1, nucb2, nucb2, nucks1, nup37, odc1, pcdh18, pdcd4, pde3b, pet100, pgam1, phf14, phldb2, phpt1, piezo2, pla2g4b, pla2g4c, plekhh2, plrg1, pltp, ppan, ppap2a, ppil3, ppp1r7, ppp1r9a, ppp2r1a, ppp3r1, prss27, psat1, prc1, psmd12, ptgr1, ptk7, rab14, rad51ap1, rai14, rbfa, rbfox2, rcn3, recql, rhobtb3, rims1, rnmt, rnps1, ror2, rrbp1, rrm2, rtfdc1, runx1, sall4, sarnp, seh1l, sept9, setd7, sh3pxd2a, shoc2, skp1, slc16a14, slc27a2, smarca5, smarcad1, smc2, smc4, sncaip, sned1, sostdc1, sparc, spc24, spc25, spp1, srek1ip1, srp14, srpx, st3gal1, stard9, stmn1, strada, sumo3, surf4, syncrip, tcf4, tctn2, tdg, tgfb2, thumpd3, tm9sf3, tmbim4, tmem263, tmsb15b, tnc, top2a, trmt112, trnaf-gaa, trpc6, ttk, tuba1a, tuba1b, twsg1, u2af2, ube2e2, ublcp1, uchl1, uchl5, uggt2, utp15, vbp1, vrk1, wdr75, whsc1, ywhab, ywhaz, G.13621, G.15595, G.17569, G.20206, G.20270, G.20534, G.9772*	*col1a2, ednra, fap, fkbp9, gpx7, hhipl2, lamb1, ppic, prickle1, pxdn, synpo, tagln3, vcan, wls,* G.18007, G.19196	*adamts9, cdh2, col3a1, dmrt2, fads1, fbln2, fgd5, igfbp4, kera, lama1, li-ac-30, li-ac-37, limch1, mdk, mepe, mylk, ogn, or5v1, pdgfra, pdzrn4, prr16, prss23, pxylp1, s100a9, sall1, st6gal2, sulf1, G.12004, G.4738*

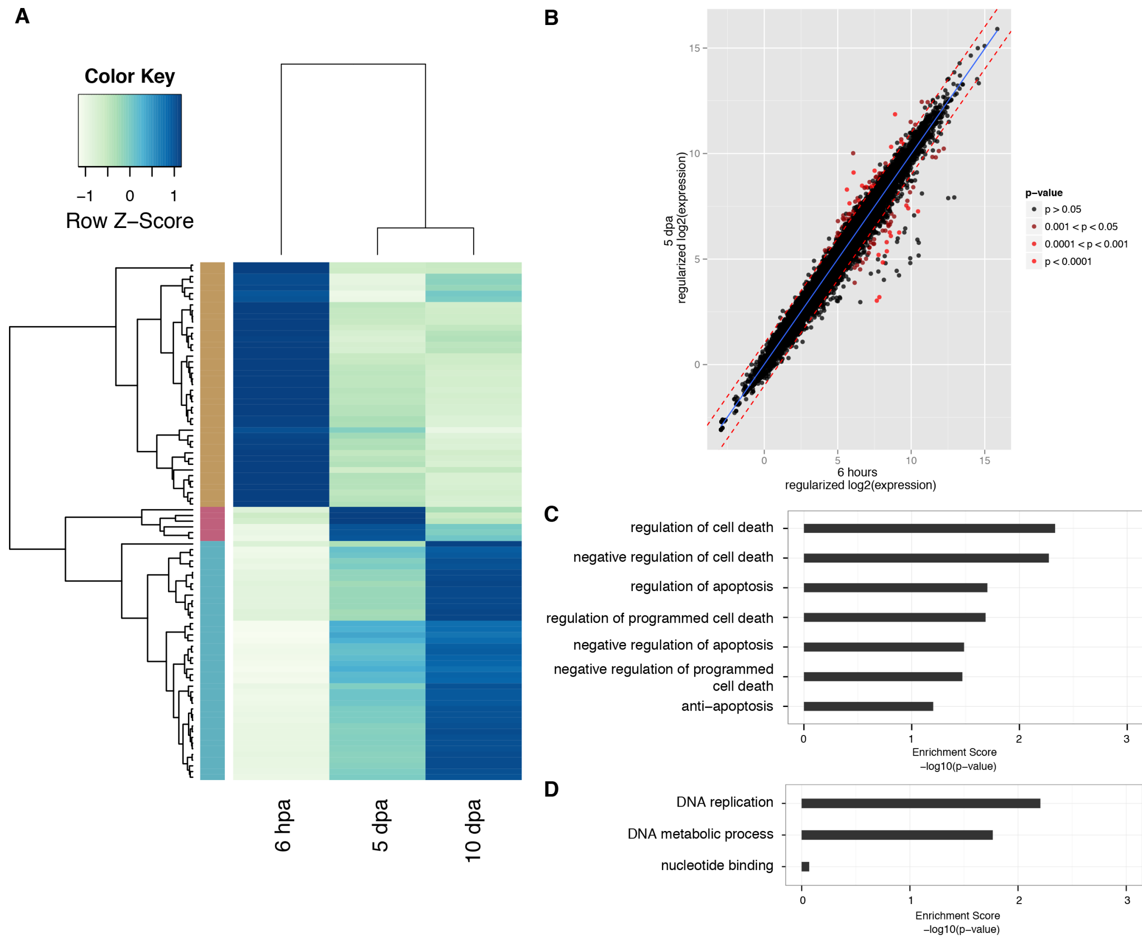

Figure 11. 91 differentially expressed genes were identified between 6 hpa and 5 dpa. (A) Heatmap of differentially expressed genes. (B) Scatterplot of expression between 6 hpa and 5 dpa. Differentially expressed genes (adjusted $p < 0.05$) are red, and the red dashed lines represent 2-fold change. Overall gene expression is highly correlated (Spearman's rank = 0.987). (C) DAVID functional annotation clusters for genes up-regulated at 6 hpa. (D) DAVID functional annotation clusters for genes up-regulated at 5 dpa. Gene ontology biological processes and molecular function categories were used for analysis.

Table 9

DAVID functional gene ontology clusters up-regulated at 6 hpa and 5 dpa

	Annotation Cluster 1	**Enrichment Score: 1.74**		
	Term	Description	P-Value	Genes
6 hpa	GO:0010941	regulation of cell death	0.0047	*traf1, ier3, slc25a4, ptgs2, mcl1, serpinb2, cat*
	GO:0060548	negative regulation of cell death	0.0053	*ier3, slc25a4, mcl1, serpinb2, cat*
	GO:0042981	regulation of apoptosis	0.0198	*traf1, ier3, ptgs2, mcl1, serpinb2, cat*
	GO:0043067	regulation of programmed cell death	0.0206	*traf1, ier3, ptgs2, mcl1, serpinb2, cat*
	GO:0043066	negative regulation of apoptosis	0.0327	*ier3, mcl1, serpinb2, cat*
	GO:0043069	negative regulation of programmed cell death	0.0338	*ier3, mcl1, serpinb2, cat*
	GO:0006916	anti-apoptosis	0.0632	*ier3, mcl1, serpinb2*

	Annotation Cluster 1	**Enrichment Score: 1.35**		
	Term		P-Value	Genes
5 dpa	GO:0006260	DNA replication	0.0062	*tyms, top2a, mcm6, dut*
	GO:0006259	DNA metabolic process	0.0171	*tyms, top2a, igfbp4, mcm6, dut*
	GO:0000166	nucleotide binding	0.8520	*tyms, ddx60, top2a, mcm6*

outgrowth of the regenerating tail. When compared to 10 dpa, only 15 differentially expressed genes were identified at 5 dpa (Figure 12A-B). There are some genes, however, that have high expression at 5 dpa (Figure 11A; 12A). These include cathepsin K (*ctsk*) and acid phosphatase 5, tartrate resistant (*acp5*), which are involved in bone remodeling and resorption (Bossard, Tomaszek, & Thompson, 1996; Oddie et al., 2000), beta-keratins, which form a structural component of reptilian skin (Valle, Nardi, Toffolo, & Niero, 2007), and transglutaminase 3 (*tgm3*), which acts as a protein crosslinker in skin (Kalinin, Marekov, & Steinert, 2001). This is consistent with the ECM remodeling and wound epithelium formation seen pre-outgrowth. Additionally, orthologs of two antimicrobial proteins found in other squamates, omwaprin-a, which was identified in the inland taipan snake, *Oxyuranus microlepidotus*, and has antibacterial properties against gram-positive bacteria (Nair, Fry, Alewood, Kumar, & Kini, 2007), and carwaprin-b, which was identified in the rough-scaled snake, *Tropidechis carinatus* (St Pierre et al., 2008), are significantly expressed at 5 dpa. This is consistent with other studies showing the presence of antimicrobial peptides in the regenerating tail stump 1-6 days following tail loss (Alibardi et al., 2012). The significant DAVID functional cluster at this stage contains terms involved in DNA replication (Figure 11D; Table 9).

333 differentially expressed genes (adjusted $p < 0.05$) were identified between 5 dpa and 10 dpa, 30 of which are highly significant ($p < 0.0001$) (Figure 12A-B; Table 8). Most of these genes are highly expressed at 10 dpa (Figure 2A), a stage that immediately precedes regenerative outgrowth. The top three functional gene ontology clusters identified by DAVID are represented by extracellular matrix organization, mitosis/cell cycle regulation, and skeletal system development (Figure 11C; Table 9). Up-regulation of mitosis is consistent with the high level of proliferation seen at this stage (Figure 13) and is suggestive of stem cell-mediated regeneration. This is in direct contrast with the

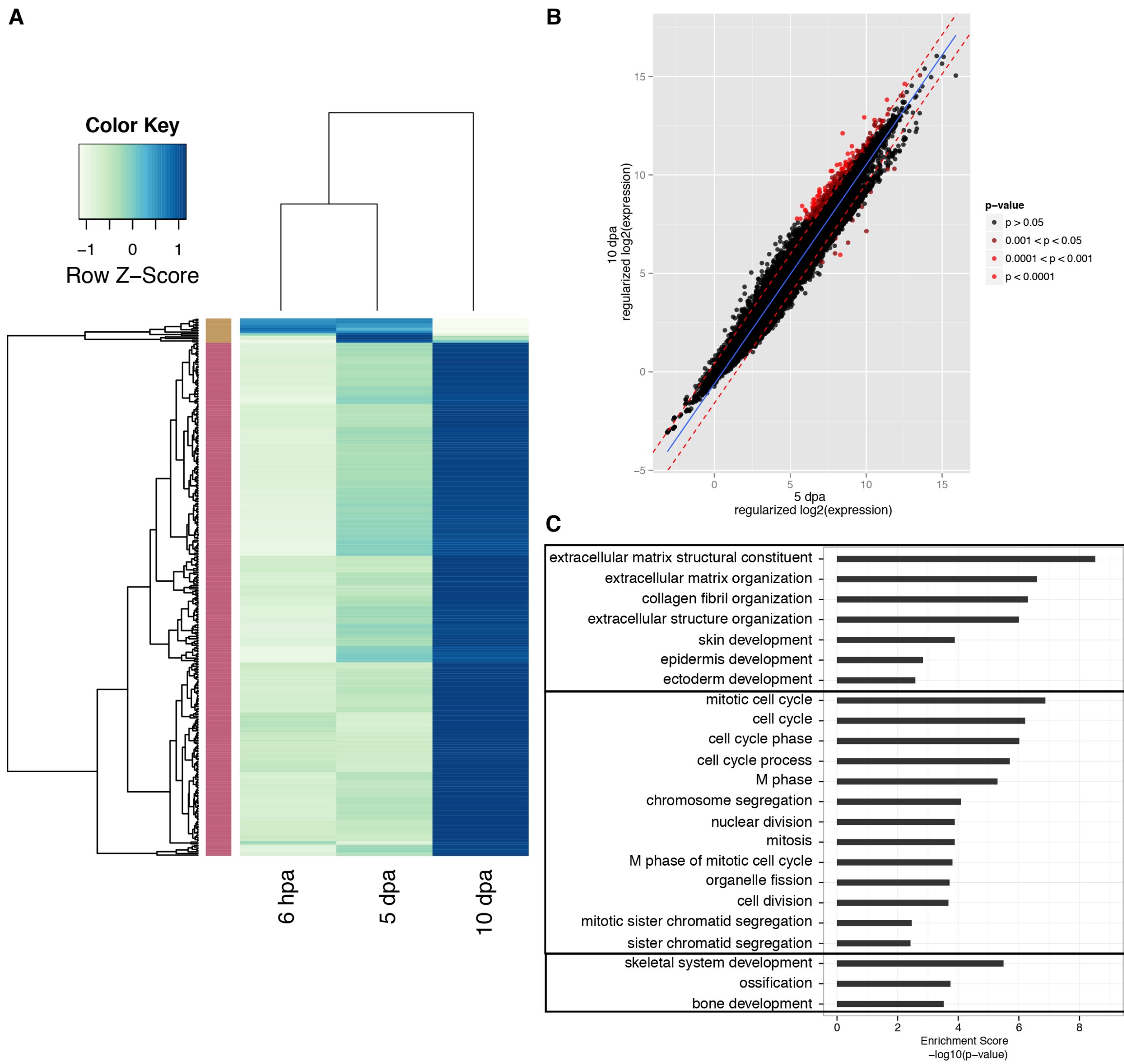

Figure 12. 333 differentially expressed genes were identified between 5 dpa and 10 dpa. (A) Heatmap of differentially expressed genes, most of which are highly expressed at 10 dpa. (B) Scatterplot of expression between 5 dpa and 10 dpa. Differentially expressed genes (adjusted $p < 0.05$) are red, and the red dashed lines represent 2-fold change. Overall gene expression is highly correlated (Spearman's rank = 0.969). (C) DAVID functional annotation clusters for genes up-regulated at 10 dpa. Gene ontology biological processes and molecular function categories were used for analysis.

dedifferentiation model of regeneration seen in salamander; blastema cells enter the cell cycle, but there is relatively low level of mitosis during blastema formation (Kelly & Tassava, 1973; Tassava, Bennett, & Zitnik, 1974). There are also a number of genes involved in blood cell formation and proliferation significantly expressed at this stage, including endothelin family members *ednra* and *edn3*, midkine (*mdk*), runt-related transcription factor 1 (*runx1*), *runx1* regulator *cbfb*, and CD34, which is consistent with the vasculature underlying the wound epidermis that is evident at this stage (Dzierzak & Speck, 2008; Goldie, 1999; Qin et al., 2015; Reynolds et al., 2004; Tracey & Speck, 2000; Chapter 2).

