Regeneration

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REVIEW

The regeneration blastema of lizards: an amniote model for the study of appendage replacement

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Abstract

Although amniotes (reptiles, including birds, and mammals) are capable of replacing certain tissues, complete appendage regeneration is rare. Perhaps the most striking example is the lizard tail. Tail loss initiates a spontaneous epimorphic (blastema-mediated) regenerative program, resulting in a fully functional but structurally non-identical replacement. Here we review lizard tail regeneration with a particular focus on the blastema. In many lizards, the original tail has evolved a series of fracture planes, anatomical modifications that permit the tail to be self-detached or autotomized. Following tail loss, the wound site is covered by a specialized wound epithelium under which the blastema develops. An outgrowth of the spinal cord, the ependymal tube, plays a key role in governing growth (and likely patterning) of the regenerate tail. In some species (e.g., geckos), the blastema forms as an apical aggregation of proliferating cells, similar to that of urodeles and teleosts. For other species (e.g., anoles) the identification of a proliferative blastema is less obvious, suggesting an unexpected diversity in regenerative mechanisms among tail-regenerating lizards.

Keywords
blastema, cell proliferation, lizard, regeneration, wound epithelium

> "This cellular mass acts as a growing-point to the new tail, and from it the various structures are developed" (White 1915, p. 472).

The lizard tail blastema

The most obvious example of multi-tissue regeneration among amniotes occurs following tail loss in lizards. Detachment of a portion of the tail initiates a cascade of events beginning with clot formation and re-epithelialization, followed by scar-free wound healing and the formation of a mass of proliferating cells (Woodland 1920; Werner 1967; Bellairs & Bryant 1985; McLean & Vickaryous 2011; Delorme et al. 2012). As for other regeneration-competent species, including urodeles (e.g., axolotls and newts) and teleosts (e.g., zebrafish), this aggregation of cells—the blastema—is widely interpreted as the primary source of tissues comprising the replacement tail (Fig. 1; Kragl et al. 2009; Grotek et al. 2013). Ultimately, continued growth and differentiation of the blastema leads to the formation of a fully functional (reviewed in Higham et al. 2013; Jagnandan et al. 2014) and superficially similar regenerate tail. Unlike other regeneration-competent models, the regenerate tail of lizards is not a perfect replica of the original. In particular, the regenerate tail demonstrates a modified pattern of scalation, differences in the tissue composition of the skeletal and central nervous systems, and a novel arrangement of skeletal muscles. Hence, the lizard blastema represents a highly successful natural experiment in functional multi-tissue restoration without the need for a strict recapitulation of developmental programs or patterning. As the closest living relatives of mammals capable of scar-free wound healing and entire appendage regeneration, lizards are becoming recognized as important biomedical models (Alibardi 2010; McLean & Vickaryous 2011; Delorme et al. 2012; Hutchins et al. 2014). Here we survey the literature detailing the lizard tail blastema with the goal of summarizing what it is, and what it is not, and what remains to be determined.

Tail loss and the initiation of regeneration

Among lizards, tail regeneration is an adaptation common to many, but not all, species. In particular, it is a phenomenon

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Figure 1. Gross morphology of the blastema of the leopard gecko (*Eublepharis macularius*) in dorsal–lateral view.

common to many groups including scincids (skinks), gekkotans (geckos), lacertids (wall lizards), and anoles. Most species are capable of tail regeneration at all stages of post-natal life (Woodland 1920; Moffat & Bellairs 1964; Bellairs & Bryant 1985). The regeneration program is spontaneously initiated once the tail has been detached. In most instances, this detachment is the result of caudal autotomy, a voluntary ability to self-sever a portion of the tail at a predetermined location. For the majority of lizards these locations (called fracture planes) are intravertebral and split the tail vertebra into cranial and caudal components (Cox 1969; Bellairs & Bryant 1985; McLean & Vickaryous 2011). Beginning at the vertebra, fracture planes radiate outwards to pass through and subdivide the surrounding tissues, including adipose tissue, skeletal muscle, and the dermis. Those tissues not subdivided by the fracture plane, including the spinal cord, spinal nerves, blood vessels, lymphatics, and the epidermis, are ruptured when the tail is detached. While autotomy provides an obvious complement to regeneration, it is not required. It is now well understood that many species are capable of replacing a portion of the tail lost due to surgical amputation outside the fracture planes (e.g., Woodland 1920; Werner 1967; Delorme et al. 2012). Hence, the capacity for regeneration appears to be an intrinsic property of the tail.

