Tail regeneration in the Tokay Gecko (Gekko gecko)

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Chapter 5. *In Situ* Hybridization Study of Gene Expression in Tokay Gecko Tail Regeneration

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Abstract

Lizards (including geckos) can regenerate their tail after shedding it (autotomy). Lizards show the 'epimorphic' type of regeneration which does not involve reorganization of the uninjured part (as in morphallaxis) but instead show regeneration from an outgrowth of stem cells organized into a compact mass or blastema. Lizard tail regeneration involves multiple types of histogenesis: replacement of vertebrae with cartilage (chondrogenesis), formation of new vasculature (angiogenesis), new muscle development (myogenesis), adipose tissue regeneration (adipogenesis), and innervation by axons (axonogenesis). In this chapter, we examined the spatial and temporal expression of genes involved in histogenesis and tissue patterning during tokay gecko tail regeneration. We performed *in situ* hybridization on sections of tails at 4, 8, 16, 20, 24, 28, and 49 days post-autotomy. The results showed that the regenerating tail of the tokay gecko expressed relatively few axonogenesis genes. Moreover, we found the unique expression pattern of cartilage and skeletal muscle development in the regenerating tail. In addition, we also found temporally collinear expression of posterior HOXC genes in the regenerating tail. These results are consistent with a model in which the regeneration of the tail in the tokay gecko involves an early activation of a stem cell population, likely comprising satellite cells and fibroblasts, that retain positional information from the original tail. This positional memory allows the regeneration of a replacement tail that is consistent with the axial level of autotomy. This likely contrasts with regeneration in the axolotl and zebrafish where a new positional coordinate system is established *de novo*, as seen in normal development.

Introduction

Regeneration is the ability of organisms to replace their damaged cells, tissue or organ with a new functional one. Lizards (including geckos) show remarkable tail regeneration after undergoing autotomy. Autotomy is the self-defense mechanism in which a lizard can shed part of its tail to distract predators (Congdon et al., 1974). This mechanism is morphologically adapted with the presence of bone fracture planes and autotomy septa along the tail tissues that make it easier for the tail to be shed (Lozito and Tuan, 2017).

Tail regeneration in lizards is of the 'epimorphic' type of regeneration because it does not involve reorganization of the uninjured part (as in morphallaxis) but instead involves regeneration from an outgrowth from stem cells organized into a compact mass or blastema (Lozito et al., 2016). The process of tail regeneration in lizards consist of the following conserved major stages (Alibardi, 2014; Lozito and Tuan, 2017; McLean and Vickaryous, 2011): (1) degeneration (wound healing); (2) proliferation (blastema formation); (3) differentiation (tail elongation); and (4) maturation. Note that these stages are also described as including the formation of an apical ectodermal cap over the blastema, but we have not found such a structure in this thesis (see Chapter 2).

These stages involve the replacement of lost vertebrae with a cartilage tube (chondrogenesis), formation of new vasculature (angiogenesis), new muscle development (myogenesis), adipose tissue regeneration (adipogenesis), and innervation by axons (axonogenesis). In geckos, tail regeneration results in a new tail structure that lacks vertebrae and true tendons (Lozito and Tuan, 2017). Instead, the unsegmented cartilage tube is formed to functionally replace the vertebrae by chondrogenesis as response to hedgehog signaling (Alibardi, 2010; Londono et al., 2017; McLean and Vickaryous, 2011). Furthermore, the spinal cord is replaced by an ependymal tube growing inside the lumen of the cartilage tube (Alibardi, 2010; Londono et al., 2017; McLean and Vickaryous, 2011). The ependymal tube is thought to be derived from the epithelial lining of the central canal of the uninjured spinal cord (Alibardi, 2014; Alibardi, 2015; Lozito and Tuan, 2015; Lozito and Tuan, 2016). The regenerating tail is innervated by nerve fibers that enter from the dorsal root ganglia located in the normal (uninjured) tail (Cristino et al., 2000; McLean and Vickaryous, 2011; Tokuyama et al., 2018).

Finally, the skeletal muscle in the regenerated tail is arranged in a ring and not in four blocks as it was in the original (Alibardi, 2010; Londono et al., 2017; McLean and Vickaryous, 2011). The regeneration of muscle in lizard tails begins with the aggregation and differentiation of blastema cells into individual myoblasts (Lozito and Tuan, 2017). Investigations of the regenerating tails of the green anole and the Common Garden Skink have demonstrated that these myoblasts then fuse into bundles of myotubes (Alibardi, 1995). Additionally, the non-fusing cells present among the myotubes

contribute to the formation of the connective tissue myosepta (Alibardi, 1995). However, the mechanism of segmentation of muscle without somite formation in lizard tail regeneration remains unclear. It is interesting to ask whether *lfng* and *hes7* are involved in muscle segmentation or not, because they are known to be involved in somite segmentation in the embryo (Bessho et al., 2001; Chen et al., 2005; Kageyama et al., 2012; Okubo et al., 2012)

These features indicate that the regeneration process only partially replicates the embryonic development of the tail, and likely relies on distinct mechanisms to redevelop the lost structures. Therefore, in this chapter, we have analyzed the spatial and temporal expression of genes involved in axonogenesis, chondrogenesis, myogenesis, segmentation and tissue patterning during tokay gecko tail regeneration using *in situ* hybridization on tissue sections.