Developmental genes in common with the 25 dpa regenerating tail tip. During appendage regeneration in the frog, salamander, and zebrafish, signaling between the wound epithelium and underlying mesenchyme interact to promote proliferation and outgrowth (R. N. Christensen et al., 2002; Ghosh et al., 2008; Han et al., 2001; Kawakami et al., 2006; Y. Lee et al., 2009; Poss et al., 2000; Yokoyama et al., 2011). Therefore, we sought to characterize genes in the lizard that are upregulated immediately prior to outgrowth (10 dpa) as well as mid-outgrowth (25 dpa) during tail regeneration. We previously identified 129 genes differentially expressed in the 25 dpa regenerating tail tip, and 22 of these are also significantly upregulated at 10 dpa (Chapter 2; Table 11). These genes regulate the wound healing response as well as a number of skeletal system developmental processes, including chondrogenesis and vasculogenesis and represent genes involved in embryonic development. This group of genes includes developmental transcription factors *sall1* and *sall4*, which regulate embryonic stem cell pluripotency (Karantzali et al., 2011; Tanimura, Saito, Ebisuya, Nishida, & Ishikawa, 2013) and have been identified in limb development and regeneration in salamanders and *Xenopus* froglets (A. W. Neff et al., 2005; R. Stewart et al., 2013). Endothelin family

Table 10

DAVID functional gene ontology clusters up-regulated at 10 dpa

Annotation Cluster 1	Enrichment Score: 5.25		
Term	**Description**	**PValue**	**Genes**
GO:0005201	extracellular matrix structural constituent	3.01E-09	*lum, col3a1, mepe, emilin2, col5a2, col5a1, lama1, fbln1, fbln2, col1a2, col6a2, col12a1, lamc1, lamb1*
GO:0030198	extracellular matrix organization	2.52E-07	*lum, col3a1, nid1, dcn, col5a2, col5a1, tgfb2, col6a2, pdgfra, col1a2, col12a1, lamc1, adamts2*
GO:0030199	collagen fibril organization	5.06E-07	*lum, col3a1, col1a2, col12a1, col5a2, adamts2, col5a1, tgfb2*
GO:0043062	extracellular structure organization	9.94E-07	*tnc, lum, col3a1, nid1, dcn, cdh2, col5a2, col5a1, tgfb2, col1a2, col6a2, pdgfra, col12a1, lamc1, adamts2*
GO:0043588	skin development	1.31E-04	*col3a1, col1a2, lef1, col5a2, adamts2, col5a1*
GO:0008544	epidermis development	0.0015	*col7a1, atp2c1, krt14, col3a1, col1a2, lef1, gli2, col5a2, adamts2, col5a1, tgfb2*
GO:0007398	ectoderm development	0.0026	*col7a1, atp2c1, krt14, col3a1, col1a2, lef1, gli2, col5a2, adamts2, col5a1, tgfb2*
Annotation Cluster 2	**Enrichment Score: 4.46**		
Term	**Description**	**PValue**	**Genes**
GO:0000278	mitotic cell cycle	1.34E-07	*kif11, prc1, dlgap5, dbf4, kif15, ttk, skp1, cdc16, smc2, smc4, eml4, spc24, spc25, ccnd1, psmd12, seh1l, kif20b, nup37, stmn1, kpna2, dnajc2, hells, bub3, acvr1*
GO:0007049	cell cycle	6.23E-07	*nbn, prc1, dbf4, jag2, ttk, cdc16, tgfb2, spc24, spc25, seh1l, nup37, dnajc2, hells, bub3, ckap2, kif11, nasp, dlgap5, kif15, strada, mnd1, skp1, smc2, smc4, mcm6, eml4, ccnd1, psmd12, kif20b, stmn1, kpna2, calm2, acvr1, myh10, sept9*
GO:0022403	cell cycle phase	9.68E-07	*nbn, kif11, prc1, dlgap5, dbf4, kif15, mnd1, ttk, cdc16, smc2, smc4, eml4, spc24, spc25, ccnd1, seh1l, kif20b, nup37, stmn1, kpna2, dnajc2, hells, bub3, acvr1*
GO:0022402	cell cycle process	1.99E-06	*nbn, prc1, dbf4, ttk, cdc16, tgfb2, spc24, spc25, seh1l, nup37, dnajc2, hells, bub3, kif11, dlgap5, kif15, mnd1, skp1, smc2, eml4, smc4, ccnd1, psmd12, kif20b, stmn1, kpna2, myh10, acvr1*
GO:0000279	M phase	5.05E-06	*nbn, kif11, prc1, dlgap5, kif15, mnd1, ttk, cdc16, smc2, smc4, eml4, spc24, spc25, seh1l, kif20b, nup37, stmn1, kpna2, hells, bub3*
GO:0007059	chromosome segregation	8.19E-05	*spc25, seh1l, dlgap5, nup37, top2a, smc2, bub3, smc4, cenph*
GO:0000280	nuclear division	1.30E-04	*kif11, dlgap5, kif15, cdc16, smc2, smc4, eml4, spc24, spc25, seh1l, kif20b, nup37, bub3, hells*
GO:0007067	mitosis	1.30E-04	
GO:0000087	M phase of mitotic cell cycle	1.56E-04	
GO:0048285	organelle fission	1.94E-04	
GO:0051301	cell division	2.13E-04	*kif11, prc1, cdc16, smc2, smc4, tgfb2, cenph, spc24, spc25, ccnd1, seh1l, kif20b, nup37, hells, myh10, sept9*
GO:0000070	mitotic sister chromatid segregation	0.0034	*seh1l, dlgap5, smc2, bub3, smc4*
GO:0000819	sister chromatid segregation	0.0038	
Annotation Cluster 3	**Enrichment Score: 4.25**		
Term	**Description**	**PValue**	**Genes**
GO:0001501	skeletal system development	3.23E-06	*twsg1, aebp1, col3a1, jag2, sparc, gli2, mmp14, mepe, mmp13, mmp2, col5a2, cbfb, eya1, col1a2, pdgfra, col12a1, ror2, igfbp4, cdh11, spp1*
GO:0001503	ossification	1.82E-04	*twsg1, sparc, mmp14, gli2, col5a2, mmp13, mmp2, cbfb, cdh11, spp1*
GO:0060348	bone development	3.01E-04	

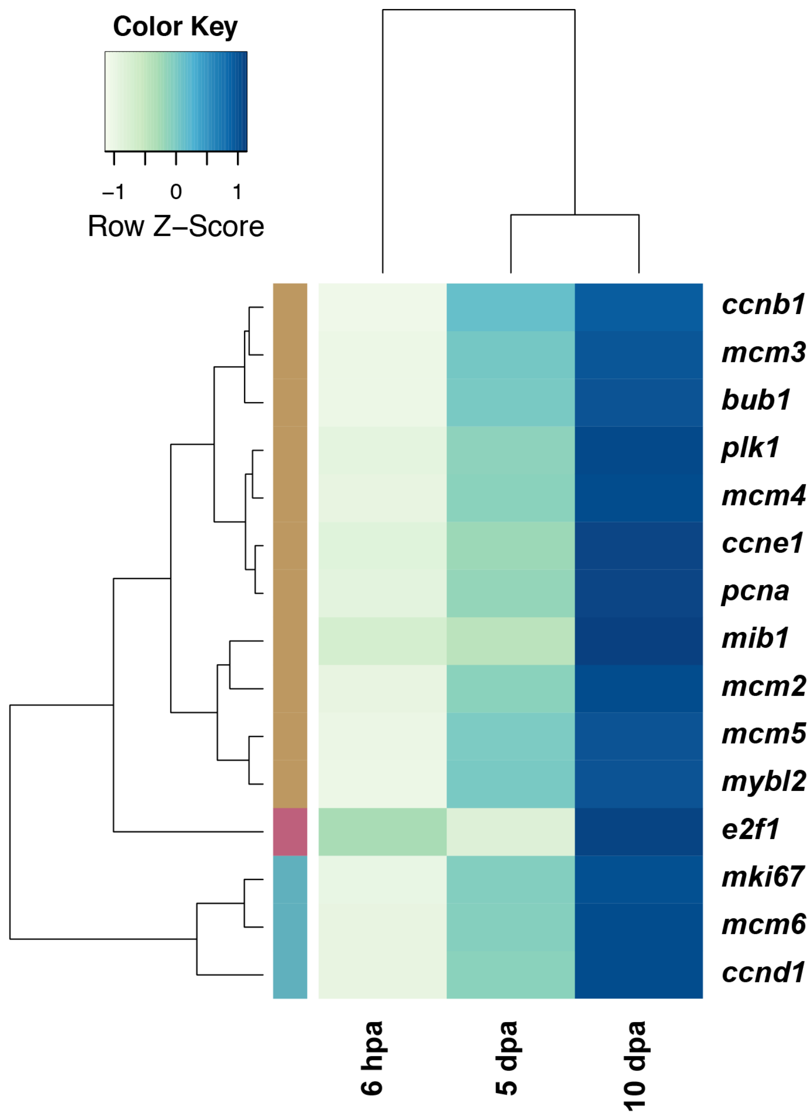

Figure 13. The regenerating tail has relatively low proliferation at 5 dpa and is highly proliferative by 10 dpa. A heatmap showing gene expression of proliferative markers in the regenerating tail.

Table 11

Differentially expressed genes at 10 dpa and the 25 dpa regenerating tail tip

ASU Gene ID	NCBI ID	Ensembl ID	Ortholog	Ortholog Long Name
ASU_Acar_G.8949	100555471	ENSACAG00000013211	*cbfb*	core-binding factor, beta subunit
ASU_Acar_G.19196	100565931	-	*edn3*	endothelin-3
ASU_Acar_G.8260	100559070	ENSACAG00000003899	*ednra*	endothelin receptor type A
ASU_Acar_G.8846	100555664	ENSACAG00000014456	*fbln1*	fibulin 1
ASU_Acar_G.4243	100551985	ENSACAG00000003374	*glis3*	GLIS family zinc finger 3
ASU_Acar_G.435	100560623	ENSACAG00000002130	*hhipl2*	HHIP-like 2
ASU_Acar_G.10506	100568251	ENSACAG00000016160	*igfbp4*	insulin-like growth factor binding protein 4
ASU_Acar_G.13871	100553004	ENSACAG00000007454	*il1r1*	interleukin 1 receptor, type I
ASU_Acar_G.141	100565875	ENSACAG00000004140	*mdk*	midkine (neurite growth-promoting factor 2)
ASU_Acar_G.8828	103278895	-	*mepe*	matrix extracellular phosphoglycoprotein
ASU_Acar_G.9462	100566168	ENSACAG00000012994	*ndnf*	neuron-derived neurotrophic factor
ASU_Acar_G.10755	100562562	ENSACAG00000011889	*or5v1*	olfactory receptor, family 5, subfamily V, member 1
ASU_Acar_G.9594	100565834	ENSACAG00000010297	*pdgfra*	platelet-derived growth factor receptor, alpha polypeptide
ASU_Acar_G.3775	100561490	ENSACAG00000004851	*ptk7*	protein tyrosine kinase 7
ASU_Acar_G.3015	100554214	ENSACAG00000000922	*ror2*	receptor tyrosine kinase-like orphan receptor 2
ASU_Acar_G.3015	100554214	ENSACAG00000000922	*ror2*	receptor tyrosine kinase-like orphan receptor 2
ASU_Acar_G.5235	100564051	-	*runx1*	runt-related transcription factor 1
ASU_Acar_G.13441	100559539	ENSACAG00000014399	*sall1*	spalt-like transcription factor 1
ASU_Acar_G.13416	100557376	ENSACAG00000012748	*sall4*	spalt-like transcription factor 4
ASU_Acar_G.10604	100562367	ENSACAG00000011641	*slc27a2*	solute carrier family 27 (fatty acid transporter), member 2
ASU_Acar_G.9105	100562360	ENSACAG00000012670	*spp1*	secreted phosphoprotein 1
ASU_Acar_G.5738	100529108	ENSACAG00000002478	*st6gal2*	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2

members *ednra* and *edn3*, which regulate vasoconstriction and cell proliferation (Goldie, 1999), and core binding factor, beta (*cbfb*), which regulates genes specific to hematopoiesis (Tracey & Speck, 2000) are significantly expressed at both stages, along with two neurotrophic factors, midkine (*mdk*) and neuron-derived neurotropic factor (*ndnf*). It is possible that factors such as these secreted from the neuroependyma and/or vasculature regulate regenerative outgrowth, considering that limb regeneration in salamanders is nerve-dependent (A. Kumar, Godwin, Gates, Garza-Garcia, & Brockes, 2007) and the neuroependyma provides positional identity in the regenerating lizard tail (Y. Wang et al., 2011).

Wound-healing and immune response genes include cytokine receptor *il1r1,* which regulates anti-inflammatory response (Beck, Izpisúa Belmonte, & Christen, 2009; González-Navajas et al., 2010; Saxena et al., 2013), the cytokine *spp1*, which regulates tissue repair and inflammation (O'Regan & Berman, 2000), platelet activator *pdgfra*, which is involved in cell migration and chemotaxis (Heredia et al., 2013; Schneider et al., 2010), and fibulin-1 (*fbln1*), which mediates platelet adhesion following injury (Godyna, Diaz-Ricart, & Argraves, 1996). Additionally, two tyrosine kinases, *ror2* and *ptk7*, that act as receptors for non-canonical Wnt signaling, specifically the planar cell polarity pathway (Wnt/PCP), are differentially expressed at both stages (Hayes, Naito, Daulat, Angers, & Ciruna, 2013; X. Lu, Borchers, Jolicoeur, Rayburn, & Baker, 2004; Minami, Oishi, Endo, & Nishita, 2010; Peradziryi, Tolwinski, & Borchers, 2012). It addition to providing positional identity, Wnt/PCP pathway signaling induced via the Wnt5a ligand, which is differentially expressed at 25 dpa (Chapter 2), upregulates pro-inflammatory cytokines and macrophages (reviewed in S. J. George, 2008).

Conclusion

While there are similar processes involved in scar-free wound healing in vertebrates, the genetic profile of pathways activated during early tail regeneration has not been described. Via whole transcriptome analysis, we have identified a number of genetic mechanisms that play a role in the early stages of tail regeneration in the lizard, including those involved in early inflammation response, extracellular matrix remodeling, positional identity, and wound epithelium formation. As early as 6 hours following autotomy, gene expression indicates an inflammatory response involving neutrophil recruitment and phagocytosis of apoptotic cells. From 6 hours to 5 days following autotomy, gene expression shows an increase in ECM remodeling and bone resorption, possibly corresponding to remodeling and contraction of tissues in the stump (Lozito & Tuan, 2015; McLean & Vickaryous, 2011; Nambiar et al., 2008). Components of the wound epithelium are also expressed at this stage, along with squamate-specific anti-microbial proteins (carwaprin-b, omwaprin-a). By 10 days, the wound epithelium is formed prior to outgrowth and blood vessels are evident immediately below the epithelium (Chapter 2; McLean & Vickaryous, 2011). Similarly, genes related to epithelium formation (i.e. keratins and keratin associated beta-proteins) and vascular growth and proliferation are expressed (*ednra*, *edn3*, *mdk*, *runx1*, *cbfb*). Additionally, many developmental genes are reactivated, and the mesenchymal cells underlying the wound epithelium are highly proliferative. Genes related to extracellular matrix organization are also highly expressed, providing the underlying structure needed for regenerative outgrowth. Positional identity may be conferred through the Wnt planar cell polarity pathway (*ror2*, *ptk7*) and/or neurotrophic factors secreted from the neuroependyma (*mdk, ndnf*). Additionally, we identified developmental genes that are differentially expressed immediately pre-outgrowth (10 dpa) and at the tip of the tail

mid-outgrowth (25 dpa), signifying the possibility that these genes are reactivated during the regenerative process. Because all of these genes have mammalian orthologs, and many are expressed during regeneration in other vertebrate model systems, they represent a group of genes and pathways that could be targeted in mammalian regeneration. We anticipate that further functional analysis of genes identified during lizard tail regeneration could be utilized for medical therapies.