The regenerative program of the lizard tail involves a highly conserved sequence of morphological events, the details of which have been reported elsewhere (Woodland 1920; Hughes & New 1959; Werner 1967; McLean & Vickaryous 2011; Delorme et al. 2012; see also Bellairs & Bryant 1985; Alibardi 2010; Gilbert et al. 2013a). Briefly, following tail loss the original tail stump resembles an open wound, with the dermis, musculature, adipose tissue, vertebrae, and spinal cord all traumatically exposed. Rapidly, a clot is formed just distal to the original spinal cord and the integument collapses around the wound site and serves to decrease the diameter of the wound (Cox 1969; McLean & Vickaryous 2011). Morphologically, these events are followed by the retraction of exposed soft tissues and the formation of a more extensive clot, covering the entire surface of the wound (Fig. 2A). Shortly thereafter cells begin to proliferate distal to the original spinal cord (sometimes within less than 24 h; McLean & Vickaryous 2011). These cells represent the first evidence of the blastema (Fig. 2A). Simultaneously, cells from the adjacent epidermis span across the surface of the wound to form the wound epithelium (Hughes & New 1959; Cox 1969; McLean & Vickaryous 2011). With the completion of re-epithelialization the clot is lost and outgrowth begins (Fig. 2B). The wound epithelium continues to proliferate, becoming thicker overall (from 3–5 layers to 7–12 cell layers; Delorme et al. 2012) and most pronounced at the tip of the blastema where it is now referred to as an apical epithelial cap (Simpson 1968). As the tail regenerates it initially resembles a dome of tissue (the diameter is greater than the length), before gradually adopting a cone-like shape (Fig. 2C). Proliferation of cells within the blastema is matched by outgrowth of the central canal of the original spinal cord, the ependymal tube. The ependymal tube initially invades the blastema and grows to a position proximally adjacent to the wound epithelium (Fig. 2C). As outgrowth continues, the regenerating tail becomes increasingly tapered, while cells of the blastema begin to differentiate in a proximal to distal gradient (McLean & Vickaryous 2011). During the final stages of regeneration, pigmentation is re-established across the regenerate tail.

As noted previously, the regenerate lizard tail is a non-identical replacement. Conspicuously, the regenerate tail does not contain vertebrae. Instead the skeletal support consists of an unsegmented, hollow cartilaginous cone (e.g., Woodland 1920; Hughes & New 1959; Alibardi 1995; McLean & Vickaryous 2011). And while the original spinal cord is composed of white and grey matter and a central canal, flanked by dorsal root ganglia, the regenerate spinal cord consists solely of a central ependymal tube surrounded by descending tracts (e.g., Kamrin & Singer 1955; Simpson 1964, 1968). Dorsal root ganglia are not regenerated. Finally, regenerate scales are typically smaller and more homogeneous in shape, and the concentric organization of

