

Chapter 2

Regeneration: Lessons from the Lizard

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2.1 Regeneration in Lizards

Regeneration of entire appendages requires complex coordination of molecular events including activation of stem cells or dedifferentiation to form proliferative cells, proliferation, and differentiation into the musculoskeletal, nervous, and epithelial tissues of the regenerated structure. The ability to regenerate entire appendages is a common trait found in teleost fish, amphibians, and squamate reptiles [1, 2]. The ability to regenerate an appendage can vary between different periods of its lifespan and between anatomical structures. These vertebrates have a common ancestor and their shared evolutionary history is reflected in their genomes, sharing multiple homologous genetic pathways that regulate developmental patterning and differentiation [3].

In the past decade, appendage regeneration research in reptiles has focused on describing tail regeneration in lizards using the green anole, *Anolis carolinensis*, (Fig. 2.1; [4–7]) and the leopard gecko, *Eublepharis macularius*, [8–11] as models. The green anole is used as model of development [12, 13], population genetics [14, 15], reproductive physiology and behavior [16, 17], and functional morphology [7, 18], and it was the first non-avian reptile to have a sequenced genome [19]. The availability of the genome makes molecular genetic studies of regeneration feasible. There have been a broad scope of studies of the green anole [20–32] that inform

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Fig. 2.1 Image of a green anole lizard with a regenerated tail. The *arrow* indicates the autotomy break point and start of the regenerated tail. Photo credit: Joel Robertson

more recent molecular, cellular, and anatomical analyses [5–7]. In alligators while tail regeneration has been reported, the structure and process of regeneration are unknown [33, 34]. The regenerated lizard tail is an extraordinary example of *de novo* development of hyaline/articular cartilage, muscle groups with tendinous attachments, skin, vasculature, and neural ependymal cells [5, 7, 9, 11]. In contrast, birds and mammals have very limited regenerative capacity. Regeneration in mammals is restricted to neonatal and juvenile individuals, including the regrowth of digit tips [35–38].

2.2 Stages of Regeneration in Lizards

Lizards represent the evolutionarily closest related group to mammals that demonstrate the ability to regenerate appendages (Fig. 2.1). Many lizard species can undergo tail autotomy followed by regeneration [33]; this is a self-induced amputation induced by physiological and/or mechanical stress leading to shedding of the tail as a predator evasion tactic. The vertebrae in the tail of many lizards have fracture planes that permit autotomy [30]. Following autotomy, there is a well described process of tail regeneration that displays aspects of a two step model of regeneration (Fig. 2.2; reviewed in [39]). In this model, there is an initial immune response following injury leading to either scar formation or full regeneration. The regenerative response includes (1) capping of the wound with a blood clot and remodeling of the ECM, (2) emergence of a wound epithelium and loss of the scab, (3) generation of proliferating cells, blood vessel formation, and thickening of the wound epithelium, and (4) growth and differentiation of tissues in the growing tail, including the neuroependyma, cartilage, and myofibers [11, 40, 41]. Studies in the leopard gecko demonstrate that tail regeneration is not limited to loss at autotomy planes; regeneration will occur whether or not the loss occurs close to the fracture plane, the tail is amputated mechanically, or it is released via autotomy [8]. In contrast to tail autotomy, the amputation of the limb leads to initial injury responses with partial formation of some tissues but ending in scar formation in the viviparous lizard *Lacerta vivipara* [42] and the common wall lizard *Podarcis muralis* [43].

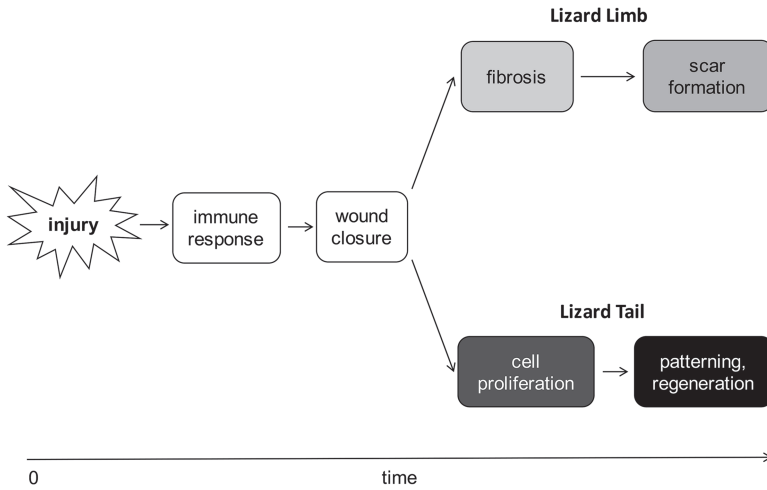


Fig. 2.2 Two step model of regeneration. Lizards are able to regenerate their tails following autotomy. However, following limb amputation, lizards display an injury response with partial regrowth but followed by fibrosis and scarring. In contrast, tail autotomy is followed by formation of the wound epithelium, ECM remodeling, then cell proliferation and patterning in regeneration