Chapter 5

CONCLUSION

Though the green anole has been used as a model for tail regeneration for decades, the lack of genomic resources has hindered its progress compared to other more traditional model organisms. Genome sequencing and our comprehensive RNA-Seq-based annotation have made it possible to investigate the genes and their microRNA regulators activated during the regenerative process in lizards. We have identified a number of developmental and repair processes that may confer scar-free wound healing in the initial stages of regeneration and regulate regenerative outgrowth, and similar gene expression studies in other model organisms allow for comparison within vertebrates.

Epimorphic versus stem cell-mediated regeneration. There are two main hypotheses for the overall mechanisms of regeneration in vertebrates. One hypothesis, called epimorphic regeneration, is that residual tissues dedifferentiate to form a blastema. The term “blastema” has its origins in salamander limb regeneration, and has since been to describe limb and fin regeneration in amphibians and teleost fish, typically presented as an alternative to tissue-specific stem cells (Brockes & Kumar, 2005; Christen, Robles, Raya, Paramonov, & Izpisúa Belmonte, 2010; Tamura, Ohgo, & Yokoyama, 2010; Tsonis, 2008). Another hypothesis is that regeneration occurs through activation of tissue-specific stem cells that were originally responsible for embryonic development and/or confer repair in the adult (Purhonen et al., 2008; Wagers & Weissman, 2004; Wagers, Sherwood, Christensen, & Weissman, 2002; Weissman, 2000). Traditionally, blastema formation is characterized by dedifferentiation of existing tissues, relatively low levels of mitosis, a mass of pluripotent proliferating cells focused at the tip of the regenerating appendage, and the absence of a vascular bed at the distal tip

(Butler & O'Brien, 1942; Echeverri & Tanaka, 2002; Hay & Fischman, 1961; Iten & Bryant, 1973; Kelly & Tassava, 1973; Mescher, 1996; Namenwirth, 1974; Peadon & Singer, 1966; M. Singer, 1974; Smith & Wolpert, 1975; Tassava et al., 1974; Thornton, 1938; B. M. Wallace & Wallace, 1973). This is in direct contrast to what has been described in the lizard tail. There is no evidence of dedifferentiation in the lizard (Chapter 2; Chapter 4; Cox, 1969; Hughes & New, 1959; S. B. Simpson, 1965), mitosis is evident immediately preceding outgrowth (Chapter 4), proliferating cells are present throughout the regenerating tail (Chapter 2; McLean & Vickaryous, 2011), and the distal tip is highly vascular (Chapter 2; Chapter4; McLean & Vickaryous, 2011). These results suggest that lizards demonstrate stem cell-mediated regeneration as opposed to epimorphic regeneration, which involves dedifferentiation and the formation of the blastema.

Additionally, a number of studies have recently confirmed the use of tissue-specific, lineage-restricted progenitor cells that contribute to the new structures in regenerating appendages in other vertebrates (Gargioli & Slack, 2004; Knopf et al., 2011; Kragl et al., 2009; Lehoczky et al., 2011; Rinkevich et al., 2011; Sandoval-Guzmán et al., 2014; Singh et al., 2012; Sousa et al., 2011; S. Stewart & Stankunas, 2012; Tu & Johnson, 2011). In the regenerating tadpole tail, SOX2-positive cells contribute to the regenerating spinal cord and PAX7 positive cells contribute to the regenerating muscle (Gaete et al., 2012; Gargioli & Slack, 2004). In the axolotl, lineage-tracing of PAX7-positive cells has revealed that muscle regeneration occurs through migration and differentiation of muscle satellite cells residing in the original limb, while in contrast, newt muscle undergoes dedifferentiation, proliferation, and redifferentiation (Kragl et al., 2009; Sandoval-Guzmán et al., 2014). In mouse digit tip regeneration, stem/progenitor cells that are lineage restricted are responsible for regeneration (Lehoczky et al., 2011; Rinkevich et al., 2011). This calls for a change in terminology; the term “blastema”

implies the presence of a dedifferentiated mass of pluripotent stem cells, with similar properties to embryonic stem cells, and for the sake of clarity, should no longer be used to define regenerative processes mediated by tissue-specific progenitor cells. There are a number of researchers in the *Xenopus* frog community in recent years that are seeking for a more specific definition for cells contributing to regenerating tissues (reviewed in (Gargioli & Slack, 2004), and other models such as lizards and salamanders should follow suit.

Future directions. Using transcriptomics, we have identified a number of genetic mechanisms, primarily those related to development as well as adult repair, regulating scar-free wound healing and regeneration in the lizard. The availability of an assembled genome and our efforts in generating a high-quality genome annotation have furthered genomic resources in this model organism. One problem that remains with the lizard model system, however, is the lack of resources for functional genomics. Because *Anolis* embryos are not as easily manipulated as those of other model systems, gene editing, knockout, and transgenics have yet to be developed in the lizard. We anticipate that in the coming years, knockout, transgenic, and/or CRISPR gene editing technologies will be adapted for *A. carolinensis*, facilitating more direct testing of genomic targets identified in this thesis and cell lineage experiments to help delineate the extent of stem cell-mediated regeneration in the lizard.

However, we do currently have other resources available. We have isolated muscle satellite cells from the lizard (Chapter 2), and continuing efforts to develop a cell culture system for *A. carolinensis* will facilitate experimental validation of targets identified by transcriptomic analysis of RNAs and microRNAs in regeneration. Proteomics can also be applied to help validate microRNA/mRNA target pairs and investigate post-transcriptional and post-translational regulation. Additionally, small

molecule inhibitors, for example β-catenin inhibitors and/or inhibitors of Wnt inhibitor GSK3B, could be applied to the tail to test the importance of pathways such as the Wnt pathway in regeneration. Therefore, we anticipate that genes identified in this thesis can be utilized to further understand the mechanism of regeneration in lizards and for application to medical therapies.

REFERENCES

Agata, K., & Inoue, T. (2012). Survey of the differences between regenerative and non-regenerative animals. *Development, Growth & Differentiation*, *54*(2), 143–152. doi:10.1111/j.1440-169X.2011.01323.x

Alföldi, J., Di Palma, F., Grabherr, M., Williams, C., Kong, L., Mauceli, E., et al. (2011). The genome of the green anole lizard and a comparative analysis with birds and mammals. *Nature*, *477*(7366), 587–591. doi:10.1038/nature10390

Alibardi, L. (1995a). Development of the axial cartilaginous skeleton in the regenerating tail of lizards. *Bulletin De l'Association Des Anatomistes*, *79*(244), 3–9.

Alibardi, L. (1995b). Muscle differentiation and morphogenesis in the regenerating tail of lizards. *Journal of Anatomy*, *186(Pt 1)*, 143–151.

Alibardi, L. (2010a). Morphological and cellular aspects of tail and limb regeneration in lizards. A model system with implications for tissue regeneration in mammals. *Advances in Anatomy, Embryology, and Cell Biology*, *207*, iii–v–x– 1–109.

Alibardi, L. (2010b). Ultrastructural features of the process of wound healing after tail and limb amputation in lizard. *Acta Zoologica, 91(3), 306–318. doi:10.1111/j.1463-6395.2009.00409.x*

Alibardi, L. (2014a). Observations on Lumbar Spinal Cord Recovery after Lesion in Lizards Indicates Regeneration of a Cellular and Fibrous Bridge Reconnecting the Injured Cord. *Journal of Developmental Biology*, *2*(4), 210–229. doi:10.3390/jdb2040210

Alibardi, L. (2014b). Ultrastructural Observations on Lumbar Spinal Cord Recovery After Lesion in Lizard Indicates Axonal Regeneration and Neurogenesis. *International Journal of Biology*, *7*(1). doi:10.5539/ijb.v7n1p122

Alibardi, L., Celeghin, A., & Valle, L. D. (2012). Wounding in lizards results in the release of beta-defensins at the wound site and formation of an antimicrobial barrier. *Developmental and Comparative Immunology*, *36*(3), 557–565. doi:10.1016/j.dci.2011.09.012

Allen, R. E., Temm-Grove, C. J., Sheehan, S. M., & Rice, G. (1997). Chapter 8 Skeletal Muscle Satellite Cell Cultures. In *Methods in cell biology* (Vol. 52, pp. 155–176). Elsevier. doi:10.1016/S0091-679X(08)60378-7

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology*, *11*(10), R106. doi:10.1186/gb-2010-11-10-r106

Anders, S., McCarthy, D. J., Chen, Y., Okoniewski, M., Smyth, G. K., Huber, W., & Robinson, M. D. (2013). Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nature Protocols*, *8*(9), 1765–1786. doi:10.1038/nprot.2013.099

Anders, S., Pyl, P. T., & Huber, W. (2014). HTSeq–A Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England),31(2), 166–9. doi:10.1093/bioinformatics/btu638*

Anderson, D. M., Arredondo, J., Hahn, K., Valente, G., Martin, J. F., Wilson-Rawls, J., & Rawls, A. (2006). Mohawk is a novel homeobox gene expressed in the developing mouse embryo. *Developmental Dynamics*, *235*(3), 792–801. doi:10.1002/dvdy.20671

Anderson, D. M., Beres, B. J., Wilson-Rawls, J., & Rawls, A. (2009). The homeobox gene Mohawk represses transcription by recruiting the sin3A/HDAC co-repressor complex. *Developmental Dynamics*, *238*(3), 572–580. doi:10.1002/dvdy.21873

Anderson, D. M., George, R., Noyes, M. B., Rowton, M., Liu, W., Jiang, R., et al. (2012). Characterization of the DNA-binding properties of the Mohawk homeobox transcription factor. *Journal of Biological Chemistry*, *287*(42), 35351–35359. doi:10.1074/jbc.M112.399386

Arnold, E. N. (1988). Caudal autotomy as a defense. *Smith VII*.

Asakura, A., Komaki, M., & Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation; Research in Biological Diversity*, *68*(4-5), 245–253.

Baggiolini, M., Walz, A., & Kunkel, S. L. (1989). Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *Journal of Clinical Investigation*, *84*(4), 1045–1049. doi:10.1172/JCI114265

Barron, L., & Wynn, T. A. (2011). Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. *European Journal of Immunology*, *41*(9), 2509–2514. doi:10.1002/eji.201141869

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, *136*(2), 215–233. doi:10.1016/j.cell.2009.01.002

Beck, C. W., Christen, B., & Slack, J. M. W. (2003). Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. *Developmental Cell*, *5*(3), 429–439.

Beck, C. W., Izpisúa Belmonte, J. C., & Christen, B. (2009). Beyond early development: *Xenopus* as an emerging model for the study of regenerative mechanisms. *Developmental Dynamics*, *238*(6), 1226–1248. doi:10.1002/dvdy.21890

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., & Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *Journal of Comparative Neurology*, *377*(4), 577–595.

Bellairs, A. A., & Bryant, S. V. (1985). Autotomy and regeneration in reptiles, 1–28.

Bely, A. E., & Nyberg, K. G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends in Ecology & Evolution*, *25*(3), 161–170. doi:10.1016/j.tree.2009.08.005

Berg, D. A., Kirkham, M., Beljajeva, A., Knapp, D., Habermann, B., Ryge, J., et al. (2010). Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. *Development*, *137*(24), 4127–4134. doi:10.1242/dev.055541

Betel, D., Koppal, A., Agius, P., Sander, C., & Leslie, C. (2010). Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biology*, *11*(8), R90. doi:10.1186/gb-2010-11-8-r90

Birol, I., Jackman, S. D., Nielsen, C. B., Qian, J. Q., Varhol, R., Stazyk, G., et al. (2009). *De novo* transcriptome assembly with ABySS, *25*(21), 2872–2877. doi:10.1093/bioinformatics/btp367

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. doi:10.1093/bioinformatics/btu170

Boothby, M., Ruddon, R. W., Anderson, C., McWilliams, D., & Boime, I. (1981). A single gonadotropin alpha-subunit gene in normal tissue and tumor-derived cell lines. *The Journal of Biological Chemistry*, *256*(10), 5121–5127.

Borgens, R. B. (1982). Mice regrow the tips of their foretoes. *Science (New York, NY)*, *217*(4561), 747–750.

Bosco, L. (1979). Expression of regenerative capacity of caudal spinal cord during the larval development of *Xenopus laevis*. *Acta Embryologiae Experimentalis*, (3), 275–285.

Bossard, M. J., Tomaszek, T. A., & Thompson, S. K. (1996). Proteolytic Activity of Human Osteoclast Cathepsin K Expression, Purification, Activation, and Substrate Identification. *Journal of Biological Chemistry*, 271(21), 12517-12524. doi:10.1074/jbc.271.21.12517

Brockes, J. P., & Kumar, A. (2005). Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science (New York, NY)*, *310*(5756), 1919–1923. doi:10.1126/science.1115200

Brockes, J. P., & Kumar, A. (2008). Comparative Aspects of Animal Regeneration. *Annual Review of Cell and Developmental Biology*, *24*(1), 525–549. doi:10.1146/annurev.cellbio.24.110707.175336

Brown, D. D., Cai, L., Das, B., Marsh-Armstrong, N., Schreiber, A. M., & Juste, R. (2005). Thyroid hormone controls multiple independent programs required for limb development in *Xenopus laevis* metamorphosis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(35), 12455–12458. doi:10.1073/pnas.0505989102

Bryant, S. V., Endo, T., & Gardiner, D. M. (2002). Vertebrate limb regeneration and the origin of limb stem cells. *The International Journal of Developmental Biology*, *46*(7), 887–896.

Buckbinder, L., & Brown, D. D. (1993). Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(9), 3820–3824.

Burge, S. W., Daub, J., Eberhardt, R., Tate, J., Barquist, L., Nawrocki, E. P., et al. (2013). Rfam 11.0: 10 years of RNA families. *Nucleic Acids Research*, *41*(Database issue), D226–32. doi:10.1093/nar/gks1005

Butler, E. G., & O'Brien, J. P. (1942). Effects of localized x-radiation on regeneration of the urodele limb. *The Anatomical Record, 84(4), 407–413. doi:10.1002/ar.1090840408*

Cairns, D. M., Liu, R., Sen, M., Canner, J. P., Schindeler, A., Little, D. G., & Zeng, L. (2012). Interplay of Nkx3.2, Sox9 and Pax3 regulates chondrogenic differentiation of muscle progenitor cells. *PLoS ONE*, *7*(7), e39642. doi:10.1371/journal.pone.0039642

Cameron, D. A. (2000). Cellular proliferation and neurogenesis in the injured retina of adult zebrafish. *Visual Neuroscience*, *17*(5), 789–797.

Campbell, L. J., & Crews, C. M. (2008). Molecular and cellular basis of regeneration and tissue repair. *Cellular and Molecular Life Sciences, 65(1), 54–63. doi:10.1007/s00018-007-7431-1*

Cao, X., Pfaff, S. L., & Gage, F. H. (2007). A functional study of miR-124 in the developing neural tube. *Genes & development, 21(5), 531–6. doi:10.1101/gad.1519207*

Caputo, V., Sinibaldi, L., Fiorentino, A., Parisi, C., Catalanotto, C., Pasini, A., et al. (2011). Brain derived neurotrophic factor (BDNF) expression is regulated by microRNAs miR-26a and miR-26b allele-specific binding. *PLoS ONE*, *6*(12), e28656. doi:10.1371/journal.pone.0028656

Carlson, B. M. (2011). Principles of Regenerative Biology.