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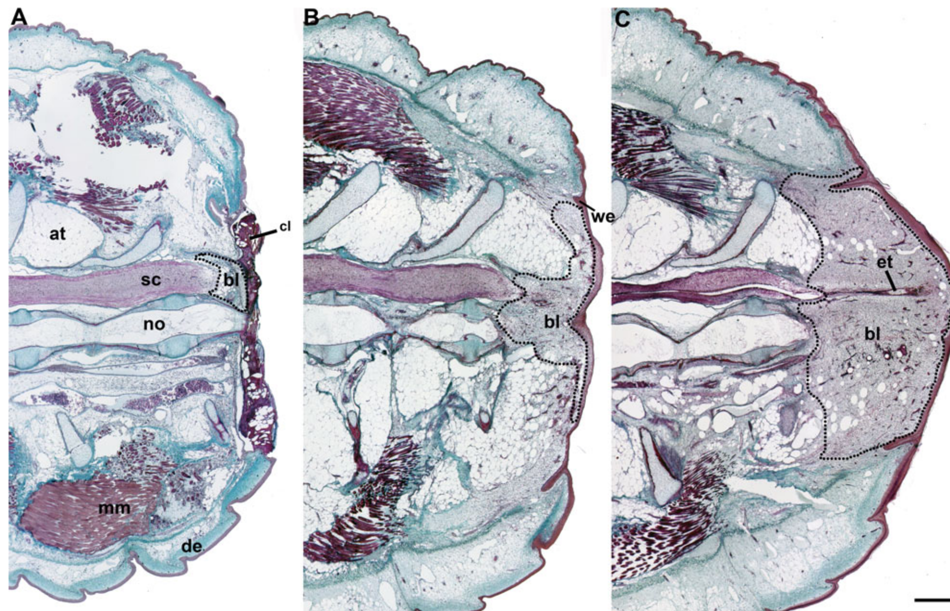

Figure 2. Histology of blastema formation during the early stages of tail regeneration in the leopard gecko (*Eublepharis macularius*). Longitudinal serial sections stained with Masson's trichrome (see McLean & Vickaryous 2011). (A) Initially the site of tail loss is capped by a temporary clot and there are no outward signs of regeneration. Note the earliest evidence of the blastema (hatched area) distal to the original spinal cord and deep to the clot (∼3 days post-autotomy). (B) Loss of the clot reveals a complete wound epithelium. Deep to this, the blastema continues to expand both distally and laterally (hatched area) (∼8 days post-autotomy). (C) With continued growth, the blastema (hatched area) begins to dominate the site of tail loss. Scale bar 500 μm. at, adipose tissue; bl, blastema; cl, clot; de, dermis; et, ependymal tube; no, notochord; mm, muscle; sc, spinal cord; we, wound epithelium.

skeletal muscle also differs (namely, regenerate muscle lacks the strict epaxial/hypaxial organization of the original).

The blastema: cells and source

Restoration of the lizard tail is an obvious example of epimorphic regeneration (or 'epimorphosis'; Morgan 1901), an injury-mediated process wherein the wound site becomes the focal point for a proliferating aggregation of cells, the blastema. In lizards, as for urodeles and teleosts, epimorphic regeneration is characterized by formation of two key structures: a wound epithelium and a blastema. The wound epithelium forms across the exposed wound site within days following tail loss. Histologically, it is characterized as a hyperplastic stratified squamous epithelium with a prominent apical thickening (the apical epithelial cap Fig. 3A) (McLean & Vickaryous 2011; Delorme et al. 2012). In addition to being thicker than the original epidermis, it also uniquely expresses the wound keratins WE6 (Fig. 3B) and K6, K16, and K17 (Alibardi & Toni 2005; Delorme et al. 2012). Removal or prevention of wound epithelium formation (e.g., by grafting original skin over the wound surface) inhibits regeneration, clearly demonstrating its essential role during tissue restoration (Whimster 1978). At present almost nothing is known about the molecular interactions between the wound epithelium and the underlying blastema.