Material and Methods

Ethics statement

The tokay gecko (*Gekko gecko*) is listed as 'least concern' in the International Union for Conservation of Nature *Red List* (IUCN, https://www.iucnredlist.org/species/195309/2378260) and also listed in Appendix II in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; https://cites.org/eng/taxonomy/term/3919). In Indonesia, the tokay gecko is not a protected species. All animals were collected in Yogyakarta, Indonesia under a license issued by the Ministry of Environment and Forestry, the Republic of Indonesia (permit number SK.83/KSDAE/SET/KSA.2/5/2021 signed on 7 May 2021) and a recommendation letter issued by The Indonesian Institute of Sciences (Lembaga Ilmu Pengetahuan Indonesia/LIPI; Recommendation Number: B- 2158 /IV/KS.01.04/3/2021 signed on 19 March 2021). The transport of samples from Indonesia to the Netherlands was conducted under CITES permit numbers 05717/IV/SATS-LN/2021 and 09160/IV/SATS-LN/2022 issued by the Ministry of Environment and Forestry, the Republic of Indonesia.

All experimental and surgical procedures needed for samples collection were done at Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia with the approval from The Ethical Committee of the Integrated Laboratory for Research and Testing (Laboratorium Penelitian dan Pengujian Terpadu/LPPT) Universitas Gadjah Mada (Ethical Clearance number: 00014/04/LPPT/IV/2021 signed on 30 April 2021).

Experimentation and samples collection

Twenty-one adult tokay geckos were anaesthetized with intramuscular ketamine injection (50–75 mg/Kg body weight) administered in the thigh prior to the autotomy procedure. Autotomy was performed by gently restraining the gecko manually, while

leaving the tail accessible. Using the thumb and index finger, a firm pinch was applied to the required autotomous region (every segment after the 5th caudal vertebra) of the tail causing the gecko to spontaneously shed its tail. The wound was painted with Betadine® iodine antiseptic solution but no dressing was applied. After the autotomy procedure was performed, the geckos were allowed to regenerate a new tail. The geckos were distributed into experimental groups based on the number of days post-autotomy, each consisting of three geckos. After ketamine-anesthesia, the tails of geckos in each group were re-autotomized 2 cm cranially from the previous autotomy site at 0, 4, 8, 16, 20, 24, 28, and 49 days post-autotomy (dpa). The regenerated tail samples were washed and further handled in phosphate-buffered saline (PBS, 4 °C) and subsequently fixed with cold 4% paraformaldehyde (pFA) in PBS.

Probe synthesis

In this project, all gene names follow the style and nomenclature of the NCBI database for *Podarcis muralis* (Common Wall Lizard). There was no published annotated genome sequence available for the tokay gecko (*Gekko gecko*) at the time. The predicted regions of the *Gekko gecko* genome for the genes were aligned using the NCBI nucleotides database, to find orthologues in other lizard genomes. Primers were designed based on the conserved regions of the aligned sequences using BLASTn. Using these primers (approximately 800 bp in length), each gene of interest was amplified using the PCR from Gekko gecko cDNA (Supplementary protocol).

The amplicons were cloned into a plasmid (*Escherichia coli* vector pCR™II-TOPO™) following the TOPO™ cloning reaction protocol (Invitrogen, Carlsbad). The bacterial clones were subsequently sequenced (BaseClear B.V., the Netherlands). Probe synthesis was carried by labelling antisense RNA probes with dioxygenin-11-UTP (Roche) according to the manufacturer's protocols.

Histology and in situ hybridization

The regenerating tail blastema samples were rinsed in RNAse-free PBS and fixed in cold 4% paraformaldehyde in PBS. The fixed samples were decalcified by soaking them in Osteosoft® (Sigma-Aldrich) for 3–5 d. The decalcified samples were then dehydrated with a graded concentration series of methanol starting from methanol 70%, 80%, 90%, 100% for 2 x 30 min. The samples were cleared 3 x 1 h (Neo-Clear®, Merck-Millipore). The samples were infiltrated 2 x 1 h molten paraffin (62 °C) and 1 x 8–18 h. The samples were finally embedded with fresh molten paraffin and solidified at room temperature.