Carlson, M. R., Bryant, S. V., & Gardiner, D. M. (1998). Expression of Msx-2 during development, regeneration, and wound healing in axolotl limbs. *The Journal of Experimental Zoology*, *282*(6), 715–723.

Cash, J. L., Christian, A. R., & Greaves, D. R. (2010). Chemerin peptides promote phagocytosis in a ChemR23- and Syk-dependent manner. *Journal of Immunology (Baltimore, Md. : 1950)*, *184*(9), 5315–5324. doi:10.4049/jimmunol.0903378

Chadwick, R. B., Bu, L., Yu, H., Hu, Y., Wergedal, J. E., Mohan, S., & Baylink, D. J. (2007). Digit tip regrowth and differential gene expression in MRL/Mpj, DBA/2, and C57BL/6 mice. *Wound Repair and Regeneration : Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, *15*(2), 275–284. doi:10.1111/j.1524-475X.2007.00216.x

Chen, J. F., Tao, Y., Li, J., Deng, Z., Yan, Z., Xiao, X., & Wang, D. Z. (2010). microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *The Journal of Cell Biology*, *190*(5), 867–879. doi:10.1016/j.cell.2007.03.030

Chen, Y. (2006). Control of muscle regeneration in the *Xenopus* tadpole tail by Pax7. *Development*, *133*(12), 2303–2313. doi:10.1242/dev.02397

Chlebowski, J. S., Przbylski, R. J., & Cox, P. G. (1973). Ultrastructural studies of lizard (I) myogenesis in vitro. *Developmental Biology*, *33*(1), 80–99.

Chodankar, R., Chang, C.-H., Yue, Z., Jiang, T.-X., Suksaweang, S., Burrus, L., et al. (2003). Shift of localized growth zones contributes to skin appendage morphogenesis: role of the Wnt/beta-catenin pathway. *The Journal of Investigative Dermatology*, *120*(1), 20–26. doi:10.1046/j.1523-1747.2003.12008.x

Christen, B., Robles, V., Raya, M., Paramonov, I., & Izpisúa Belmonte, J. C. (2010). Regeneration and reprogramming compared. *BMC Biology*, *8*, 5. doi:10.1186/1741-7007-8-5

Christensen, R. N., & Tassava, R. A. (2000). Apical epithelial cap morphology and fibronectin gene expression in regenerating axolotl limbs. *Developmental Dynamics*, *217*(2), 216–224. doi:10.1002/(SICI)1097-0177(200002)217:2<216::AID-DVDY8>3.0.CO;2-8

Christensen, R. N., Weinstein, M., & Tassava, R. A. (2002). Expression of fibroblast growth factors 4, 8, and 10 in limbs, flanks, and blastemas of *Ambystoma*. *Developmental Dynamics*, *223*(2), 193–203. doi:10.1002/dvdy.10049

Cohen, E. (2007). Woman's persistence pays off in regenerated fingertip. *Cable News Network*, 9th September.

Coolen, M., Katz, S., & Bally-Cuif, L. (2013). miR-9: a versatile regulator of neurogenesis. *Frontiers in Cellular Neuroscience*, *7*, 220. doi:10.3389/fncel.2013.00220

Cox, P. G. (1968). In vitro myogenesis of promuscle cells from the regenerating tail of the lizard, *Anolis carolinensis*. *Journal of Morphology*, *126*(1), 1–18. doi:10.1002/jmor.1051260102

Cox, P. G. (1969). Some aspects of tail regeneration in the lizard, *Anolis carolinensis*. I. A description based on histology and autoradiography. *The Journal of Experimental Zoology*, *171*(2), 127–149. doi:10.1002/jez.1401710202

Croteau, W., Davey, J. C., Galton, V. A., & St Germain, D. L. (1996). Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *Journal of Clinical Investigation*, *98*(2), 405–417. doi:10.1172/JCI118806

Cruciat, C.-M., & Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor Perspectives in Biology*, *5*(3), a015081. doi:10.1101/cshperspect.a015081

da Silva, S. M., Gates, P. B., & Brockes, J. P. (2002). The newt ortholog of CD59 is implicated in proximodistal identity during amphibian limb regeneration. *Developmental Cell*, *3*(4), 547–555.

Day, T. F., Guo, X., Garrett-Beal, L., & Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Developmental Cell*, *8*(5), 739–750. doi:10.1016/j.devcel.2005.03.016

DeChiara, T. M., Kimble, R. B., Poueymirou, W. T., Rojas, J., Masiakowski, P., Valenzuela, D. M., & Yancopoulos, G. D. (2000). Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. *Nature Genetics*, *24*(3), 271–274. doi:10.1038/73488

Delavary, B. M., van der Veer, W. M., & van Egmond, M. (2011). Macrophages in skin injury and repair. *Immunobiology,* 216(7), 753–762. doi:10.1016/j.imbio.2011.01.001

Delorme, S. L., Lungu, I. M., & Vickaryous, M. K. (2012). Scar-Free Wound Healing and Regeneration Following Tail Loss in the Leopard Gecko, *Eublepharis macularius*. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *295*(10), 1575–1595. doi:10.1002/ar.22490

Dent, J. N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *Journal of Morphology*, *110*, 61–77. doi:10.1002/jmor.1051100105

Dentice, M., Marsili, A., Ambrosio, R., Guardiola, O., Sibilio, A., Paik, J.-H., et al. (2010). The FoxO3/type 2 deiodinase pathway is required for normal mouse myogenesis and muscle regeneration. *Journal of Clinical Investigation*, *120*(11), 4021–4030. doi:10.1172/JCI43670DS1

Dey, B. K., Gagan, J., Yan, Z., & Dutta, A. (2012). miR-26a is required for skeletal muscle differentiation and regeneration in mice. *Genes & Development*, *26*(19), 2180–2191. doi:10.1101/gad.198085.112

Dial, B. E., & Fitzpatrick, L. C. (1983). Lizard Tail Autotomy: Function and Energetics of Postautotomy Tail Movement in *Scincella lateralis*. *Science (New York, NY)*, *219*(4583), 391–393. doi:10.1126/science.219.4583.391

Dill, H., Linder, B., Fehr, A., & Fischer, U. (2012). Intronic miR-26b controls neuronal differentiation by repressing its host transcript, ctdsp2. *Genes & Development*, *26*(1), 25–30. doi:10.1101/gad.177774.111

Douglas, B. S. (1972). Conservative management of guillotine amputation of the finger in children. *Australian Paediatric Journal*, *8*(2), 86–89.

Duffy, M. T., Simpson, S. B., Liebich, D. R., & Davis, B. M. (1990). Origin of spinal cord axons in the lizard regenerated tail: Supernormal projections from local spinal neurons. *Journal of Comparative Neurology*, *293*(2), 208–222. doi:10.1002/cne.902930205

Dzierzak, E., & Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology*, *9*(2), 129–136. doi:10.1038/ni1560

Echeverri, K., & Tanaka, E. M. (2002). Ectoderm to mesoderm lineage switching during axolotl tail regeneration. *Science (New York, NY)*, *298*(5600), 1993–1996. doi:10.1126/science.1077804

Eckalbar, W. L., Hutchins, E. D., Markov, G. J., Allen, A. N., Corneveaux, J. J., Lindblad-Toh, K., et al. (2013). Genome reannotation of the lizard *Anolis carolinensis* based on 14 adult and embryonic deep transcriptomes. *BMC Genomics*, *14*, 49. doi:10.1186/1471-2164-14-49

Eckalbar, W. L., Infante, C., Emmert, N., Losos, J., Rawls, A., Wilson-Rawls, J., & Kusumi, K. (2010). Identification of a divergent notch pathway delta ligand in the segmentation clock of the reptile, *Anolis carolinensis*. *Developmental Biology*, *344*(1), 528. doi:10.1016/j.ydbio.2010.05.385

Eckalbar, W. L., Lasku, E., Infante, C. R., Elsey, R. M., Markov, G. J., Allen, A. N., et al. (2012). Somitogenesis in the anole lizard and alligator reveals evolutionary convergence and divergence in the amniote segmentation clock. *Developmental Biology*, *363*(1), 308–319. doi:10.1016/j.ydbio.2011.11.021

Egar, M., Simpson, S. B., & Singer, M. (1970). The growth and differentiation of the regenerating spinal cord of the lizard, *Anolis carolinensis*. *Journal of Morphology*, *131*(2), 131–151. doi:10.1002/jmor.1051310202

Endo, T., Tamura, K., & Ide, H. (2000). Analysis of Gene Expressions during *Xenopus* Forelimb Regeneration. *Developmental Biology*, *220*(2), 296–306. doi:10.1006/dbio.2000.9641

Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., & Marks, D. S. (2003). MicroRNA targets in Drosophila. *Genome Biology*, *5*(1), R1. doi:10.1186/gb-2003-5-1-r1

Fahmy, G. H., & Sicard, R. E. (2002). A role for effectors of cellular immunity in epimorphic regeneration of amphibian limbs. *In Vivo (Athens, Greece)*, *16*(3), 179–184.

Fan, C.-M., Li, L., Rozo, M. E., & Lepper, C. (2012). Making skeletal muscle from progenitor and stem cells: development versus regeneration. *Wiley Interdisciplinary Reviews: Developmental Biology*, *1*(3), 315–327. doi:10.1002/wdev.30

Feldman, J. L., & Stockdale, F. E. (1991). Skeletal muscle satellite cell diversity: Satellite cells form fibers of different types in cell culture. *Developmental Biology*, *143*(2), 320–334. doi:10.1016/0012-1606(91)90083-F

Fernando, W. A., Leininger, E., Simkin, J., Li, N., Malcom, C. A., Sathyamoorthi, S., et al. (2011). Wound healing and blastema formation in regenerating digit tips of adult mice. *Developmental Biology*, *350*(2), 301–310. doi:10.1016/j.ydbio.2010.11.035

Filoni, S., & Paglialunga, L. (1990). Effect of denervation on hindlimb regeneration in *Xenopus laevis* larvae. *Differentiation; Research in Biological Diversity*, *43*(1), 10–19.

Fisher, R. E., Geiger, L. A., Stroik, L. K., Hutchins, E. D., George, R. M., DeNardo, D. F., et al. (2012). A histological comparison of the original and regenerated tail in the green anole, *Anolis carolinensis*. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *295*(10), 1609–1619. doi:10.1002/ar.22537

Frade, J. M., & Barde, Y. A. (1998). Microglia-derived nerve growth factor causes cell death in the developing retina. *Neuron*, *20*(1), 35–41.

Friedländer, M. R., Chen, W., Adamidi, C., Maaskola, J., Einspanier, R., Knespel, S., & Rajewsky, N. (2008). Discovering microRNAs from deep sequencing data using miRDeep. *Nature Biotechnology*, *26*(4), 407–415. doi:10.1038/nbt1394

Friedländer, M. R., Mackowiak, S. D., Li, N., Chen, W., & Rajewsky, N. (2012). miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Research*, *40*(1), 37–52. doi:10.1093/nar/gkr688

Furlow, J. D., & Neff, E. S. (2006). A developmental switch induced by thyroid hormone: *Xenopus laevis* metamorphosis. *Trends in Endocrinology and Metabolism: TEM*, *17*(2), 40–47. doi:10.1016/j.tem.2006.01.007

Gaete, M., Muñoz, R., Sánchez, N., Tampe, R., Moreno, M., Contreras, E. G., et al. (2012). Spinal cord regeneration in *Xenopus* tadpoles proceeds through activation of Sox2-positive cells. *Neural Development*, *7*, 13. doi:10.1186/1749-8104-7-13

Galliera, E., Tacchini, L., & Corsi Romanelli, M. M. (2015). Matrix metalloproteinases as biomarkers of disease: updates and new insights. *Clinical Chemistry and Laboratory Medicine : CCLM / FESCC*, *53*(3), 349–355. doi:10.1515/cclm-2014-0520

Gargioli, C., & Slack, J. M. W. (2004). Cell lineage tracing during *Xenopus* tail regeneration. *Development*, *131*(11), 2669–2679. doi:10.1242/dev.01155

Garlanda, C., Hirsch, E., Bozza, S., Salustri, A., De Acetis, M., Nota, R., et al. (2002). Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature*, *420*(6912), 182–186. doi:10.1038/nature01195

Ge, Y., Sun, Y., & Chen, J. (2011). IGF-II is regulated by microRNA-125b in skeletal myogenesis. *The Journal of Cell Biology*, *192*(1), 69–81. doi:10.1083/jcb.201007165.dv

Gemberling, M., Bailey, T. J., Hyde, D. R., & Poss, K. D. (2013). The zebrafish as a model for complex tissue regeneration. *Trends in Genetics : TIG*, *29*(11), 611–620. doi:10.1016/j.tig.2013.07.003

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology*, *5*(10), R80. doi:10.1186/gb-2004-5-10-r80

George, R. M., Biressi, S., Beres, B. J., Rogers, E., Mulia, A. K., Allen, R. E., et al. (2013). Numb-deficient satellite cells have regeneration and proliferation defects. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(46), 18549–18554. doi:10.1073/pnas.1311628110

George, S. J. (2008). Wnt pathway: a new role in regulation of inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *28*(3), 400–402. doi:10.1161/ATVBAHA.107.160952

Ghosh, S., Roy, S., Séguin, C., Bryant, S. V., & Gardiner, D. M. (2008). Analysis of the expression and function of Wnt-5a and Wnt-5b in developing and regenerating axolotl (*Ambystoma mexicanum*) limbs. *Development, Growth & Differentiation*, *50*(4), 289–297. doi:10.1111/j.1440-169X.2008.01000.x

Gilbert, E. A. B., Payne, S. L., & Vickaryous, M. K. (2013a). The Anatomy and Histology of Caudal Autotomy and Regeneration in Lizards. *Physiological and Biochemical Zoology*, *86*(6), 631–644. doi:10.1086/673889

Gilbert, R. W. D., Vickaryous, M. K., & Viloria-Petit, A. M. (2013b). Characterization of TGFβ signaling during tail regeneration in the leopard Gecko (*Eublepharis macularius*). *Developmental Dynamics*, *242*(7), 886–896. doi:10.1002/dvdy.23977

Godwin, J. W., & Brockes, J. P. (2006). Regeneration, tissue injury and the immune response. *Journal of Anatomy*, *209*(4), 423–432. doi:10.1111/j.1469-7580.2006.00626.x

Godwin, J. W., & Rosenthal, N. (2014). Scar-free wound healing and regeneration in amphibians: Immunological influences on regenerative success. *Differentiation; Research in Biological Diversity*, *87*(1-2), 66–75. doi:10.1016/j.diff.2014.02.002

Godwin, J. W., Pinto, A. R., & Rosenthal, N. A. (2013). Macrophages are required for adult salamander limb regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(23), 9415–9420. doi:10.1073/pnas.1300290110

Godwin, J., Kuraitis, D., & Rosenthal, N. (2014). The International Journal of Biochemistry & Cell Biology. *The International Journal of Biochemistry & Cell Biology*, *56*, 47–55. doi:10.1016/j.biocel.2014.10.011

Godyna, S., Diaz-Ricart, M., & Argraves, W. S. (1996). Fibulin-1 mediates platelet adhesion via a bridge of fibrinogen. *Blood*, *88*(7), 2569–2577.

Goldie, R. G. (1999). Endothelins in health and disease: an overview. *Clinical and Experimental Pharmacology & Physiology*, *26*(2), 145–148.