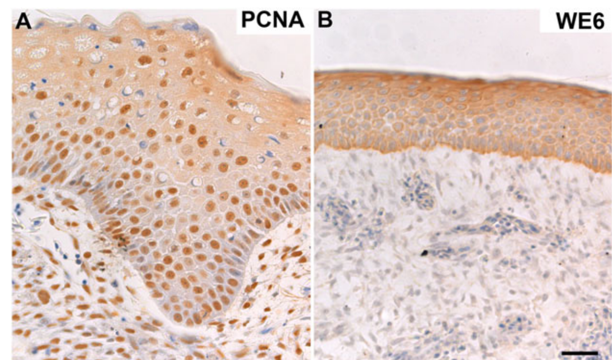

Figure 3. Immunohistochemical staining (DAB visualization) of protein expression in the wound epithelium during tail regeneration in the leopard gecko (*Eublepharis macularius*) (see Delorme et al. 2012). Numerous cells in the stratified layers of the wound epithelium express (A) proliferating cell nuclear antigen (PCNA), a marker of cells in the S phase of the cell cycle (most commonly, proliferating cells) (note the positive cell nuclei), and (B) WE6, a wound keratin marker unique to the wound epithelium. Scale bar 20 μm.

The second key element of epimorphic regeneration is the blastema. In the context of Morgan's original

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definition, the blastema represents the "proliferation of material [that] precedes the development of the new part" (Morgan 1901, p. 23). Since that time, the term has been repeatedly refined but almost inevitably includes reference to the blastema cells being dedifferentiated (Simpson 1965; Burgess 1967; Bellairs & Bryant 1985; Carlson 2007) or possibly undifferentiated (Butler 1935; Gurley & Sanchez-Alvarado 2008; Tweedell 2010; Wu et al. 2013). Among various regeneration-competent species other than lizards, including *Xenopus* tadpoles (tails; Lin et al. 2007; Gaete et al. 2013; Lee-Liu et al. 2014), axolotls (limbs; Kragl et al. 2009), zebrafish (tails; reviewed in Poss et al. 2003) and mice (digit tips; Rinkevich et al. 2011), it has been established that the blastema is a heterogeneous population of lineage-restricted cells. These cells retain a memory of their origin and are not capable of switching between germ layers (Lin et al. 2007; Kragl et al. 2009; Rinkevich et al. 2011). More recently it has been determined that the exact source of proliferating lineage-restricted blastema cells may vary even between closely related taxa. For example, the source of regenerating skeletal muscle differs in the salamander species *Notophthalmus viridescens* (newts) and *Ambystoma mexicanum* (axolotls) (Sandoval-Guzman et al. 2014). Following limb amputation, new skeletal muscle is derived from dedifferentiated PAX7− myofibres in newts and PAX7+ satellite cells in axolotls (Sandoval-Guzman et al. 2014). Furthermore, tissue replacement may involve more than one mode of regeneration. It has recently been established in zebrafish that regeneration of the heart ventricle following cryoinjury involves both localized blastema formation and larger-scale compensatory growth (Sallin et al. 2015). Although it hardly matters from the perspective of the animal, recognition of the diversity and variability of the regenerative mechanism provides a remarkable illustration of how regeneration has evolved (see Maden 2013) and underscores the need for additional comparative studies.

What about the lizard blastema? Although the specific source remains unknown, it seems reasonable to accept that, like other regeneration-competent vertebrates, the contributing cells are lineage-restricted. It is also clear that the mechanism of activation and proliferation of source cells is dynamic, injury-mediated, and, at the very least, involves the formation of a blastema. Prior to tail loss the majority of mitotically active cells (as evidenced by immunostaining for proliferating cell nuclear antigen [PCNA]) are associated with physiological maintenance functions (e.g., keratinocytes of the basal layer of the epidermis and cells of the hematopoietic tissues; McLean & Vickaryous 2011). Almost immediately following tail loss additional populations of cells begin to proliferate, including keratinocytes within most strata of the wound epithelium and an accumulation of mesenchymal-like cells contributing to the newly formed blastema (Fig. 4A). These cell populations remain distinctly PCNA-positive as the wound epithelium seals off the site of autotomy and begins to thicken (forming the apical epithelial cap), and as blastema grows to form the cone-like outgrowth presaging the new tail. Tritiated thymidine experiments have also shown that following tail loss cells of the wound epithelium maintain a high labeling index that is maintained throughout all stages of regeneration (Cox 1969). As tissues begin to differentiate within the new tail, mature (and mitotically inactive) cells and tissues replace the majority of once proliferating mesenchymal-like blastema cells, although chondroblasts and myoblasts continue to show a high labeling index for tritiated thymidine until the tail is fully formed (Cox 1969; Alibardi 1995). Concomitant with differentiation the wound epithelium thins, and the number of keratinocytes expressing PCNA diminishes (gradually returning to the basal population only).