2.3 Molecular Mechanisms of Lizard Regeneration

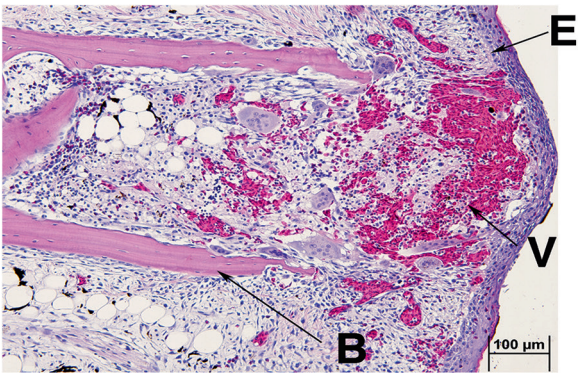
Prior to the outgrowth observed in regeneration, the damaged tissue is covered by a wound epithelium for scar-free wound healing [44, 45]. This wound epithelium expands in thickness to twice that of the original epidermis in the lizard and newt [8, 11, 40]. In the newt, this structure has been called the apical epithelial cap (AEC), in reference to the apical ectodermal ridge (AER) formed at the edge of the limb bud development [46–48].

Remodeling and clean-up of the damaged tissues takes place before the onset of outgrowth in regeneration. Key to this process is the reorganization of extracellular matrix (ECM) to create a new scaffolding matrix for the regenerated appendage [49, 50]. Remodeling of the ECM is a characteristic of the scar-free wound healing that occurs prior to regeneration, as opposed to a fibrotic, non-regenerative response [51, 52]. Several factors regulating scar-free wound healing have been identified. Matrix metalloproteases (MMPs), which have been observed in the regenerating tail of the green anole lizard [6] and the leopard gecko [8], likely contribute to ECM remodeling. In addition to ECM remodeling, regulation of the inflammatory response and inhibition of fibrosis are key early steps that permit scar-free regeneration [53–58]. Studies in the Italian wall lizard (*Podarcis sicula*) have identified infiltration of granulocytes and monocytes/macrophages into the autotomized tail stump [59, 60]. Given their role in regulation of inflammation, ECM remodeling, fibroblast formation, angiogenesis, and peripheral nerve innervation, macrophages are of particular interest [61–64]. Macrophages regulate proliferation of endothelial cells, keratinocytes, and fibroblasts [65] as well as stimulate the production of immune cytokines including PDGFs, IGFs, FGFs, TGFs, CSFs, hepatocyte growth factors, colony-stimulating factors, and Wnt ligands [66].

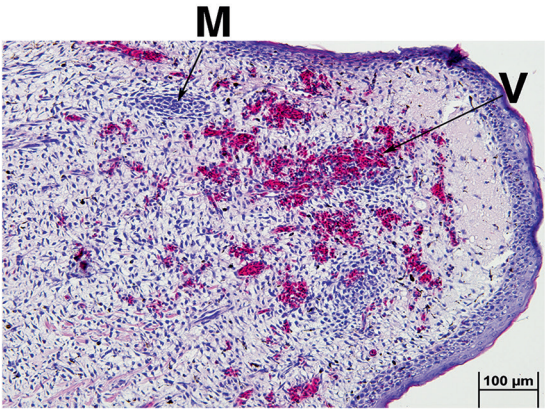
The formation of the blastema is well described in the regenerating limbs and fins of amphibians and teleost fish. A blastema is traditionally defined as the dedifferentiated coalescence of pluripotent proliferating cells concentrated at the tip of a regenerating appendage. Importantly, there is a lack of a vascular bed found at the distal tip [67–77]. More recently, studies in amphibians have found that the traditional view of the blastema as a mass of pluripotential, dedifferentiated cells is not entirely accurate. Further, the cellular composition of this structure can vary by stage and species [78, 79]. For example, in the newt, *Notophthalmus viridescens*, studies with Cre/loxP mediated lineage tracing found that mature muscle of an amputated limb dedifferentiated and formed PAX7-negative proliferating cells that could be found in the blastema. However, these cells contributed solely to regenerating muscle [80]. Whereas, in the axolotl, *Ambystoma mexicanum*, these same lineage tracing approaches demonstrated that the remaining muscle did not dedifferentiate, nor contribute any cells to the blastema. Muscle regeneration in this salamander occurs through PAX7-positive satellite cells, the resident stem cell population found in muscle [80]. This was also observed when transplanted GFP-positive cells were used to track cells in regenerating axolotl limbs. These studies demonstrated that all cells that contributed to the blastema retained their original embryological fate and contributed only to those tissues. Cells that were derived from lateral plate mesoderm only contributed to dermis, and skeleton and muscle precursors that are derived from presomitic mesoderm only became muscle [78]. Interestingly, in the Japanese newt, *Cynops pyrrhogaster*, post-metamorphosis muscle regeneration in amputated limbs occurs through muscle dedifferentiation, but pre-metamorphosis PAX7-positive satellite cells regenerate muscle post-amputation [79].