González-Navajas, J. M., Law, J., Nguyen, K. P., Bhargava, M., Corr, M. P., Varki, N., et al. (2010). Interleukin 1 receptor signaling regulates DUBA expression and facilitates Toll-like receptor 9-driven antiinflammatory cytokine production. *Journal of Experimental Medicine*, *207*(13), 2799–2807. doi:10.1084/jem.20101326

Graw, J. (2010). Chapter Ten - Eye Development. In P. Koopman, *Organogenesis in Development* (Vol. 90, pp. 343–386). Academic Press. doi:10.1016/S0070-2153(10)90010-0

Gross, J., & Lapiere, C. M. (1962). Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proceedings of the National Academy of Sciences of the United States of America*, *48*, 1014–1022.

Haas, B. J. (2003). Improving the *Arabidopsis* genome annotation using maximal transcript alignment assemblies. *Nucleic Acids Research*, *31*(19), 5654–5666. doi:10.1093/nar/gkg770

Haas, H. J. (1962). Studies on mechanisms of joint and bone formation in the skeleton rays of fish fins. *Developmental Biology*, *5*, 1–34.

Halim, S., Stone, C. A., & Devaraj, V. S. (1998). The Hyphecan cap: a biological fingertip dressing. *Injury*, *29*(4), 261–263.

Hamamori, Y., Sartorelli, V., Ogryzko, V., Puri, P. L., Wu, H. Y., Wang, J. Y., et al. (1999). Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell*, *96*(3), 405–413.

Han, M. J., An, J. Y., & Kim, W. S. (2001). Expression patterns of Fgf-8 during development and limb regeneration of the axolotl. *Developmental Dynamics*, *220*(1), 40–48. doi:10.1002/1097-0177(2000)9999:9999<::AID-DVDY1085>3.0.CO;2-8

Han, M., Yang, X., Lee, J., Allan, C. H., & Muneoka, K. (2008). Development and regeneration of the neonatal digit tip in mice. *Developmental Biology*, *315*(1), 125–135. doi:10.1016/j.ydbio.2007.12.025

Han, M., Yang, X., Taylor, G., Burdsal, C. A., Anderson, R. A., & Muneoka, K. (2005). Limb regeneration in higher vertebrates: developing a roadmap. *Anatomical Record. Part B, New Anatomist*, *287*(1), 14–24. doi:10.1002/ar.b.20082

Harty, M., Neff, A. W., King, M. W., & Mescher, A. L. (2003). Regeneration or scarring: an immunologic perspective. *Developmental Dynamics*, *226*(2), 268–279. doi:10.1002/dvdy.10239

Hay, E. D., & Fischman, D. A. (1961). Origin of the blastema in regenerating limbs of the newt *Triturus viridescens*. An autoradiographic study using tritiated thymidine to follow cell proliferation and migration. *Developmental Biology*, *3*, 26–59.

Hayes, M., Naito, M., Daulat, A., Angers, S., & Ciruna, B. (2013). Ptk7 promotes non-canonical Wnt/PCP-mediated morphogenesis and inhibits Wnt/β-catenin-dependent cell fate decisions during vertebrate development. *Development*, *140*(8), 1807–1818. doi:10.1242/dev.090183

Hellsten, U., Harland, R. M., Gilchrist, M. J., Hendrix, D., Jurka, J., Kapitonov, V., et al. (2010). The genome of the Western clawed frog *Xenopus tropicalis*. *Science (New York, NY)*, *328*(5978), 633–636. doi:10.1126/science.1183670

Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., et al. (2013). Type 2 Innate Signals Stimulate Fibro/Adipogenic Progenitors to Facilitate Muscle Regeneration. *Cell*, *153*(2), 376–388. doi:10.1016/j.cell.2013.02.053

Holman, E. C., Campbell, L. J., Hines, J., & Crews, C. M. (2012). Microarray Analysis of microRNA Expression during Axolotl Limb Regeneration. *PLoS ONE*, *7*(9), e41804. doi:10.1371/journal.pone.0041804.s001

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, *37*(1), 1–13. doi:10.1093/nar/gkn923

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. doi:10.1038/nprot.2008.211

Huang, P., He, Z., Ji, S., Sun, H., Xiang, D., Liu, C., et al. (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature*, *475*(7356), 386–389. doi:10.1038/nature10116

Hughes, A., & New, D. (1959). Tail regeneration in the geckonid lizard, *Sphaerodactylus*. *Journal of Embryology and Experimental Morphology*, *7*, 281–302.

Hui, S. P., Sengupta, D., Lee, S. G. P., Sen, T., Kundu, S., Mathavan, S., & Ghosh, S. (2014). Genome wide expression profiling during spinal cord regeneration identifies comprehensive cellular responses in zebrafish. *PLoS ONE*, *9*(1), e84212. doi:10.1371/journal.pone.0084212

Hutchison, C., Pilote, M., & Roy, S. (2007). The axolotl limb: a model for bone development, regeneration and fracture healing. *Bone*, *40*(1), 45–56. doi:10.1016/j.bone.2006.07.005

Illingworth, C. M. (1974). Trapped fingers and amputated finger tips in children. *Journal of Pediatric Surgery*, *9*(6), 853–858.

Iten, L. E., & Bryant, S. V. (1973). Forelimb regeneration from different levels of amputation in the newt, *Notophthalmus viridescens*: length, rate, and stages. *Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen. doi:10.1007/BF00575834*

J, G., & M, S. (1992). The fish fin regenerate. *Monographs in Developmental Biology*, *23*, 62–72.

John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., & Marks, D. S. (2004). Human MicroRNA targets. *PLoS Biology*, *2*(11), e363. doi:10.1371/journal.pbio.0020363

Kahn, E. B., & Simpson, S. B. (1974). Satellite cells in mature, uninjured skeletal muscle of the lizard tail. *Developmental Biology*, *37*(1), 219–223.

Kalinin, A., Marekov, L. N., & Steinert, P. M. (2001). Assembly of the epidermal cornified cell envelope. *Journal of Cell Science*, *114*(Pt 17), 3069–3070.

Kamrin, R. P., & Singer, M. (1955). The influence of the spinal cord in regeneration of the tail of the lizard, *Anolis carolinensis*. *Journal of Experimental Zoology*. *128*, 611–627.

Karantzali, E., Lekakis, V., Ioannou, M., Hadjimichael, C., Papamatheakis, J., & Kretsovali, A. (2011). Sall1 regulates embryonic stem cell differentiation in association with nanog. *Journal of Biological Chemistry*, *286*(2), 1037–1045. doi:10.1074/jbc.M110.170050

Kato, T., Miyazaki, K., Shimizu-Nishikawa, K., Koshiba, K., Obara, M., Mishima, H. K., & Yoshizato, K. (2003). Unique expression patterns of matrix metalloproteinases in regenerating newt limbs. *Developmental Dynamics*, *226*(2), 366–376. doi:10.1002/dvdy.10247

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I., & Izpisua Belmonte, J. C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes & Development*, *20*(23), 3232–3237. doi:10.1101/gad.1475106

Kelly, D. J., & Tassava, R. A. (1973). Cell division and ribonucleic acid synthesis during the initiation of limb regeneration in larval axolotls (*Ambystoma mexicanum*). *The Journal of Experimental Zoology*, *185*(1), 45–54. doi:10.1002/jez.1401850105

Kim, H. K. (2006). Muscle-specific microRNA miR-206 promotes muscle differentiation. *The Journal of Cell Biology*, *174*(5), 677–687. doi:10.1083/jcb.200603008

King, M. W., Neff, A. W., & Mescher, A. L. (2012). The developing *Xenopus* limb as a model for studies on the balance between inflammation and regeneration. *Anatomical Record (Hoboken, N.J. : 2007)*, *295*(10), 1552–1561. doi:10.1002/ar.22443

Kintner, C. R., & Brockes, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating limb regeneration. *Nature*, *308*(5954), 67–69.

Kizil, C., Kaslin, J., Kroehne, V., & Brand, M. (2012). Adult neurogenesis and brain regeneration in zebrafish. *Developmental Neurobiology*, *72*(3), 429–461. doi:10.1002/dneu.20918

Knapp, D., Schulz, H., Rascon, C. A., Volkmer, M., Scholz, J., Nacu, E., et al. (2013). Comparative transcriptional profiling of the axolotl limb identifies a tripartite regeneration-specific gene program. *PLoS ONE*, *8*(5), e61352. doi:10.1371/journal.pone.0061352.s011

Knopf, F., Hammond, C., Chekuru, A., Kurth, T., Hans, S., Weber, C. W., et al. (2011). Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Developmental Cell*, *20*(5), 713–724. doi:10.1016/j.devcel.2011.04.014

Koning, M., Werker, P. M. N., van der Schaft, D. W. J., Bank, R. A., & Harmsen, M. C. (2012). MicroRNA-1 and MicroRNA-206 Improve Differentiation Potential of Human Satellite Cells: A Novel Approach for Tissue Engineering of Skeletal Muscle. *Tissue Engineering Part A*, *18*(9-10), 889–898. doi:10.1089/ten.tea.2011.0191

Koning, R., Zovko, S., Bárcena, M., Oostergetel, G., Koerten, H., Galjart, N., et al. (2008). Cryo electron tomography of vitrified fibroblasts: Microtubule plus ends in situ. *Journal of Structural Biology*, *161*(3), 459–468.

Korneluk, R. G., & Anderson, M. J. (1982). Stage dependency of forelimb regeneration on nerves in postmetamorphic froglets of *Xenopus laevis*. *Journal of Experimental Zoology, 220*(3), 331–342 .

Korneluk, R. G., & Liversage, R. A. (1984). Tissue regeneration in the amputated forelimb of *Xenopus laevis* froglets. *Canadian Journal of Zoology, 62(12), 2383–2391. doi:10.1139/z84-351*

Koshiba-Takeuchi, K., Mori, A. D., Kaynak, B. L., Cebra-Thomas, J., Sukonnik, T., Georges, R. O., et al. (2009). Reptilian heart development and the molecular basis of cardiac chamber evolution. *Nature*, *461*(7260), 95–98. doi:10.1038/nature08324

Kostakopoulou, K., Vogel, A., Brickell, P., & Tickle, C. (1996). "Regeneration" of wing bud stumps of chick embryos and reactivation of Msx-1 and Shh expression in response to FGF-4 and ridge signals. *Mechanisms of Development*, *55*(2), 119–131.

Koutsoulidou, A., Mastroyiannopoulos, N. P., Furling, D., Uney, J. B., & Phylactou, L. A. (2011). Expression of miR-1, miR-133a, miR-133b andmiR-206 increases during development of human skeletal muscle. *BMC Developmental Biology*, *11*(1), 34. doi:10.1186/1471-213X-11-34

Kozomara, A., & Griffiths-Jones, S. (2010). miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Research*, *39*(Database), D152–D157. doi:10.1093/nar/gkq1027

Kozomara, A., & Griffiths-Jones, S. (2013). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, *42*(D1), D68–D73. doi:10.1093/nar/gkt1181

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., & Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature*, *460*(7251), 60–65. doi:10.1038/nature08152

Kroesen, B. J., Teteloshvili, N., & Czepiel, K. S. (2015). Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology*. doi:10.1111/imm.12367

Krüger, J., & Rehmsmeier, M. (2006). RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Research*, *34*(Web Server issue), W451–4. doi:10.1093/nar/gkl243

Kumar, A., & Brockes, J. P. (2012). Nerve dependence in tissue, organ, and appendage regeneration. *Trends in Neurosciences*, *35*(11), 691–699. doi:10.1016/j.tins.2012.08.003

Kumar, A., Godwin, J. W., Gates, P. B., Garza-Garcia, A. A., & Brockes, J. P. (2007). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science (New York, NY)*, *318*(5851), 772–777. doi:10.1126/science.1147710

Kusumi, K., & Fisher, R. E. (2012). Studying Mechanisms of Regeneration in Amphibian and Reptilian Vertebrate Models. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *295*(10), 1529–1531. doi:10.1002/ar.22541

Kusumi, K., Kulathinal, R. J., Abzhanov, A., Boissinot, S., Crawford, N. G., Faircloth, B. C., et al. (2011). Developing a community-based genetic nomenclature for anole lizards. *BMC Genomics*, *12*(1), 554. doi:10.1186/1471-2164-12-554

Kusumi, K., May, C. M., & Eckalbar, W. L. (2013). A large-scale view of the evolution of amniote development: insights from somitogenesis in reptiles. *Current Opinion in Genetics & Development*, *23*(4), 491–497. doi:10.1016/j.gde.2013.02.011

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, *129*(7), 1401–1414. doi:10.1016/j.cell.2007.04.040

Lang, R. A., & Bishop, J. M. (1993). Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell*, *74*(3), 453–462.

Lee, L., Lau, P., & Chan, C. (1995). A simple and efficient treatment for fingertip injuries. *The Journal of Hand Surgery: Journal of the British Society for Surgery of the Hand*, *20*(1), 63–71. doi:10.1016/S0266-7681(05)80019-1

Lee, W. S., Jain, M. K., Arkonac, B. M., Zhang, D., Shaw, S. Y., Kashiki, S., et al. (1998). Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. *Circulation Research*, *82*(8), 845–851.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M., & Poss, K. D. (2005). Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. *Development*, *132,* 5173–5183.

Lee, Y., Hami, D., De Val, S., Kagermeier-Schenk, B., Wills, A. A., Black, B. L., et al. (2009). Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. *Developmental Biology*, *331*(2), 270–280. doi:10.1016/j.ydbio.2009.05.545

Lees, S. J., Rathbone, C. R., & Booth, F. W. (2006). Age-associated decrease in muscle precursor cell differentiation. *American Journal of Physiology. Cell Physiology*, *290*(2), C609–15. doi:10.1152/ajpcell.00408.2005

Lehoczky, J. A., Robert, B., & Tabin, C. J. (2011). Mouse digit tip regeneration is mediated by fate-restricted progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(51), 20609–20614. doi:10.1073/pnas.1118017108

Li, Z., Hassan, M. Q., Volinia, S., van Wijnen, A. J., Stein, J. L., Croce, C. M., et al. (2008). A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(37), 13906–13911. doi:10.1073/pnas.0804438105

Lin, G., Chen, Y., & Slack, J. M. W. (2013). Imparting Regenerative Capacity to Limbs by Progenitor Cell Transplantation. *Developmental Cell*, *24*(1), 41–51. doi:10.1016/j.devcel.2012.11.017

Liu, C., Teng, Z.-Q., Santistevan, N. J., Szulwach, K. E., Guo, W., Jin, P., & Zhao, X. (2010). Epigenetic Regulation of miR-184 by MBD1 Governs Neural Stem Cell Proliferation and Differentiation. *Stem Cell*, *6*(5), 433–444. doi:10.1016/j.stem.2010.02.017

Lobov, I. B., Rao, S., Carroll, T. J., Vallance, J. E., Ito, M., Ondr, J. K., et al. (2005). WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature*, *437*(7057), 417–421. doi:10.1038/nature03928

Loke, J. C., Stahlberg, E. A., Strenski, D. G., Haas, B. J., Wood, P. C., & Li, Q. Q. (2005). Compilation of mRNA polyadenylation signals in *Arabidopsis* revealed a new signal element and potential secondary structures. *Plant Physiology*, *138*(3), 1457–1468. doi:10.1104/pp.105.060541

Looso, M., Preussner, J., Sousounis, K., Bruckskotten, M., Michel, C. S., Lignelli, E., et al. (2013). A *de novo* assembly of the newt transcriptome combined with proteomic validation identifies new protein families expressed during tissue regeneration. *Genome Biology*, *14*(2), R16. doi:10.1186/gb-2013-14-2-r16

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. doi:10.1186/s13059-014-0550-8

Love, N. R., Chen, Y., Bonev, B., Gilchrist, M. J., Fairclough, L., Lea, R., et al. (2011). Genome-wide analysis of gene expression during *Xenopus tropicalis* tadpole tail regeneration. *BMC Developmental Biology*, *11*(1), 70. doi:10.1186/1471-213X-11-70

Lovern, M. B., & Wade, J. (2003). Yolk testosterone varies with sex in eggs of the lizard, *Anolis carolinensis*. *Journal of Experimental Zoology. Part a, Comparative Experimental Biology*, *295*(2), 206–210. doi:10.1002/jez.a.10225

Lozano-Velasco, E., & Galiano-Torres, J. (2014). miR-27 and miR-125 Distinctly Regulate Muscle-Enriched Transcription Factors in Cardiac and Skeletal Myocytes. *BioMed Research International*.