Various lines of evidence support the presence of resident stem/progenitor populations within the original lizard tail, although details of their contributions are poorly understood. Using histological methods, putative satellite cells (fusiform in shape with no distinct nucleolus, condensed chromatin forming characteristic bands around the periphery of the nucleus, and a juxtanuclear Golgi complex) have been identified in original skeletal muscle (Kahn & Simpson 1974). Whereas these cells appear to be evenly distributed throughout the musculature, their presence at the interface between the myomeres and the intervening myosepta/fracture planes suggests that they may be activated (and thus contribute to the formation of the blastema) in response to autotomy (Kahn & Simpson 1974). Consistent with these findings, a recent bromodeoxyuridine (BrdU) pulse-chase experiment reported that cells within the myosepta/fracture planes, as well as cells of the periosteum and the ependymal tube of the spinal cord, are label retaining for as long as 22 days (Alibardi 2014a). Other evidence for putative stem progenitor cells in the original lizard tail comes from studies investigating the expression of the intermediate filament nestin. This protein is common to various precursor cell types during embryonic development, but is gradually replaced by tissue-specific intermediate filaments following differentiation (Michalczyk & Ziman 2005). Among adults expression of nestin is rare, with the notable exception of stem progenitor cells in the central nervous system (Lendahl et al. 1990) and hair follicles (Li et al. 2003). During lizard tail regeneration, nestin has been reported within keratinocytes of the basal layer of the wound epithelium, as well as regenerating myocytes, axons, and ependymal cells (Zhou et al. 2013; Alibardi 2014b). Ependymal cells are also reported to express neuron-specific enolase (NSE) as differentiation begins. Both nestin and NSE co-localize with BrdU (as a marker of proliferation) in ependymal cells. Taken together these results suggest that the ependymal tube includes populations of potentially slow cycling stem/progenitor cells (Zhou et al. 2013).

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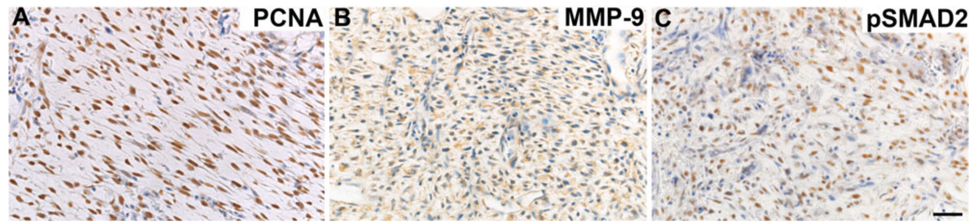

Figure 4. Immunohistochemical staining (DAB visualization) of protein expression in the tail blastema during regeneration in the leopard gecko (*Eublepharis macularius*) (see Delorme et al. 2012). Numerous cells of the blastema express (A) proliferating cell nuclear antigen (PCNA), a marker of cells in the S phase of the cell cycle (most commonly, proliferating cells); (B) matrix metalloproteinase 9 (MMP-9), an enzyme associated with extracellular matrix remodeling; and (C) phosphorylated SMAD2 (pSMAD2), indicating that the canonical transforming growth factor β (TGFβ) signaling pathway has been activated. Scale bar 20 μm.