The paraffin blocks were then sectioned longitudinally at 6–8 µm on a rotary microtome. The sections were mounted on poly-L-lysine coated slides and left overnight at 37 °C. Alternating sections were taken for *in situ* hybridization or histological staining, respectively. For *in situ* hybridization we based our protocol on those in (Acloque et al.,

2008). The sections were deparaffinized with xylene 3 x 7 min and rehydrated using a graded ethanol series (100% 3 x 2 min; 90%, 80%, 70% for 1 min each). After that, pre-treatment was performed using PBST (phosphate buffered saline with triton) 3 x 5 min, proteinase K (10 ng/ml) 10 min, PBST 3 x 5 min, 4% paraformaldehyde (pFA) 10 min, and PBST 2 x 5 min. Then, the sections were covered with pre-hybridization mix (without probe) for ≥1 h at 60 °C, and incubated with probe mix (1000-1500 ng/mL) at 60 °C overnight with a cover slip. Next day, the sections were washed using washing solution A (2 x SSC (standard sodium citrate) 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate), 50% formamide) 3 x 30 min and TBST ([tris (hydroxymethyl)aminomethane] buffered saline with triton) 3 x 10 min. Anti-DIG-AP detection was carried out using 10% sheep serum in TBS (tris buffered saline) for 1 h and continued with anti-DIG-AP (1:1000 concentration) in 10% sheep serum in TBST at 4 °C overnight. Finally, the sections were covered with TBST 3 x 10 min and NTT (NaCl tris buffer with tween) pH 9.5 3 x 10 min. Staining was conducted using BM purple (Roche) and was checked regularly under the microscope for up to two days. When the desired, degree of color was achieved, the staining was stopped. The samples were then dehydrated by quickly dipping in graded ethanols (70%, 80%, 90%, 3 x 100%), and coverslip using Eukit®.

Results

At 8 dpa, there were scattered mesenchymal cells beneath the wound epithelium and most of these cells were *col1a2* expressing (Figure 5-1A, B, C). Together with this mesenchymal tissue we observed structures that resembled adult skeletal muscle (arrowhead in Figure 5-1B, C). By 16–20 dpa, *col1a2* was widely expressed in the blastema, except in developing muscle and cartilage (Figure 5-1, D–I). At 24 dpa, *col1a2* became expressed in the myosepta (Figure 5-1 K, L). We found no expression of *col1a2* in the uninjured (adult) tail (Figure 5-1A, D, G, J, M).

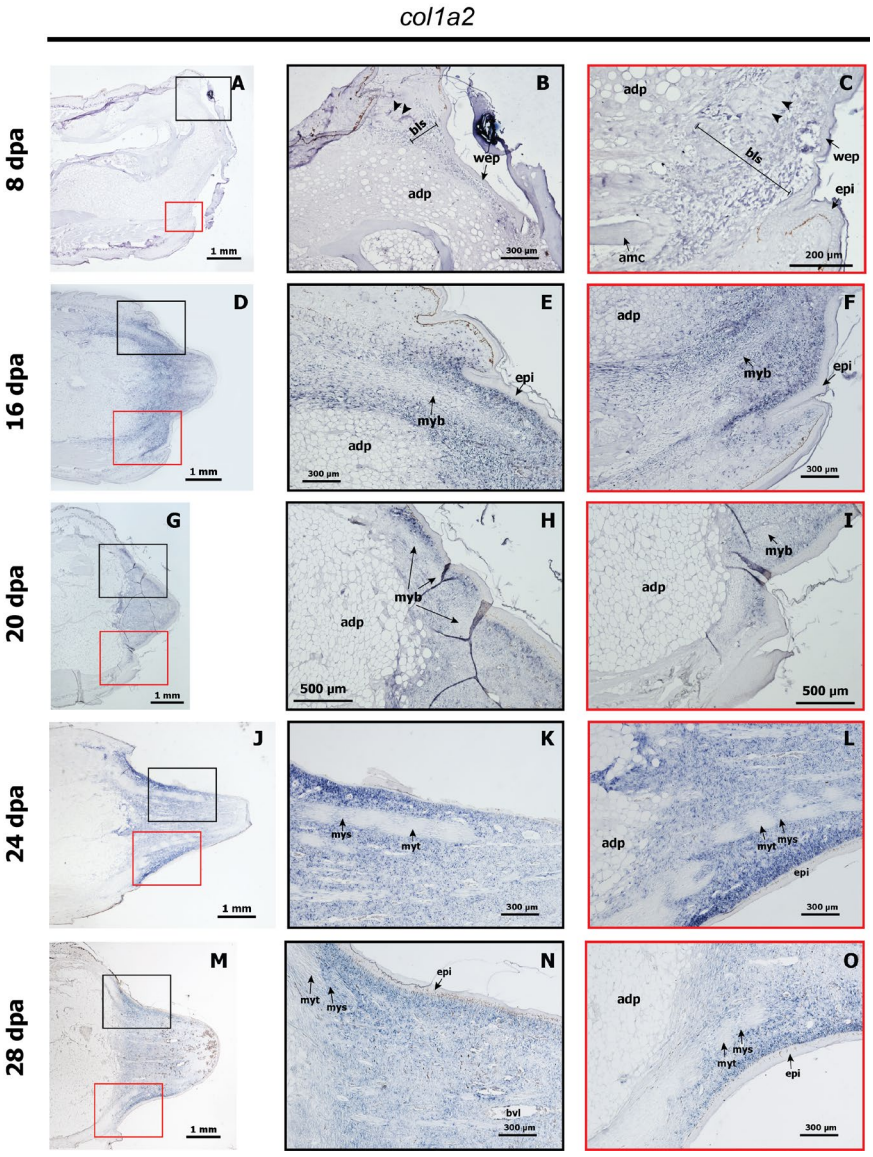

Figure 5-1. **Expression of *col1a2* is specific to developing connective tissue in the tokay gecko regeneration blastema.** Note that *col1a2* is expressed in the blastema but not at all in the original tail tissue. *Key*: adp, adipose tissue; amc, adult muscle cells; bls, blastema; bvl, blood vessel; ct, cartilage tube; ep, epidermis; ery, erythrocytes; leu, leukocytes; pbl, pre-blastema; myb, myoblast; mys, myoseptum; myt, myotubes; nvf, nerve fiber; wep, wound epithelium.