Lozito, T. P., & Tuan, R. S. (2015). Developmental Biology. *Developmental Biology*, 1–14. doi:10.1016/j.ydbio.2014.12.036

Lu, X., Borchers, A., Jolicoeur, C., Rayburn, H., & Baker, J. C. (2004). PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. *Nature, 430(6995), 93–8. doi:10.1038/nature02677*

Lucas, T., Waisman, A., Ranjan, R., Roes, J., Krieg, T., Müller, W., et al. (2010). Differential roles of macrophages in diverse phases of skin repair. *Journal of Immunology (Baltimore, Md : 1950)*, *184*(7), 3964–3977. doi:10.4049/jimmunol.0903356

Lyson, T. R., Sperling, E. A., Heimberg, A. M., Gauthier, J. A., King, B. L., & Peterson, K. J. (2012). MicroRNAs support a turtle + lizard clade. *Biology Letters*, *8*(1), 104–107. doi:10.1111/j.1525-142X.2010.00452.x

Maden, M., Manwell, L. A., & Ormerod, B. K. (2013). Proliferation zones in the axolotl brain and regeneration of the telencephalon. *Neural Development*, *8*(1), 1. doi:10.1186/1749-8104-8-1

Maderson, P. F., & Licht, P. (1968). Factors influencing rates of tail regeneration in the lizard *Anolis carolinensis*. *Experientia*, *24*(10), 1083–1086.

Maiorano, N., & Mallamaci, A. (2009). Promotion of embryonic cortico-cerebral neuronogenesis by miR-124. *Neural Development*, *4*(1), 40. doi:10.1186/1749-8104-4-40

Makanae, A., Mitogawa, K., & Satoh, A. (2014). Co-operative Bmp- and Fgf-signaling inputs convert skin wound healing to limb formation in urodele amphibians. *Developmental Biology*, 1–33. doi:10.1016/j.ydbio.2014.09.021

Makeyev, E. V., Zhang, J., Carrasco, M. A., & Maniatis, T. (2007). The MicroRNA miR-124 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA Splicing. *Molecular Cell*, *27*(3), 435–448. doi:10.1016/j.molcel.2007.07.015

Makino, S., Whitehead, G. G., Lien, C.-L., Kim, S., Jhawar, P., Kono, A., et al. (2005). Heat-shock protein 60 is required for blastema formation and maintenance during regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14599–14604. doi:10.1073/pnas.0507408102

Manceau, M., Gros, J., Savage, K., Thomé, V., McPherron, A., Paterson, B., & Marcelle, C. (2008). Myostatin promotes the terminal differentiation of embryonic muscle progenitors. *Genes & Development*, *22*(5), 668.

Marín-Teva, J. L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., & Mallat, M. (2004). Microglia promote the death of developing Purkinje cells. *Neuron*, *41*(4), 535–547.

Martini, R., Fischer, S., López-Vales, R., & David, S. (2008). Interactions between Schwann cells and macrophages in injury and inherited demyelinating disease. *Glia*, *56*(14), 1566–1577. doi:10.1002/glia.20766

Mccarthy, J. J., & Esser, K. A. (2006). MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *Journal of Applied Physiology*, *102*(1), 306–313. doi:10.1152/japplphysiol.00932.2006

McCusker, C., & Gardiner, D. M. (2011). The axolotl model for regeneration and aging research: a mini-review. *Gerontology*, *57*(6), 565–571. doi:10.1159/000323761

McLean, K. E., & Vickaryous, M. K. (2011). A novel amniote model of epimorphic regeneration: the leopard gecko, *Eublepharis macularius*. *BMC Developmental Biology*, *11*(1), 50. doi:10.1186/1471-213X-11-50

Mercer, S. E., Cheng, C.-H., Atkinson, D. L., Krcmery, J., Guzman, C. E., Kent, D. T., et al. (2012). Multi-tissue microarray analysis identifies a molecular signature of regeneration. *PLoS ONE*, *7*(12), e52375. doi:10.1371/journal.pone.0052375

Mercola, M. (2012). Cardiovascular biology: A boost for heart regeneration. *Nature*, *492*(7429), 360–362. doi:10.1038/nature11763

Mescher, A. L. (1996). The cellular basis of limb regeneration in urodeles. *The International Journal of Developmental Biology*, *40*(4), 785–795.

Mescher, A. L., & Neff, A. W. (2006). Limb regeneration in amphibians: immunological considerations. *The Scientific World Journal*, *6 Suppl 1*, 1–11. doi:10.1100/tsw.2006.323

Michaelson, M. D., Mehler, M. F., Xu, H., Gross, R. E., & Kessler, J. A. (1996). Interleukin-7 is trophic for embryonic neurons and is expressed in developing brain. *Developmental Biology*, *179*(1), 251–263. doi:10.1006/dbio.1996.0255

Min, H., & Yoon, S. (2010). Got target? Computational methods for microRNA target prediction and their extension. *Experimental & Molecular Medicine*, *42*(4), 233–244. doi:10.3858/emm.2010.42.4.032

Minami, Y., Oishi, I., Endo, M., & Nishita, M. (2010). Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. *Developmental Dynamics*, *239*(1), 1–15. doi:10.1002/dvdy.21991

Miska, E. A., Alvarez-Saavedra, E., Townsend, M., Yoshii, A., Šestan, N., Rakic, P., et al. (2004). Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biology*, *5*(9), R68. doi:10.1186/gb-2004-5-9-r68

Mohammad, K. S., & Neufeld, D. A. (2000). Denervation retards but does not prevent toetip regeneration. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society, 8(4), 277–81.*

Mohammad, K. S., Day, F. A., & Neufeld, D. A. (1999). Bone growth is induced by nail transplantation in amputated proximal phalanges. *Calcified Tissue International*, *65*(5), 408–410.

Monaghan, J. R., Athippozhy, A., Seifert, A. W., Putta, S., Stromberg, A. J., Maden, M., et al. (2012). Gene expression patterns specific to the regenerating limb of the Mexican axolotl. *Biology Open*, *1*(10), 937–948. doi:10.1242/bio.20121594

Monaghan, J. R., Epp, L. G., Putta, S., Page, R. B., Walker, J. A., Beachy, C. K., et al. (2009). Microarray and cDNA sequence analysis of transcription during nerve-dependent limb regeneration. *BMC Biology*, *7*(1), 1. doi:10.1186/1741-7007-7-1

Montuelle, S. J., Daghfous, G., & Bels, V. L. (2008). Effect of locomotor approach on feeding kinematics in the green anole (*Anolis carolinensis*). *Journal of Experimental Zoology. Part a, Ecological Genetics and Physiology*, *309*(9), 563–567. doi:10.1002/jez.484

Montuelle, S. J., Herrel, A., Libourel, P.-A., Reveret, L., & Bels, V. L. (2009). Locomotor-feeding coupling during prey capture in a lizard (*Gerrhosaurus major*): effects of prehension mode. *Journal of Experimental Biology*, *212*(Pt 6), 768–777. doi:10.1242/jeb.026617

Morrison, J. I., Borg, P., & Simon, A. (2010). Plasticity and recovery of skeletal muscle satellite cells during limb regeneration. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *24*(3), 750–756. doi:10.1096/fj.09-134825

Morrison, J. I., Lööf, S., He, P., & Simon, A. (2006). Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population. *The Journal of Cell Biology*, *172*(3), 433–440. doi:10.1083/jcb.200509011

Mulcahy, H., O'Rourke, K. P., Adams, C., Molloy, M. G., & O'Gara, F. (2006). LST1 and NCR3 expression in autoimmune inflammation and in response to IFN-gamma, LPS and microbial infection. *Immunogenetics*, *57*(12), 893–903. doi:10.1007/s00251-005-0057-2

Muneoka, K., & Bryant, S. V. (1982). Evidence that patterning mechanisms in developing and regenerating limbs are the same. *Nature*, *298*(5872), 369–371.

Muneoka, K., Allan, C. H., Yang, X., Lee, J., & Han, M. (2008). Mammalian regeneration and regenerative medicine. *Birth Defects Research. Part C, Embryo Today : Reviews*, *84*(4), 265–280. doi:10.1002/bdrc.20137

Murawala, P., Tanaka, E. M., & Currie, J. D. (2012). Regeneration: The ultimate example of wound healing. *Seminars in Cell and Developmental Biology*, *23*(9), 954–962. doi:10.1016/j.semcdb.2012.09.013

Nabrit, S. M. (1929). The Rôle of the Fin Rays in the Regeneration in the Tail-Fins of Fishes in *Fundulus* and Goldfish. *Biological Bulletin, 56*(4), 235–266.

Nacu, E., & Tanaka, E. M. (2011). Limb Regeneration: A New Development? *Annual Review of Cell and Developmental Biology*, *27*(1), 409–440. doi:10.1146/annurev-cellbio-092910-154115

Nacu, E., Glausch, M., Le, H. Q., Damanik, F. F. R., Schuez, M., Knapp, D., et al. (2013). Connective tissue cells, but not muscle cells, are involved in establishing the proximo-distal outcome of limb regeneration in the axolotl. *Development*, *140*(3), 513–518. doi:10.1242/dev.081752

Nair, D. G., Fry, B. G., Alewood, P., Kumar, P. P., & Kini, R. M. (2007). Antimicrobial activity of omwaprin, a new member of the waprin family of snake venom proteins. *Biochemical Journal*, *402*(1), 93–104. doi:10.1042/BJ20060318

Nakamura, K., Maki, N., Trinh, A., Trask, H. W., Gui, J., Tomlinson, C. R., & Tsonis, P. A. (2010). miRNAs in Newt Lens Regeneration: Specific Control of Proliferation and Evidence for miRNA Networking. *PLoS ONE*, *5*(8), e12058. doi:10.1371/journal.pone.0012058.s001

Nambiar, V. V., Bhatt, I. Y., Deshmukh, P. A., & Jape, R. R. (2008). Assessment of extracellular matrix remodeling during tail regeneration in the lizard *Hemidactylus flaviviridis*. *Journal of Endocrinology and Reproduction, 12*(2), 67–72.

Namenwirth, M. (1974). The inheritance of cell differentiation during limb regeneration in the axolotl. *Developmental Biology*, *41*(1), 42–56.

Neale, D. B., Wegrzyn, J. L., Stevens, K. A., Zimin, A. V., Puiu, D., Crepeau, M. W., et al. (2014). Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology*, *15*(3), R59. doi:10.1186/gb-2014-15-3-r59

Nechiporuk, A., Poss, K. D., Johnson, S. L., & Keating, M. T. (2003). Positional cloning of a temperature-sensitive mutant emmental reveals a role for sly1 during cell proliferation in zebrafish fin regeneration. *Developmental Biology*, *258*(2), 291–306.

Neff, A. W., King, M. W., Harty, M. W., Nguyen, T., Calley, J., Smith, R. C., & Mescher, A. L. (2005). Expression of *Xenopus* XlSALL4 during limb development and regeneration. *Developmental Dynamics*, *233*(2), 356–367. doi:10.1002/dvdy.20363

Neufeld, D. A., & Zhao, W. (1995). Bone regrowth after digit tip amputation in mice is equivalent in adults and neonates. *Wound Repair and Regeneration : Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, *3*(4), 461–466. doi:10.1046/j.1524-475X.1995.30410.x

Nucera, S., Biziato, D., & De Palma, M. (2011). The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. *The International Journal of Developmental Biology*, *55*(4-5), 495–503. doi:10.1387/ijdb.103227sn

O'Regan, A., & Berman, J. S. (2000). Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *International journal of experimental pathology, 81(6), 373–90. doi:10.1046/j.1365-2613.2000.00163.x*

Oddie, G. W., Schenk, G., Angel, N. Z., Walsh, N., Guddat, L. W., de Jersey, J., et al. (2000). Structure, function, and regulation of tartrate-resistant acid phosphatase. *Bone*, *27*(5), 575–584.

Ogai, K., Nakatani, K., Hisano, S., Sugitani, K., Koriyama, Y., & Kato, S. (2014). Function of Sox2 in ependymal cells of lesioned spinal cords in adult zebrafish. *Neuroscience Research*, 1–4. doi:10.1016/j.neures.2014.07.010

Ohgo, S., Itoh, A., Suzuki, M., Satoh, A., Yokoyama, H., & Tamura, K. (2010). Analysis of hoxa11 and hoxa13 expression during patternless limb regeneration in *Xenopus*. *Developmental Biology*, *338*(2), 148–157. doi:10.1016/j.ydbio.2009.11.026

Otteson, D. C., & Hitchcock, P. F. (2003). Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. *Vision Research*, *43*(8), 927–936.