The regenerative axis

Current evidence points towards the centrally located ependymal tube as the primary axis of lizard tail regeneration. Cells of the ependymal tube serve to organize and guide unmyelinated descending tracts from the original tail distally into the regenerate (Simpson 1968; Egar et al. 1970; Alibardi & Miolo 1990; Duffy et al. 1990) and probably induce and direct outgrowth of the new tail. During regeneration, the ependymal tube is one of the first identifiable structures to appear within the blastema (as early as 4 days post-autotomy), outgrowing from the stump of the original spinal cord (Kamrin & Singer 1955; Hughes & New 1959; Simpson 1964; McLean & Vickaryous 2011). If the spinal nerves from the original tail are transected and prevented from passing into the blastema, but the spinal cord is left intact, regeneration occurs. If the original spinal cord adjacent to the site of tail loss is ablated and/or blocked from outgrowing, with or without spinal nerves, regeneration fails (Kamrin & Singer 1955; Simpson 1964). More specifically, it is the ependymal cells lining the central canal of the original spinal cord that appear to be crucial for initiating regeneration; damage to the white and grey matter alone (but maintaining the ependymal population) is not sufficient to preclude ependymal tube outgrowth into the regenerating blastema (Simpson 1964). Furthermore, segments of the ependymal tube can initiate blastema formation and regenerative outgrowth even when transplanted to ectopic locations on the tail (Simpson 1964; Whimster 1978) and elsewhere (Bryant & Wozny 1974). For example, segments of cartilaginous cone containing ependymal tube from the regenerated tails of Yucca night lizards (*Xantusia vigilis*) were transplanted into the stumps of previously amputated hindlimbs. In 82% of limbs receiving the ependymal transplants, regenerative outgrowth was stimulated (Bryant & Wozny 1974). Although the exact mechanism of action by which the ependymal tube mediates regeneration remains unclear, it is almost certain to be the source of one or more trophic factors (e.g., Kamrin & Singer 1955; Simpson 1968; Egar et al. 1970). One possible candidate is acidic fibroblast growth factor (FGF1) (Alibardi & Lovicu 2010). In addition, elongation of the ependymal tube (and the surrounding nerve fiber tracts) may also be facilitated by SNAP25, a SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein known to be involved during development of the central nervous system (Wang et al. 2012). In geckos, SNAP25 has been shown to enhance astrocyte process elongation in vitro and has been hypothesized to promote elongation and outgrowth during tail regeneration (Wang et al. 2012).

The ependymal tube has also been identified as playing a fundamental role in patterning of the regenerate tail (Simpson 1964; Bellairs & Bryant 1985; Wang et al. 2011). Its central location within the blastema (and ultimately the regenerate tail) is highly suggestive of a role in concentric patterning (Kamrin & Singer 1955; Alibardi 2010). Furthermore, experimental manipulations indicate that the ependyma may be required for the induction of cartilage (Simpson 1964). More recently, CD59, a cell-surface marker and a known determinant of proximal−distal cell identity, was cloned from the gecko *Gekko japonicas* (Wang et al. 2011). *CD59* transcripts are present in the original (adult) spinal cord and during regeneration their expression increases at both 1 day and 2 weeks following tail loss (Wang et al. 2011). In vitro experiments demonstrate that over-expression of CD59 caused cells of proximal blastema to engulf more distal populations (Wang et al. 2011). Although the recapitulation of highly conserved developmental mechanisms such as cell-to-cell interactions and the release of polarizing transcription factors such as sonic hedgehog (Shh) are indicated, to date these predictions await investigation (French et al. 1976; Torok et al. 1999).