The regenerating tail expressed relatively few genes involved in axon development (axonogenesis)

The regenerated gecko tail has only an ependymal tube in place of a spinal cord. In order to understand why development of the nervous system is so limited in the regenerated, we studied the expression of eight axonogenesis-related genes, namely: *ngf*, *unc5a*, *nefh*, *notch3*, *efna*, *ntf3*, *gap43*, and *sema3a* (Figure S 5-2). This should also help us determine whether axonogenesis in the regenerating tail makes use of the same genes that are deployed in normal embryonic development.

We found that only three of these axonogenesis genes, *unc5a*, *nefh*, and *notch3*, were expressed in the regenerating tail blastema (Figure 5-2). At 20 dpa, there was slight expression of *unc5a* and *nefh* in the myotubes of the regenerating tail (Figure 5-2C, F). There was no expression of *notch3* at this stage (Figure S 5-2 E, F). At 28 dpa, *unc5a* and *nefh* showed clear expression in the developing skeletal muscle myotubes (Figure 5-2I, L). Moreover, *notch3* was weakly expressed in myotubes in the proximal part of the blastema, at this stage (Figure 5-2O). In addition, expression of *unc5a* and *nefh* was detected in cell bodies in the dorsal root ganglia (DRG) of the original (uninjured) tail at 20 and 28 dpa (Figure 5-2B, E, H, K). Even though the other genes (*ngf*, *efna*, *ntf3*, *gap43*, and *sema3a)* were not expressed in the blastema, we know that the probe worked because these genes were expressed strongly in the dorsal root ganglia and dermis of the uninjured tail, in the same histological sections (Figure S 5-2).