Paiva, K. B. S., & Granjeiro, J. M. (2014). Bone tissue remodeling and development: focus on matrix metalloproteinase functions. *Archives of Biochemistry and Biophysics*, *561*, 74–87. doi:10.1016/j.abb.2014.07.034

Panaite, P.-A., & Barakat-Walter, I. (2010). Thyroid hormone enhances transected axonal regeneration and muscle reinnervation following rat sciatic nerve injury. *Journal of Neuroscience Research*, *88*(8), 1751–1763. doi:10.1002/jnr.22344

Park, J. E., & Barbul, A. (2004). Understanding the role of immune regulation in wound healing. *The American Journal of Surgery, 187(5A), 11S–16S.* doi:10.1016/S0002-9610(03)00296-4

Parks, W. C., Wilson, C. L., & López-Boado, Y. S. (2004). Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature Reviews. Immunology*, *4*(8), 617–629. doi:10.1038/nri1418

Pasquinelli, A. E. (2012). MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nature Publishing Group*, *13*(4), 271–282. doi:10.1038/nrg3162

Peadon, A. M., & Singer, M. (1966). The blood vessels of the regenerating limb of the adult newt, *Triturus*. *Journal of Morphology*, *118*(1), 79–89. doi:10.1002/jmor.1051180106

Peradziryi, H., Tolwinski, N. S., & Borchers, A. (2012). The many roles of PTK7: a versatile regulator of cell-cell communication. *Archives of Biochemistry and Biophysics*, *524*(1), 71–76. doi:10.1016/j.abb.2011.12.019

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., & Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science (New York, NY)*, *331*(6020), 1078–1080. doi:10.1126/science.1200708

Poss, K. D., Shen, J., Nechiporuk, A., McMahon, G., Thisse, B., Thisse, C., & Keating, M. T. (2000). Roles for Fgf signaling during zebrafish fin regeneration. *Developmental Biology*, *222*(2), 347–358. doi:10.1006/dbio.2000.9722

Poss, K. D., Wilson, L. G., & Keating, M. T. (2002). Heart regeneration in zebrafish. *Science (New York, NY)*, *298*(5601), 2188–2190. doi:10.1126/science.1077857

Pourrajab, F., Babaei Zarch, M., BaghiYazdi, M., Hekmatimoghaddam, S., & Zare-Khormizi, M. R. (2014). MicroRNA-based system in stem cell reprogramming; differentiation/dedifferentiation. *The International Journal of Biochemistry & Cell Biology*, *55*, 318–328. doi:10.1016/j.biocel.2014.08.008

Pownall, M. E., & Isaacs, H. V. (2010). FGF Signalling in Vertebrate Development. *Colloquium Series on Developmental Biology. (Morgan and Claypool).*

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., et al. (2012). The Pfam protein families database. *Nucleic Acids Research*, *40*(Database issue), D290–301. doi:10.1093/nar/gkr1065

Purhonen, S., Palm, J., Rossi, D., Kaskenpää, N., Rajantie, I., Ylä-Herttuala, S., et al. (2008). Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(18), 6620–6625. doi:10.1073/pnas.0710516105

Qin, X., Jiang, Q., Matsuo, Y., Kawane, T., Komori, H., Moriishi, T., et al. (2015). Cbfb regulates bone development by stabilizing runx family proteins. *Journal of Bone and Mineral Research : the Official Journal of the American Society for Bone and Mineral Research*, *30*(4), 706–714. doi:10.1002/jbmr.2379

Rehmsmeier, M., Steffen, P., Hochsmann, M., & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *Rna*, *10*(10), 1507–1517. doi:10.1261/rna.5248604

Reimer, M. M., Sörensen, I., Kuscha, V., Frank, R. E., Liu, C., Becker, C. G., & Becker, T. (2008). Motor neuron regeneration in adult zebrafish. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *28*(34), 8510–8516. doi:10.1523/JNEUROSCI.1189-08.2008

Revardel, J. L., & Chebouki, F. (1987). Étude de la réponse l'amputation des phalanges chez la souris: rôle morphogénétique des épithéliums, stimulation de la chondrogense. *Canadian Journal of Zoology, 65*(12), 3166–3176.

Reynolds, A. R., Reynolds, L. E., Nagel, T. E., Lively, J. C., Robinson, S. D., Hicklin, D. J., et al. (2004). Elevated Flk1 (vascular endothelial growth factor receptor 2) signaling mediates enhanced angiogenesis in beta3-integrin-deficient mice. *Cancer Research*, *64*(23), 8643–8650. doi:10.1158/0008-5472.CAN-04-2760

Rhind, N., Chen, Z., Yassour, M., Thompson, D. A., Haas, B. J., Habib, N., et al. (2011). Comparative functional genomics of the fission yeasts. *Science (New York, NY)*, *332*(6032), 930–936. doi:10.1126/science.1203357

Rinkevich, Y., Lindau, P., Ueno, H., Longaker, M. T., & Weissman, I. L. (2011). Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. *Nature*, *476*(7361), 409–413. doi:10.1038/nature10346

Ritzman, T. B., Stroik, L. K., Julik, E., Hutchins, E. D., Lasku, E., Denardo, D. F., et al. (2012). The gross anatomy of the original and regenerated tail in the green anole (*Anolis carolinensis*). *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *295*(10), 1596–1608. doi:10.1002/ar.22524

Roberts, J. C., Warren, R. B., Griffiths, C. E. M., & Ross, K. (2013). Expression of microRNA-184 in keratinocytes represses argonaute 2. *Journal of Cellular Physiology*, *228*(12), 2314–2323. doi:10.1002/jcp.24401

Robertson, G., Schein, J., Chiu, R., Corbett, R., Field, M., Jackman, S. D., et al. (2010). *De novo* assembly and analysis of RNA-seq data, *7*(11), 909–912. doi:10.1038/nmeth.1517

Rollinger-Holzinger, I., Eibl, B., Pauly, M., Griesser, U., Hentges, F., Auer, B., et al. (2000). LST1: a gene with extensive alternative splicing and immunomodulatory function. *Journal of Immunology (Baltimore, Md. : 1950)*, *164*(6), 3169–3176.

Rosenwald, M. (2010). A doctor, a pig, and a magical pixie dust that could regrow fingers. *Esquire*, 18th September.

Roy, S., & Gardiner, D. M. (2002). Cyclopamine induces digit loss in regenerating axolotl limbs. *The Journal of Experimental Zoology*, *293*(2), 186–190. doi:10.1002/jez.10110

Roy, S., Gardiner, D. M., & Bryant, S. V. (2000). Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of Shh. *Developmental Biology*, *218*(2), 199–205. doi:10.1006/dbio.1999.9556

Ruffell, D., Mourkioti, F., Gambardella, A., Kirstetter, P., Lopez, R. G., Rosenthal, N., & Nerlov, C. (2009). A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(41), 17475–17480. doi:10.1073/pnas.0908641106

Ryffel, G. U. (2003). Tagging muscle cell lineages in development and tail regeneration using Cre recombinase in transgenic *Xenopus*. *Nucleic Acids Research*, *31*(8), 44e–44. doi:10.1093/nar/gng044

Said, S., Parke, W., & Neufeld, D. A. (2004). Vascular supplies differ in regenerating and nonregenerating amputated rodent digits. *The Anatomical Record. Part a, Discoveries in Molecular, Cellular, and Evolutionary Biology*, *278*(1), 443–449. doi:10.1002/ar.a.20034

Sakuma, K., Nishikawa, J., Nakao, R., Watanabe, K., Totsuka, T., Nakano, H., et al. (2003). Calcineurin is a potent regulator for skeletal muscle regeneration by association with NFATc1 and GATA-2. *Acta Neuropathologica*, *105*(3), 271–280. doi:10.1007/s00401-002-0647-0

Samson, M., Edinger, A. L., Stordeur, P., Rucker, J., Verhasselt, V., Sharron, M., et al. (1998). ChemR23, a putative chemoattractant receptor, is expressed in monocyte-derived dendritic cells and macrophages and is a coreceptor for SIV and some primary HIV-1 strains. *European Journal of Immunology*, *28*(5), 1689–1700. doi:10.1002/(SICI)1521-4141(199805)28:05<1689::AID-IMMU1689>3.0.CO;2-I

Sandoval-Guzmán, T., Wang, H., Khattak, S., Schuez, M., Roensch, K., Nacu, E., et al. (2014). Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell*, *14*(2), 174–187. doi:10.1016/j.stem.2013.11.007

Santamaría, J. A., & Becerra, J. (1991). Tail fin regeneration in teleosts: cell-extracellular matrix interaction in blastemal differentiation. *Journal of Anatomy*, *176*, 9–21.

Santos-Ruiz, L., Santamaría, J. A., Ruiz-Sánchez, J., & Becerra, J. (2002). Cell proliferation during blastema formation in the regenerating teleost fin. *Developmental Dynamics*, *223*(2), 262–272. doi:10.1002/dvdy.10055

Sanuki, R., Onishi, A., Koike, C., Muramatsu, R., Watanabe, S., Muranishi, Y., et al. (2011). miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nature Publishing Group*, *14*(9), 1125–1134. doi:10.1038/nn.2897

Satoh, A., Bryant, S. V., & Gardiner, D. M. (2012). Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (*Ambystoma mexicanum*). *Developmental Biology*, *366*(2), 374–381. doi:10.1016/j.ydbio.2012.03.022

Satoh, A., Endo, T., Abe, M., Yakushiji, N., Ohgo, S., Tamura, K., & Ide, H. (2006). Characterization of *Xenopus* digits and regenerated limbs of the froglet. *Developmental Dynamics*, *235*(12), 3316–3326. doi:10.1002/dvdy.20985

Satoh, A., Graham, G. M. C., Bryant, S. V., & Gardiner, D. M. (2008). Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (*Ambystoma mexicanum*). *Developmental Biology*, *319*(2), 321–335. doi:10.1016/j.ydbio.2008.04.030

Satoh, A., Ide, H., & Tamura, K. (2005). Muscle formation in regenerating *Xenopus* froglet limb. *Developmental Dynamics*, *233*(2), 337–346. doi:10.1002/dvdy.20349

Satoh, A., James, M. A., & Gardiner, D. M. (2009). The role of nerve signaling in limb genesis and agenesis during axolotl limb regeneration. *The Journal of Bone and Joint Surgery. American Volume*, *91 Suppl 4*, 90–98. doi:10.2106/JBJS.I.00159

Satoh, A., Makanae, A., Hirata, A., & Satou, Y. (2011). Blastema induction in aneurogenic state and *Prrx-1* regulation by MMPs and FGFs in *Ambystoma mexicanum* limb regeneration. *Developmental Biology*, *355*(2), 263–274. doi:10.1016/j.ydbio.2011.04.017

Saxena, A., Chen, W., Su, Y., Rai, V., Uche, O. U., Li, N., & Frangogiannis, N. G. (2013). IL-1 induces proinflammatory leukocyte infiltration and regulates fibroblast phenotype in the infarcted myocardium. *Journal of Immunology (Baltimore, Md. : 1950)*, *191*(9), 4838–4848. doi:10.4049/jimmunol.1300725

Schneider, L., Cammer, M., Lehman, J., Nielsen, S. K., Guerra, C. F., Veland, I. R., et al. (2010). Directional cell migration and chemotaxis in wound healing response to PDGF-AA are coordinated by the primary cilium in fibroblasts. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *25*(2-3), 279–292. doi:10.1159/000276562

Sehm, T., Sachse, C., Frenzel, C., & Echeverri, K. (2009). miR-196 is an essential early-stage regulator of tail regeneration, upstream of key spinal cord patterning events. *Developmental Biology*, *334*(2), 468–480. doi:10.1016/j.ydbio.2009.08.008

Seifert, A. W., Kiama, S. G., Seifert, M. G., Goheen, J. R., Palmer, T. M., & Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (*Acomys*). *Nature*, *489*(7417), 561–565. doi:10.1038/nature11499

Shen, Y., Ji, G., Haas, B. J., Wu, X., Zheng, J., Reese, G. J., & Li, Q. Q. (2008). Genome level analysis of rice mRNA 3'-end processing signals and alternative polyadenylation. *Nucleic Acids Research*, *36*(9), 3150–3161. doi:10.1093/nar/gkn158

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J. M., & Birol, I. (2009). ABySS: a parallel assembler for short read sequence data. *Genome Research*, *19*(6), 1117–1123. doi:10.1101/gr.089532.108

Simpson, S. B. (1964). Analysis of Tail Regeneration in the Lizard *Lygosoma* laterale. I. Initiation of Regeneration and Cartilage Differentiation: The Role of Ependyma. *Journal of Morphology*, *114*, 425–435. doi:10.1002/jmor.1051140305

Simpson, S. B. (1965). Regeneration of the lizard tail. *Regeneration in Animals and Related Problems. North-Holland, Amsterdam*, 431–443.

Simpson, S. B. (1968). Morphology of the regenerated spinal cord in the lizard, *Anolis carolinensis*. *Journal of Comparative Neurology*, *134*(2), 193–210. doi:10.1002/cne.901340207

Simpson, S. B., & Duffy, M. T. (1994). The lizard spinal cord: a model system for the study of spinal cord injury and repair. *Progress in Brain Research*, *103*, 229–241.

Singer, M. (1974). Neurotrophic control of limb regeneration in the newt. *Annals of the New York Academy of Sciences*, 308–322.

Singer, M., Weckesser, E. C., Géraudie, J., Maier, C. E., & Singer, J. (1987). Open finger tip healing and replacement after distal amputation in rhesus monkey with comparison to limb regeneration in lower vertebrates. *Anatomy and Embryology*, *177*(1), 29–36.

Singh, S. P., Holdway, J. E., & Poss, K. D. (2012). Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Developmental Cell*, *22*(4), 879–886. doi:10.1016/j.devcel.2012.03.006

Sjogren, R. J. O., Egan, B., Katayama, M., Zierath, J. R., & Krook, A. (2014). Temporal analysis of reciprocal miRNA-mRNA expression patterns predicts regulatory networks during differentiation in human skeletal muscle cells. *Physiological Genomics*. doi:10.1152/physiolgenomics.00037.2014

Slack, J. M. W., Lin, G., & Chen, Y. (2007). Molecular and Cellular Basis of Regeneration and Tissue Repair. *Cellular and Molecular Life Sciences*, *65*(1), 54–63. doi:10.1007/s00018-007-7431-1

Smith, A. R., & Wolpert, L. (1975). Nerves and angiogenesis in amphibian limb regeneration. *Nature*, *257*(5523), 224–225.

Sousa, S., Afonso, N., Bensimon-Brito, A., Fonseca, M., Simões, M., Leon, J., et al. (2011). Differentiated skeletal cells contribute to blastema formation during zebrafish fin regeneration. *Development*, *138*(18), 3897–3905. doi:10.1242/dev.064717

St Pierre, L., Earl, S. T., Filippovich, I., Sorokina, N., Masci, P. P., de Jersey, J., & Lavin, M. F. (2008). Common evolution of waprin and kunitz-like toxin families in Australian venomous snakes. *Cellular and Molecular Life Sciences*, *65*(24), 4039–4054. doi:10.1007/s00018-008-8573-5

Stefani, G., & Slack, F. J. (2008). Small non-coding RNAs in animal development. *Nature Reviews Molecular Cell Biology*, *9*(3), 219–230. doi:10.1038/nrm2347

Stefater, J. A., Ren, S., Lang, R. A., & Duffield, J. S. (2011). Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. *Trends in Molecular Medicine*, *17*(12), 743–752. doi:10.1016/j.molmed.2011.07.009

Stewart, R., Rascon, C. A., Tian, S., Nie, J., Barry, C., Chu, L.-F., et al. (2013). Comparative RNA-seq analysis in the unsequenced axolotl: the oncogene burst highlights early gene expression in the blastema. *PLoS Computational Biology*, *9*(3), e1002936. doi:10.1371/journal.pcbi.1002936

Stewart, S., & Stankunas, K. (2012). Limited dedifferentiation provides replacement tissue during zebrafish fin regeneration. *Developmental Biology*, *365*(2), 339–349. doi:10.1016/j.ydbio.2012.02.031

Stocum, D. L., & Cameron, J. A. (2011). Looking proximally and distally: 100 years of limb regeneration and beyond. *Developmental Dynamics*, *240*(5), 943–968. doi:10.1002/dvdy.22553

Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N., & Moon, R. T. (2006). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development*, *134*(3), 479–489. doi:10.1242/dev.001123

Sugiura, T., Tazaki, A., Ueno, N., Watanabe, K., & Mochii, M. (2009). *Xenopus* Wnt-5a induces an ectopic larval tail at injured site, suggesting a crucial role for noncanonical Wnt signal in tail regeneration. *Mechanisms of Development*, *126*(1-2), 56–67. doi:10.1016/j.mod.2008.10.002

Suksaweang, S., Lin, C.-M., Jiang, T.-X., Hughes, M. W., Widelitz, R. B., & Chuong, C.-M. (2004). Morphogenesis of chicken liver: identification of localized growth zones and the role of β-catenin/Wnt in size regulation. *Developmental Biology*, *266*(1), 109–122. doi:10.1016/j.ydbio.2003.10.010

Summerbell, D., & Tickle, C. (1977). Pattern formation along the antero-posterior axis of the chick limb bud. *Vertebrate Limb and Somite Morphogenesis: The Third Symposium of the British Society for Developmental Biology*, 41–53.