Regulating regeneration

Research to date has shown that the length of the regenerate tail is directly proportional to how much of the original tail remains (Bryant & Bellairs 1967) and that positional

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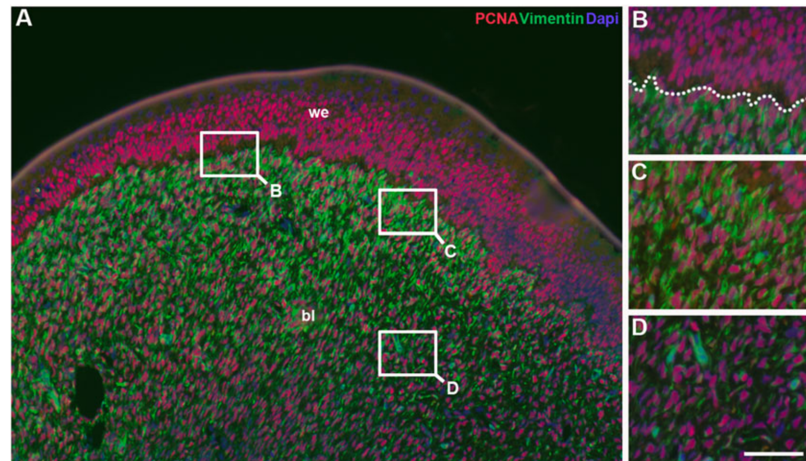

Figure 5. Immunofluorescent staining of proliferating cell nuclear antigen (PCNA) and vimentin in the blastema during tail regeneration in the leopard gecko (*Eublepharis macularius*) (see supplementary methods). PCNA (red) labels cells in the S phase of the cell cycle (most commonly, proliferating cells); vimentin (green) is an intermediate filament characteristic of mesenchymal cells; DAPI (blue) is a nuclear stain. (A), (B) Cells of the blastema are positive for both PCNA and vimentin, while cells of the wound epithelium (separated by a hatched line) in (B) are positive for PCNA only. Vimentin demonstrates a gradient of expression within the blastema, being most abundant towards the apex (C) and diminishing proximally (D). Scale bar 10 μm. bl, blastema; we, wound epithelium.

information present in the stump governs (at least in part) the size of the regenerate appendage (French et al. 1976; Haynie & Bryant 1976; Whimster 1978; Day & Lawrence 2000). The time required to fully regenerate the tail is heavily influenced by the lizard's age, available nutrition and environmental factors (e.g., ambient temperature; Noble & Bradley 1933; Hughes & New 1959; Moffat & Bellairs 1964). It also varies broadly between species (Kamrin & Singer 1955; Bryant & Bellairs 1976; McLean & Vickaryous 2011). Although the signaling pathways involved have not been explored in detail, it seems likely that regeneration in lizards is regulated by similar growth signaling pathways as seen in other groups (e.g., JAK/STAT, JNK, and Wnt/β-catenin; Sun & Irvine 2014). For example, in *Drosophila* matrix metalloproteinases (MMPs) are regulated by JNK signaling during cell differentiation and blastema formation (Sun & Irvine 2014). During lizard tail regeneration both the wound epithelium and blastema express MMP-9 (Fig. 4B; Delorme et al. 2012), an important protein involved in degrading components of the extracellular matrix, thus preventing scar formation. It may also be involved in promoting epithelial to mesenchymal interactions (Yang et al. 1999). More direct evidence comes from the study of transforming growth factor β (TGFβ)/activin ligands. TGFβ/activin signaling is widely recognized as playing an important role during regeneration in numerous species (e.g., Jazwinksa et al. 2007; Levesque et al. 2007), possibly through its ability to control and promote the epithelial−mesenchymal transitions, thus allowing cells to move from a stationary to motile state. The canonical TGFβ/activin pathway is regulated by phosphorylation of SMAD2. Hence, phosphorylated SMAD2 is considered an accurate readout of TGFβ/activin signaling. During lizard tail regeneration, many cells of the blastema are positive for phosphorylated SMAD2 (Fig. 4C; Gilbert et al. 2013b). Interestingly, these cells express neither TGFβ3 (Delorme et al. 2012) nor TGFβ1 (Gilbert et al. 2013b). As demonstrated by quantitative reverse transcription polymerase chain reaction, the only TGFβ/activin ligand that is significantly upregulated during this time is *activin-βA* (Gilbert et al. 2013b). Related to this, the same blastema cell population also upregulates the transcriptional repressors (and epithelial−mesenchymal transition markers) *Snail1* and *Snail2* (=*Slug*) (Gilbert et al. 2013b).