Clearly de-differentiation as a source of proliferating progenitor cells is not the rule, and this is consistent with observations from studies of *A. carolinensis* tail regeneration [81–84]. In histological sections it was noted that differentiating muscle was apparent as early as 15 days post autotomy (dpa); regenerating tails in this species demonstrate significant distal outgrowth until 65 dpa [5]. By 20 dpa, there was differentiating muscle from the distal tip to the proximal breakpoint, but there was no obvious zone of proliferating progenitors at the tip [6]. Interestingly, the distal tip of the regenerating tail is also highly vascularized (Fig. 2.3) [6]. Cartilage, which replaces the missing skeleton, and the ependymal cells that regenerate the spinal cord, extend from the breakpoint to the distal tip of the early regenerating tail (20 dpa) [6]. Proliferating cells were found throughout the regenerating anole tail when assayed using an antibody that recognized MCM2, a protein expressed in cells that are replicating their genome in preparation to divide. Subsequent transcriptome analysis of genes involved in proliferation complemented these data; it was found that these genes were expressed at similar levels all along the tail. Interestingly, the lowest level of expression was found in the region of the distal tip [6]. Similarly in the leopard gecko, proliferating cells were found throughout the regenerating tail, instead of restricted to the distal tip, and the distal tip is vascularized as well [11]. In these lizards a true blastema does not seem to exist.

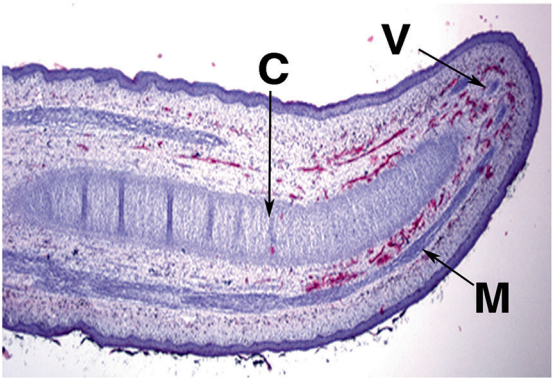
Fig. 2.3 Histology of early regenerating *A. carolinensis* tail. Sagittal sections through the tips of the early regenerating tail stained with H&E. At 10 dpa the outgrowth of the tail has not started but the epithelium (E) has regenerated and the distalmost portion is highly vascularized (V). At 15 dpa outgrowth has started, there are muscle (M) groups developing near the distal tip and the vascular network is still prominent. At 30 dpa, there is well developed muscle and cartilage (C). The vascular network has extended



10 dpa



15 dpa



30 dpa

Studies in salamanders demonstrated that satellite cells, an existing progenitor population in muscle, were responsible for muscle regeneration in amputated limbs [78]. Based on our observations, regeneration in anole lizard tails employs a similar strategy. Transcriptome analysis of proximal to distal gene expression in the early regenerating tail (25 dpa) demonstrated that there was significant expression of markers of satellite cells and muscle development. These genes include important regulatory factors such as the marker of mammalian satellite cells paired box domain 7 (*pax7*), the myogenic transcriptional regulator MyoD (*myod1*), myocyte enhancer factor 2C (*mef2c*) a cofactor of the myogenic regulators, *twist1*, and Mohawk (*mxk*). The tail also expresses genes that regulate muscle development such as nuclear factor of activated T cells 1 (*nfatc1*), which regulates skeletal muscle fiber type and negatively regulates MyoD, paraxis (*pcf15*) a transcription factor that regulates compartmentalization of the somite, and myostatin (*mstn*), a TGF β family member and negative regulator of muscle cell growth [6].