Sun, L., Luo, H., Bu, D., Zhao, G., Yu, K., Zhang, C., et al. (2013). Utilizing sequence intrinsic composition to classify protein-coding and long non-coding transcripts. *Nucleic Acids Research*, *41*(17), e166. doi:10.1093/nar/gkt646

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS ONE*, *6*(7), e21800. doi:10.1371/journal.pone.0021800

Suzuki, M., Satoh, A., Ide, H., & Tamura, K. (2005). Nerve-dependent and -independent events in blastema formation during *Xenopus* froglet limb regeneration. *Developmental Biology*, *286*(1), 361–375. doi:10.1016/j.ydbio.2005.08.021

Takahashi, Y., Takagi, A., Hiraoka, S., Koseki, H., Kanno, J., Rawls, A., & Saga, Y. (2007). Transcription factors Mesp2 and Paraxis have critical roles in axial musculoskeletal formation. *Developmental Dynamics*, *236*(6), 1484–1494. doi:10.1002/dvdy.21178

Takeo, M., Chou, W. C., Sun, Q., Lee, W., Rabbani, P., Loomis, C., et al. (2013). Wnt activation in nail epithelium couples nail growth to digit regeneration. *Nature*, *499*(7457), 228–232. doi:10.1038/nature12214

Takeo, M., Lee, W., & Ito, M. (2015). Wound healing and skin regeneration. *Cold Spring Harbor Perspectives in Medicine*, *5*(1), a023267. doi:10.1101/cshperspect.a023267

Tamura, K., Ohgo, S., & Yokoyama, H. (2010). Limb blastema cell: a stem cell for morphological regeneration. *Development, Growth & Differentiation*, *52*(1), 89–99. doi:10.1111/j.1440-169X.2009.01144.x

Tanaka, E. M., & Reddien, P. W. (2011). The cellular basis for animal regeneration. *Developmental Cell*, *21*(1), 172–185. doi:10.1016/j.devcel.2011.06.016

Tanimura, N., Saito, M., Ebisuya, M., Nishida, E., & Ishikawa, F. (2013). Stemness-related factor Sall4 interacts with transcription factors Oct-3/4 and Sox2 and occupies Oct-Sox elements in mouse embryonic stem cells. *Journal of Biological Chemistry*, *288*(7), 5027–5038. doi:10.1074/jbc.M112.411173

Tassava, R. A., Bennett, L. L., & Zitnik, G. D. (1974). DNA synthesis without mitosis in amputated denervated forelimbs of larval axolotls. *The Journal of Experimental Zoology*, *190*(1), 111–116. doi:10.1002/jez.1401900110

Tatsumi, R., Yamada, M., Katsuki, Y., Okamoto, S., Ishizaki, J., Mizunoya, W., et al. (2006). Low-pH preparation of skeletal muscle satellite cells can be used to study activation in vitro. *The International Journal of Biochemistry & Cell Biology*, *38*(10), 1678–1685. doi:10.1016/j.biocel.2006.04.003

Tattikota, S. G., Rathjen, T., McAnulty, S. J., Wessels, H.-H., Akerman, I., van de Bunt, M., et al. (2014). Argonaute2 Mediates Compensatory Expansion of the Pancreatic b Cell. *Cell Metabolism*, *19*(1), 122–134. doi:10.1016/j.cmet.2013.11.015

Thatcher, E. J., Paydar, I., Anderson, K. K., & Patton, J. G. (2008). Regulation of zebrafish fin regeneration by microRNAs. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(47), 18384–18389. doi:10.1073/pnas.0803713105

Thornton, C. S. (1938). The histogenesis of the regenerating fore limb of larval *Amblystoma* after exarticulation of the humerus. *Journal of Morphology*, *62*(2), 219–241. doi:10.1002/jmor.1050620204

Tidball, J. G., & Wehling-Henricks, M. (2007). Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *The Journal of Physiology*, *578*(Pt 1), 327–336. doi:10.1113/jphysiol.2006.118265

Tollis, M., & Boissinot, S. (2014). Genetic variation in the green anole lizard (*Anolis carolinensis*) reveals island refugia and a fragmented Florida during the quaternary. *Genetica*, *142*(1), 59–72. doi:10.1007/s10709-013-9754-1

Torok, M. A., Gardiner, D. M., Shubin, N. H., & Bryant, S. V. (1998). Expression of HoxD genes in developing and regenerating axolotl limbs. *Developmental Biology*, *200*(2), 225–233. doi:10.1006/dbio.1998.8956

Tracey, W. D., & Speck, N. A. (2000). Potential roles for RUNX1 and its orthologs in determining hematopoietic cell fate. *Seminars in Cell and Developmental Biology*, *11*(5), 337–342. doi:10.1006/scdb.2000.0186

Trapnell, C., Hendrickson, D. G., Sauvageau, M., Goff, L., Rinn, J. L., & Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology*, *31*(1), 46–53. doi:10.1038/nbt.2450

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, *7*(3), 562–578. doi:10.1038/nprot.2012.016

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., et al. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, *28*(5), 516–520. doi:10.1038/nbt.1621

Tsonis, P. A. (2008). Stem cells and blastema cells. *Current Stem Cell Research & Therapy*, *3*(1), 53–54.

Tsonis, P. A., Call, M. K., Grogg, M. W., Sartor, M. A., Taylor, R. R., Forge, A., et al. (2007). MicroRNAs and regeneration: Let-7 members as potential regulators of dedifferentiation in lens and inner ear hair cell regeneration of the adult newt. *Biochemical and Biophysical Research Communications*, *362*(4), 940–945. doi:10.1016/j.bbrc.2007.08.077

Tu, S., & Johnson, S. L. (2011). Fate restriction in the growing and regenerating zebrafish fin. *Developmental Cell*, *20*(5), 725–732. doi:10.1016/j.devcel.2011.04.013

Tucker, A. S., & Slack, J. (1995). The *Xenopus laevis* tail-forming region. *Development, 121*, 249–262.

Turner, J. E., & Singer, M. (1973). Some morphological and ultrastructural changes in the ependyma of the amputation stump during early regeneration of the tail in the lizard, *Anolis carolinensis*. *Journal of Morphology, 140*(3), 257–269. doi:10.1002/jmor.1051400302

Undi, R. B., Kandi, R., & Gutti, R. K. (2013). MicroRNAs as Haematopoiesis Regulators. *Advances in Hematology*, *2013*, 695754. doi:10.1155/2013/695754

Valle, L. D., Nardi, A., Toffolo, V., & Niero, C. (2007). Cloning and characterization of scale beta-keratins in the differentiating epidermis of geckoes show they are glycine-proline-serine-rich proteins with a central motif homologous to avian beta-keratins. *Developmental dynamics : an official publication of the American Association of Anatomists*, *236*(2), 374–88. doi:10.1002/dvdy.21022

van Rooij, E., Liu, N., & Olson, E. N. (2008). MicroRNAs flex their muscles. *Trends in Genetics*, *24*(4), 159–166. doi:10.1016/j.tig.2008.01.007

Vidal, P., & Dickson, M. G. (1993). Regeneration of the distal phalanx. A case report. *Journal of Hand Surgery (Edinburgh, Scotland)*, *18*(2), 230–233.

Vinarsky, V., Atkinson, D. L., Stevenson, T. J., Keating, M. T., & Odelberg, S. J. (2005). Normal newt limb regeneration requires matrix metalloproteinase function. *Developmental Biology*, *279*(1), 86–98. doi:10.1016/j.ydbio.2004.12.003

Wade, J. (2012). Sculpting reproductive circuits: relationships among hormones, morphology and behavior in anole lizards. *General and Comparative Endocrinology*, *176*(3), 456–460. doi:10.1016/j.ygcen.2011.12.011

Wagers, A. J., & Weissman, I. L. (2004). Plasticity of adult stem cells. *Cell, 116(5), 639–48.*

Wagers, A. J., Sherwood, R. I., Christensen, J. L., & Weissman, I. L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science (New York, NY)*, *297*(5590), 2256–2259. doi:10.1126/science.1074807

Wallace, B. M., & Wallace, H. (1973). Participation of grafted nerves in amphibian limb regeneration. *Journal of Embryology and Experimental Morphology*, *29*(3), 559–570.

Wallis, JW, Aerts, J, Groenen, M., & Crooijmans, R. (2004). A physical map of the chicken genome. *Nature, 432*, 761–764.

Wang, Y., Wang, R., Jiang, S., Zhou, W., Liu, Y., Wang, Y., et al. (2011). Gecko CD59 Is Implicated in Proximodistal Identity during Tail Regeneration. *PLoS ONE*, *6*(3), e17878. doi:10.1371/journal.pone.0017878.g008

Wehner, D., Cizelsky, W., Vasudevaro, M. D., Özhan, G., Haase, C., Kagermeier-Schenk, B., et al. (2014). Wnt/b-Catenin Signaling Defines Organizing Centers that Orchestrate Growth and Differentiationof the Regenerating Zebrafish Caudal Fin. *CellReports*, *6*(3), 467–481. doi:10.1016/j.celrep.2013.12.036

Weissman, I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell, 100(1), 157–68.*

Wheeler, B. M., Heimberg, A. M., Moy, V. N., Sperling, E. A., Holstein, T. W., Heber, S., & Peterson, K. J. (2009). The deep evolution of metazoan microRNAs. *Evolution & Development*, *11*(1), 50–68. doi:10.1111/j.1525-142X.2008.00302.x

Whimster, I. W. (1978). Nerve supply as a stimulator of the growth of tissues including skin. *Clinical and Experimental Dermatology, 3*(4), 389–410.

Whitehead, G. G., Makino, S., Lien, C.-L., & Keating, M. T. (2005). fgf20 is essential for initiating zebrafish fin regeneration. *Science (New York, NY)*, *310*(5756), 1957–1960. doi:10.1126/science.1117637

Williams, A. H., Liu, N., van Rooij, E., & Olson, E. N. (2009). MicroRNA control of muscle development and disease. *Current Opinion in Cell Biology*, *21*(3), 461–469. doi:10.1016/j.ceb.2009.01.029

Wilson-Rawls, J., Molkentin, J. D., Black, B. L., & Olson, E. N. (1999). Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. *Molecular and Cellular Biology*, *19*(4), 2853–2862.

Wordley, C., Slate, J., & Stapley, J. (2011). Mining online genomic resources in *Anolis carolinensis* facilitates rapid and inexpensive development of cross-species microsatellite markers for the *Anolis* lizard genus. *Molecular Ecology Resources*, *11*(1), 126–133. doi:10.1111/j.1755-0998.2010.02863.x

Wu, C.-H., Tsai, M.-H., Ho, C.-C., Chen, C.-Y., & Lee, H.-S. (2013a). *De novo* transcriptome sequencing of axolotl blastema for identification of differentially expressed genes during limb regeneration. *BMC Genomics*, *14*(1), 1–1. doi:10.1186/1471-2164-14-434

Wu, Q., Wang, C., Guo, L., Ge, Q., & Lu, Z. (2013b). Clinica Chimica Acta. *Clinica Chimica Acta*, *415*(C), 239–244. doi:10.1016/j.cca.2012.11.002

Yablonka-Reuveni, Z., Seger, R., & Rivera, A. J. (1999). Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, *47*(1), 23–42.

Yakushiji, N., Suzuki, M., Satoh, A., Sagai, T., Shiroishi, T., Kobayashi, H., et al. (2007). Correlation between Shh expression and DNA methylation status of the limb-specific Shh enhancer region during limb regeneration in amphibians. *Developmental Biology*, *312*(1), 171–182. doi:10.1016/j.ydbio.2007.09.022

Yang, E. V., & Bryant, S. V. (1994). Developmental regulation of a matrix metalloproteinase during regeneration of axolotl appendages. *Developmental Biology*, *166*(2), 696–703.

Yang, E. V., Gardiner, D. M., Carlson, M. R., Nugas, C. A., & Bryant, S. V. (1999). Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. *Developmental Dynamics*, *216*(1), 2–9. doi:10.1002/(SICI)1097-0177(199909)216:1<2::AID-DVDY2>3.0.CO;2-P

Yin, V. P., Lepilina, A., Smith, A., & Poss, K. D. (2012). Regulation of zebrafish heart regeneration by miR-133. *Developmental Biology*, *365*(2), 319–327. doi:10.1016/j.ydbio.2012.02.018

Yin, V. P., Thomson, J. M., Thummel, R., Hyde, D. R., Hammond, S. M., & Poss, K. D. (2008). Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish. *Genes & Development*, *22*(6), 728–733. doi:10.1101/gad.1641808

Yokoyama, H. (2008). Initiation of limb regeneration: the critical steps for regenerative capacity. *Development, Growth & Differentiation*, *50*(1), 13–22. doi:10.1111/j.1440-169X.2007.00973.x

Yokoyama, H., Maruoka, T., Ochi, H., Aruga, A., Ohgo, S., Ogino, H., & Tamura, K. (2011). Different requirement for Wnt/β-catenin signaling in limb regeneration of larval and adult *Xenopus*. *PLoS ONE*, *6*(7), e21721. doi:10.1371/journal.pone.0021721

Yokoyama, H., Ogino, H., Stoick-Cooper, C. L., Grainger, R. M., & Moon, R. T. (2007). Wnt/β-catenin signaling has an essential role in the initiation of limb regeneration. *Developmental Biology*, *306*(1), 170–178. doi:10.1016/j.ydbio.2007.03.014

Yoo, A. S., Staahl, B. T., Chen, L., & Crabtree, G. R. (2009). MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. *Nature*, *460*(7255), 642–646. doi:10.1038/nature08139

Yoshimura, T., & Oppenheim, J. J. (2011). Chemokine-like receptor 1 (CMKLR1) and chemokine (C–C motif) receptor-like 2 (CCRL2); Two multifunctional receptors with unusual properties. *Experimental Cell Research, 317*, 674–684.

Yu, Y.-M., Gibbs, K. M., Davila, J., Campbell, N., Sung, S., Todorova, T. I., et al. (2011). MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. *European Journal of Neuroscience*, *33*(9), 1587–1597. doi:10.1111/j.1460-9568.2011.07643.x

Yue, R., Li, H., Liu, H., Li, Y., Wei, B., Gao, G., et al. (2012). Thrombin receptor regulates hematopoiesis and endothelial-to-hematopoietic transition. *Developmental Cell*, *22*(5), 1092–1100. doi:10.1016/j.devcel.2012.01.025

Zhang, Q., Sakamoto, K., & Wagner, K.-U. (2014). D-type Cyclins are important downstream effectors of cytokine signaling that regulate the proliferation of normal and neoplastic mammary epithelial cells. *Molecular and Cellular Endocrinology*, *382*(1), 583–592. doi:10.1016/j.mce.2013.03.016

Zhao, C., Sun, G., Li, S., Lang, M.-F., Yang, S., Li, W., & Shi, Y. (2010). MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(5), 1876–1881. doi:10.1073/pnas.0908750107

Zika, J. M. (1969). A histological study of the regenerative response in a lizard, *Anolis carolinensis*. *The Journal of Experimental Zoology*, *172*(1), 1–8. doi:10.1002/jez.1401720102