But is it a blastema?

Although the role of the lizard tail blastema during regeneration has never been questioned, its characterization as a "blastema" proper (comparable to those of urodeles) has. The primary basis for this concern centers on the apparent lack of proliferation by the early-formed mesenchymal-like cell mass otherwise accepted as the blastema (Cox 1969; Hutchins et al. 2014). It is worth noting that both these studies investigated tail regeneration in *Anolis carolinensis*. In contrast, investigations employing gecko models (e.g., Hughes & New 1959; McLean & Vickaryous 2011; Delorme et al. 2012; Gilbert et al. 2013b) have documented just the opposite: an abundance of proliferating mesenchymal-like cells in the early-formed tail blastema (Fig. 5). Indeed, recent studies using the gecko *Eublepharis macularius* have determined that, similar to urodeles, mesenchymal-like cells of tail blastema are not only proliferative (as evidenced by immunostaining with PCNA, a marker also used by Hutchins et al. 2014) and express the enzyme MMP-9 (involved in extracellular matrix remodeling), but also activate the canonical (SMAD-mediated) TGFβ/activin pathway (Fig. 4; McLean & Vickaryous 2011; Delorme et al. 2012; Gilbert et al. 2013b). Taken as a whole, these data suggest that aspects of the regenerative program have evolved and diversified in lizards (as for urodeles; Sandoval-Guzman et al. 2014). They may also explain why rates of regeneration vary so widely among species—for example, under ideal environmental

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conditions the tail of *A. carolinensis* requires at least 60 days to fully regenerate (Fisher et al. 2012) whereas that of *E. macularius* requires only 30 (McLean &Vickaryous 2011).

Clearly many obvious questions remain. Among the most pressing is to establish the cellular source of new tissues, and the underlying mechanism leading to blastema formation (dedifferentiation or stem/progenitor cell recruitment). How does the ependymal tube regulate regeneration, and are the same trophic factors conserved across lizard species and even other regeneration-competent non-lizard groups? And what are the adaptive advantages for lizards to recreate a similar but structurally non-identical tail? Continued investigations of the lizard tail hold great promise as a powerful tool for biologists and biomedical scientists alike.

Acknowledgments

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Supporting Information

Immunofluorescence

Slides were rehydrated to water and rinsed three times in 1 × PBS (5 min each). Sections were then blocked for 60 min in 5% normal goat serum (Jackson Immuno Research Laboratories) diluted in 1 × PBS for 1 h at room temperature. Sections were incubated overnight at 4°C in primary antibodies diluted in 1 × PBS (1:50 mouse anti-vimentin, Developmental Studies Hybridoma Bank; and 1:100 rabbit anti-PCNA, Santa Cruz Biotechnology); negative controls were incubated in 1 × PBS. Slides were then rinsed three times in 1 × PBS (5 min each) and then incubated in secondary antibodies diluted in 1 × PBS (1:200 Alexa Fluor-488 labeled goat anti-mouse, Life Technologies, and 1:200 Cy3 labeled goat anti-rabbit, Jackson Immuno Research Laboratories) for 1 h at room temperature. Slides were rinsed three times in 1 × PBS (5 min each) followed by the addition of 4′,6-diamidino-2-phenylindole diluted in 1 × PBS (1:10000 DAPI, Life Technologies). Slides were rinsed again three times in 1 × PBS (5 min each) and then cover slipped using fluorescent mounting medium (Dako Canada).

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