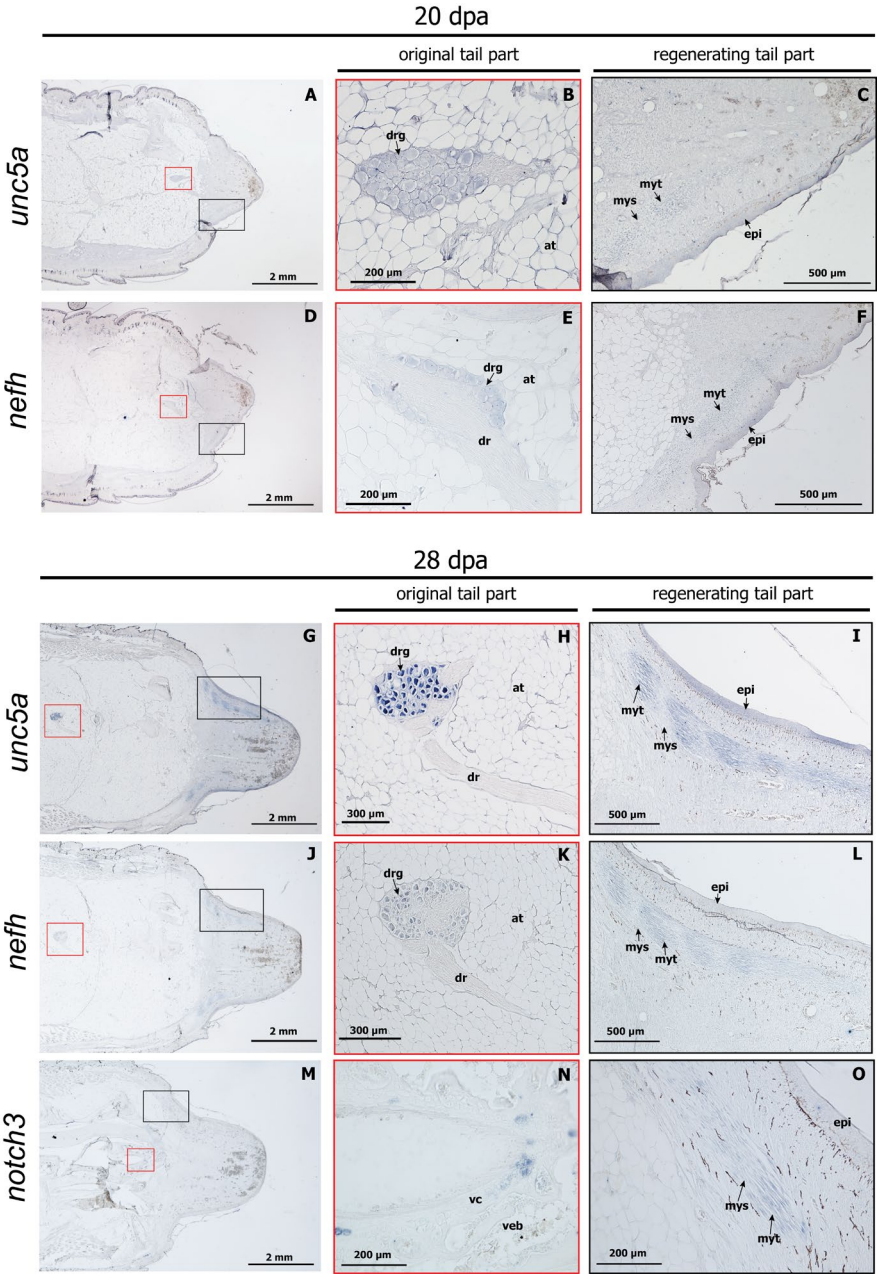

Figure 5-2. **The regenerating tail expresses relatively few genes involved in axon development (axonogenesis)**. (A) The expression of *unc5a* and *nefh* in 20 dpa regenerating tail. In situ hybridization on tissue sections. (B) The expression of *unc5a*, *nefh*, and *notch3* in 28 dpa regenerating tail. Key: at, adipose tissue; dr, dorsal root; drg, dorsal root ganglia, epi, epidermis; myt, myotube; mys, myoseptum.

Chondrogenesis in the regenerating tail is uncoupled from segmentation

The absence of vertebrae in the regenerated tail is a major unexplained phenomenon. One possible explanation could be that genes normally associated with chondrogenesis in embryonic development of the vertebrae, are not expressed in the regenerating tail. In this section we examine this hypothesis by looking at the expression of the chondrogenesis-related genes *sox9*, *wnt5a*, *wnt3*, *bmp1* and *bmp3*. Using *in situ* hybridization on tissue sections, we find *sox9* expression in the cartilage tube from 20–49 dpa, and in myotubes at 20 dpa only (Figure 5-3). *Wnt5a* was expressed in the cartilage tube, myotubes and dermis at the 20 and 49 dpa (Figure 5-4A, B, C). *Wnt3* was expressed in myotubes, the pre-cartilaginous cartilage tube, and epidermis at 20 and 30 dpa (Figure 5-4D–I). By 49 dpa the cartilage tube showed strong expression of *bmp3* in its proximal part; expression of *bmp3* in the myotubes was weak by this stage (Figure 5-5A, B, C). *Col2a1*, a cartilage marker, was expressed specifically in the cartilage tube at 24 dpa (Figure 5-5D, E).

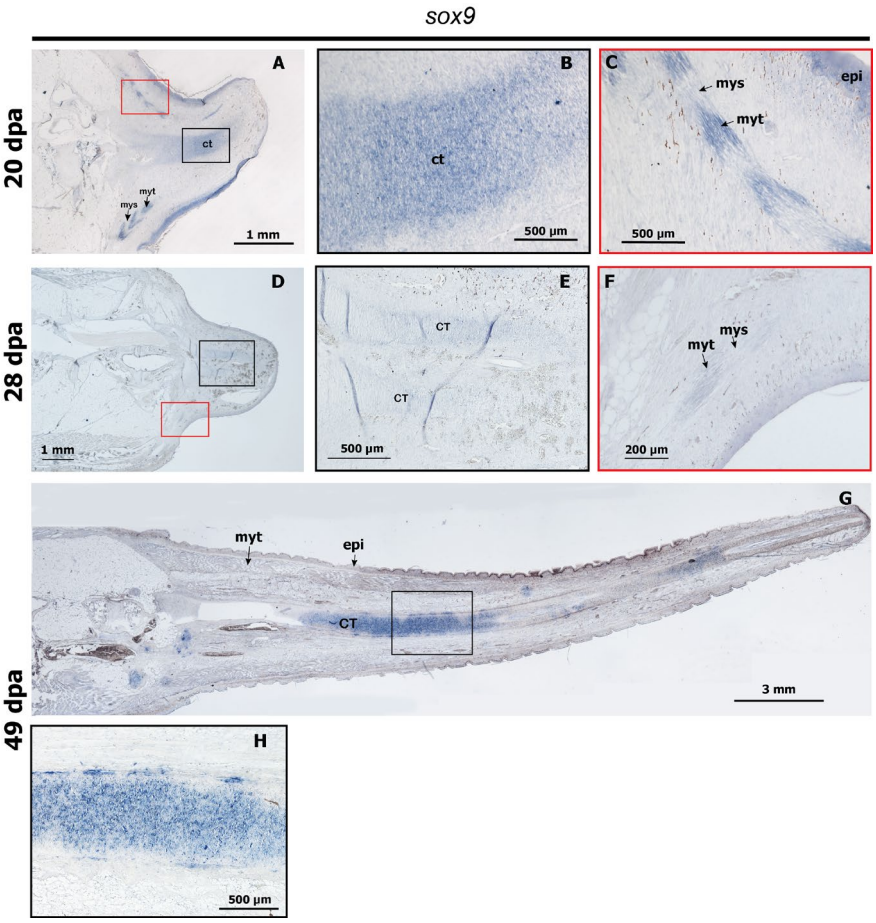

Figure 5-3. **Expression of *sox9* in the regenerating tail of tokay gecko**. *Key*: epi, epidermis; ct, cartilage tube; myt, myotube; mys, myoseptum.