Another gene that was significantly up-regulated in the regenerating anole tail was *twist1*. This gene encodes a basic helix-loop-helix transcription factor that in mammals is involved in limb patterning and Saethre-Chozen syndrome [85–90]. There are three Twist family members and *Twist1* and *Twist3* were found in specific populations of cells in the ambystoma limb blastema [91]. Using single cell PCR, it was found that blastemal cells that expressed *Twist1* and *Sox9* and were derived from, and will become, cartilage whereas *Myf5* positive cells that will become muscle did not co-express *Twist1* or *Twist3*. Consistently, *Twist1* and *Twist3* co-expressing cells were destined to become dermis and were derived from this tissue [91]. In the anole tail, *twist1* was significantly up-regulated in the regenerating tail [6], a challenge for future studies in the lizard will be to identify the source of stem/progenitor cells for different musculoskeletal tissues in the regenerating tail.

Studies in *Xenopus* frog tadpoles and salamanders suggest that nerve signaling is a crucial positional cue driving regeneration. Similarly, in lizards, damage to the spinal cord proximal to the regenerating tail inhibits the regenerative process [28, 92, 93]. In the Japanese gecko, *Gekko japonicus*, ependymal cells at the core of the regenerating tail provide positional identity to cells in the regenerating tail [94]. Studies done in *A. carolinensis* and *Scincella lateralis* have shown that the ependyma is necessary for regeneration of the cartilage [28, 81, 84]. The ependymal cells regrow directly from the spinal cord, and there is no evidence of dedifferentiation of nervous tissues in tail regeneration in many lizards examined including *A. carolinensis*, *Sphaerodactylus goniorhynchus*, *S. argus* and *Lygosoma laterale* [30, 81, 82].

2.4 Genomic Insights into Lizard Regeneration

With the availability of high throughput sequencing technologies and emergence of annotated genomes for regenerative species, gene expression studies of regeneration in reptiles have become possible [95]. In the green anole lizard,

RNA-Seq analysis has identified at least 326 genes that are differentially expressed within different regions of the regenerating tail, including regulators of muscle and cartilage development, wound response, and thyroid hormonal response, and members of the Wnt and FGF/MAPK pathways. These data can be compared to similar gene expression studies in other regenerative model organisms in order to identify common factors required for regeneration across vertebrates. Namely, studies in a number of vertebrate models have identified genes in the Wnt- Ca^{2+} pathway in both regeneration and regulation of the inflammatory response [96–98]. In the green anole lizard, *wnt5a* and the Wnt inhibitors *dkk2* and *cerberus* were elevated in the distal tip of the regenerating tail [6]. *Wnt5a* and *wnt5b* are expressed in the axolotl limb blastema [99], and in the regenerating fins of zebrafish *wnt5a*, *wnt5b*, and *wnt10* are co-expressed [100]. Further studies will help to identify the role that Wnt signaling plays in creating permissible conditions for regeneration.

Given the large number of genes differentially expressed during the process of regeneration, attention has been focused on regulatory agents such as microRNAs, which are highly conserved amongst metazoans and can modulate the expression of multiple genes [101]. MicroRNAs have been found to regulate a number of biological processes, including proliferation and differentiation in cells ranging from skeletal and cardiac muscle to neurons [102], hematopoietic and embryonic stem cells [103, 104] and T cells [105], as well as repair of muscle [106]. MicroRNAs has also been found in regeneration of the limb and tail of axolotl salamanders [107, 108], lens and inner ear of newts [109, 110], and tail, spinal cord, and heart in the zebrafish [111–113]. Recently, sequencing in the green anole lizard regenerating tail and adult tissues has identified 350 putative novel and 196 known microRNAs [114]. In the regenerating tail at peak growth (25 days post autotomy), 11 differentially expressed microRNAs were identified within the growing tail, including miR-133a, miR-133b, and miR-206, a regulator of stem cell proliferation in other regenerating species. In addition, 3 novel microRNAs were identified to be elevated in the tail tip, suggesting potentially uncharacterized pathways or regulators specific to lizards may involved in regeneration.

MicroRNAs are not the only factors that may lead to differential expression of hundreds of genes; lizards and other regenerative species could potentially display genomic changes in coding or non-coding regulatory sequences such as enhancers, silencers, and insulators that account for regenerative differences. Alternately, changes in chromatin regulation between regenerative and non-regenerative vertebrates may also play a role. Further comparative studies making use of multiple model systems will allow us to distinguish between these possibilities.

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