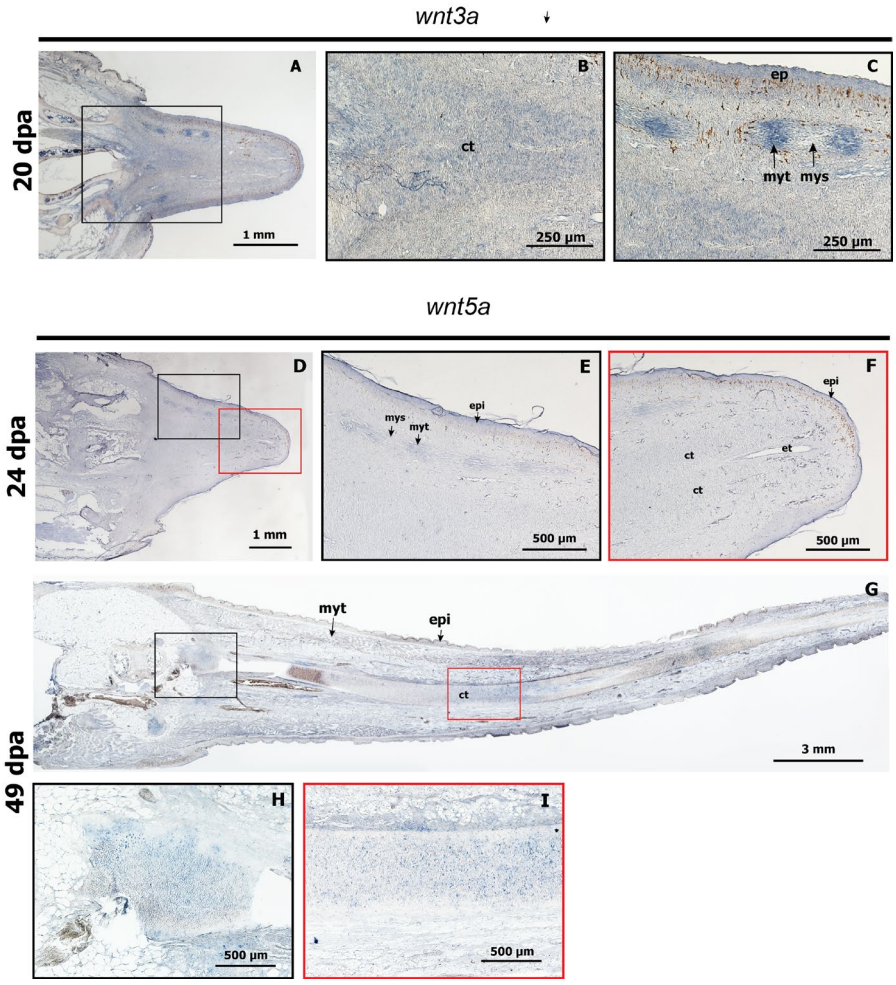

Figure 5-4. **Expression of *wnt3* and *wnt5a* in the regenerating tail**. *Key:* epi, epidermis; ct, cartilage tube; myt, myotube; mys, myoseptum.

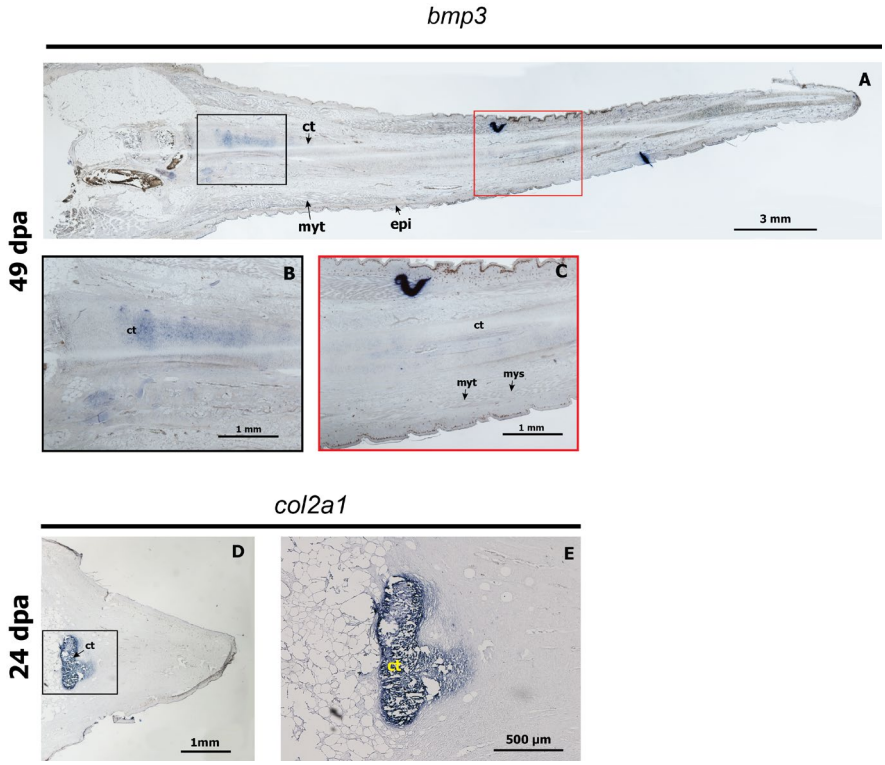

Figure 5-5. **Expression of *bmp3 col2a1* in the regenerating tail**. *Key*: epi, epidermis; ct, cartilage tube; myt, myotube; mys, myoseptum.

Tail regeneration in the tokay gecko involves a unique pattern of skeletal muscle development

The skeletal muscles of the regenerating tail are formed segmentally, as a longitudinal series of myomeres separated by fibro-connective tissue myosepta. We wanted to examine whether muscle development in the regenerating tail resembles muscle development in the embryo. To do this, we looked at the expression of genes related to muscle development, namely: *pax3*, *pax7*, *myod1*, *myog*, *ckm*, and *tnc*. We also hypothesized that the segmentation of regenerating muscles was based on the activity of genes that are normally involved in embryonic somite segmentation. Therefore, we also looked at the expression of *lfng* and *hes7*, which are known to be involved in somite segmentation. We also looked at paralogs of *hes7*, namely: *hes1*, *hes2*, *hes5*, and *hes6*, to see if they have acquired a function in regeneration (by neofunctionalization, for example).

We found expression of *pax3*, *pax7* and *myod1* in some of the mesenchymal cells under the wound epidermis (i.e. the newly-formed blastema), at 8 dpa (Figure 5-6). At 16 dpa these three genes are expressed in cells that appear to be forming myoblast

clusters (as indicated by their *myod1* expression) (Figure 5-7). These clusters are present in regions of the blastema that lack *col1a2* expression (Figure 5-1E, F). The expression of *myog, ckm* and *tnc* is first seen in myotubes at 24 dpa (Figure 5-8). Myotubes at this stage, like the myoblasts before them, lack *col1a2* expression; however, *col1a2* was expressed in the connective tissue myosepta (Figure 5-1K, L). Furthermore, *lfng* was expressed in myoblasts at 16 and 20 dpa, and in myotubes at 24 and 28 dpa (Figure 5-9). *Hes2* was expressed in myotubes and blood cells at 24 and 28 dpa (Figure 5-9). We found no expression of *hes6* in the regenerating tail (Figure S 5-3).

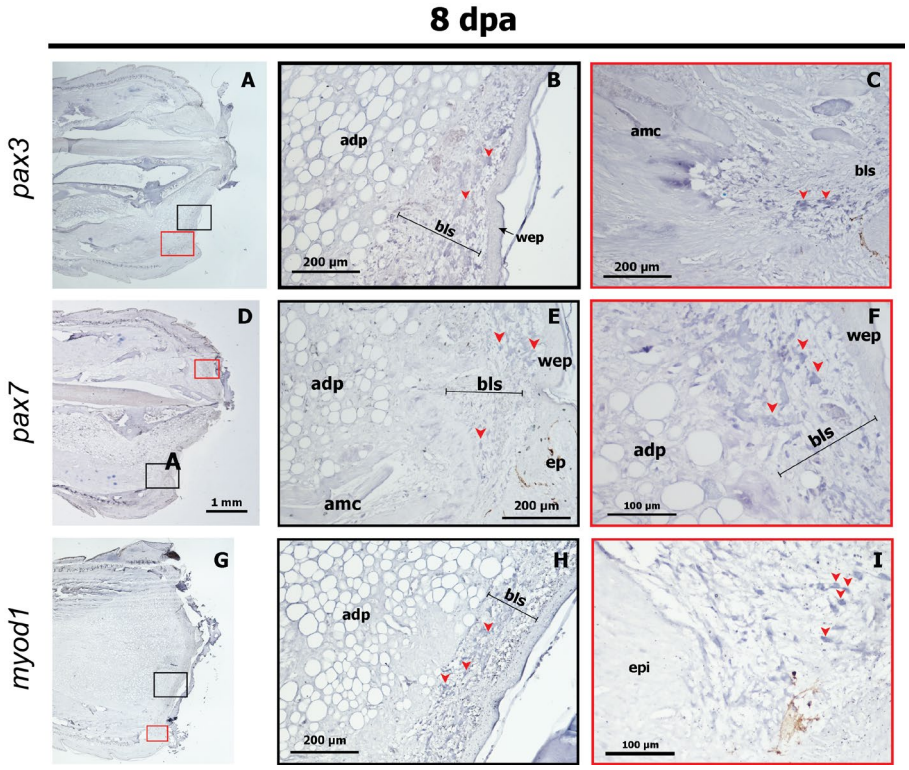

Figure 5-6. **Expression of *pax3*, *pax7* and *myod1* at 8 dpa in the regenerating tail**. *Key*: adp, adipose tissue; amc, adult muscle cells; bls, blastema; wep, wound epithelium.

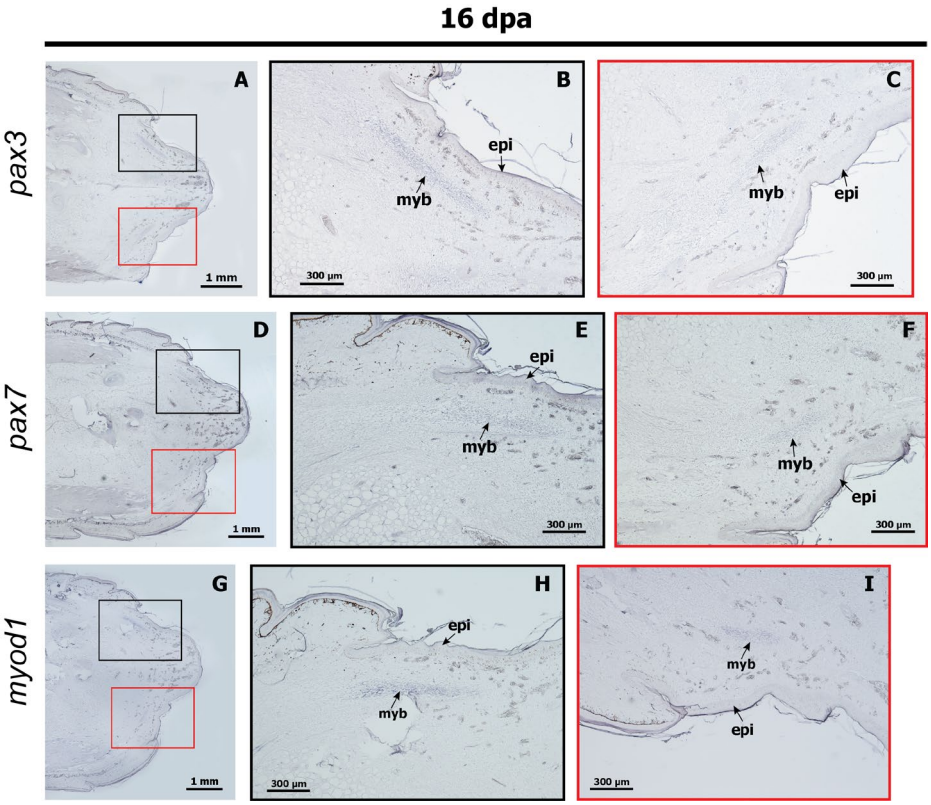

Figure 5-7. **Expression of pax3, pax7 and myod1 at 16 dpa in the regenerating tail**. *Key*: epi, epidermis; myb, myoblast.

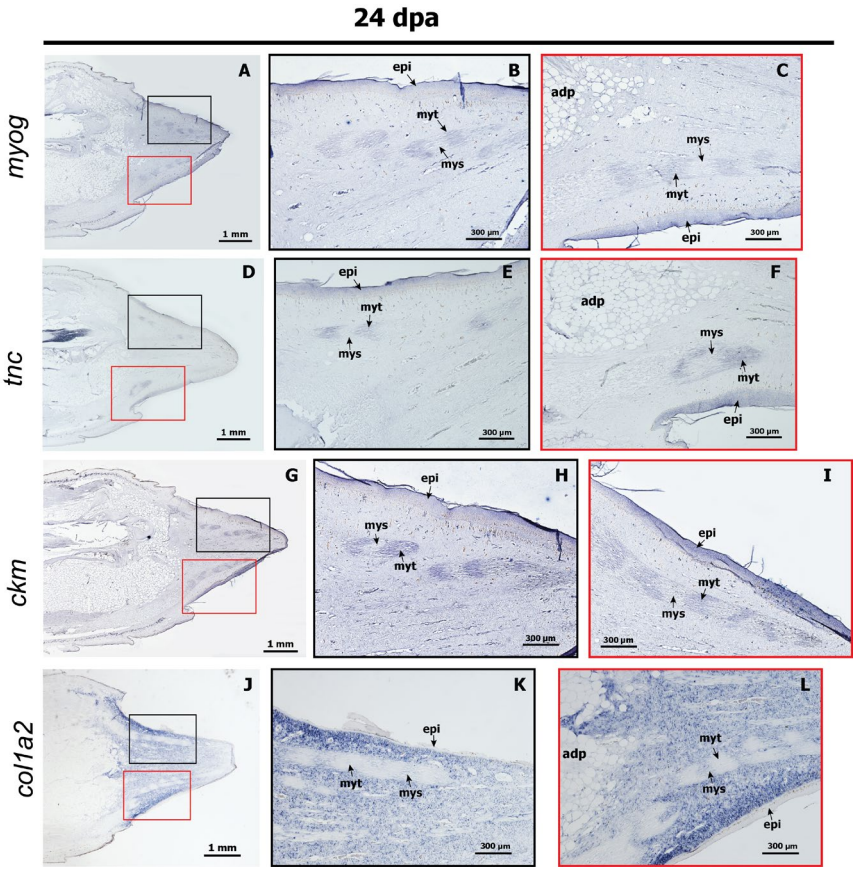

Figure 5-8. **Expression of *myog*, *tnc*, and *ckm*, at 24 dpa in the regenerating tail**. *Key*: myt, myotube; mys, myoseptum.

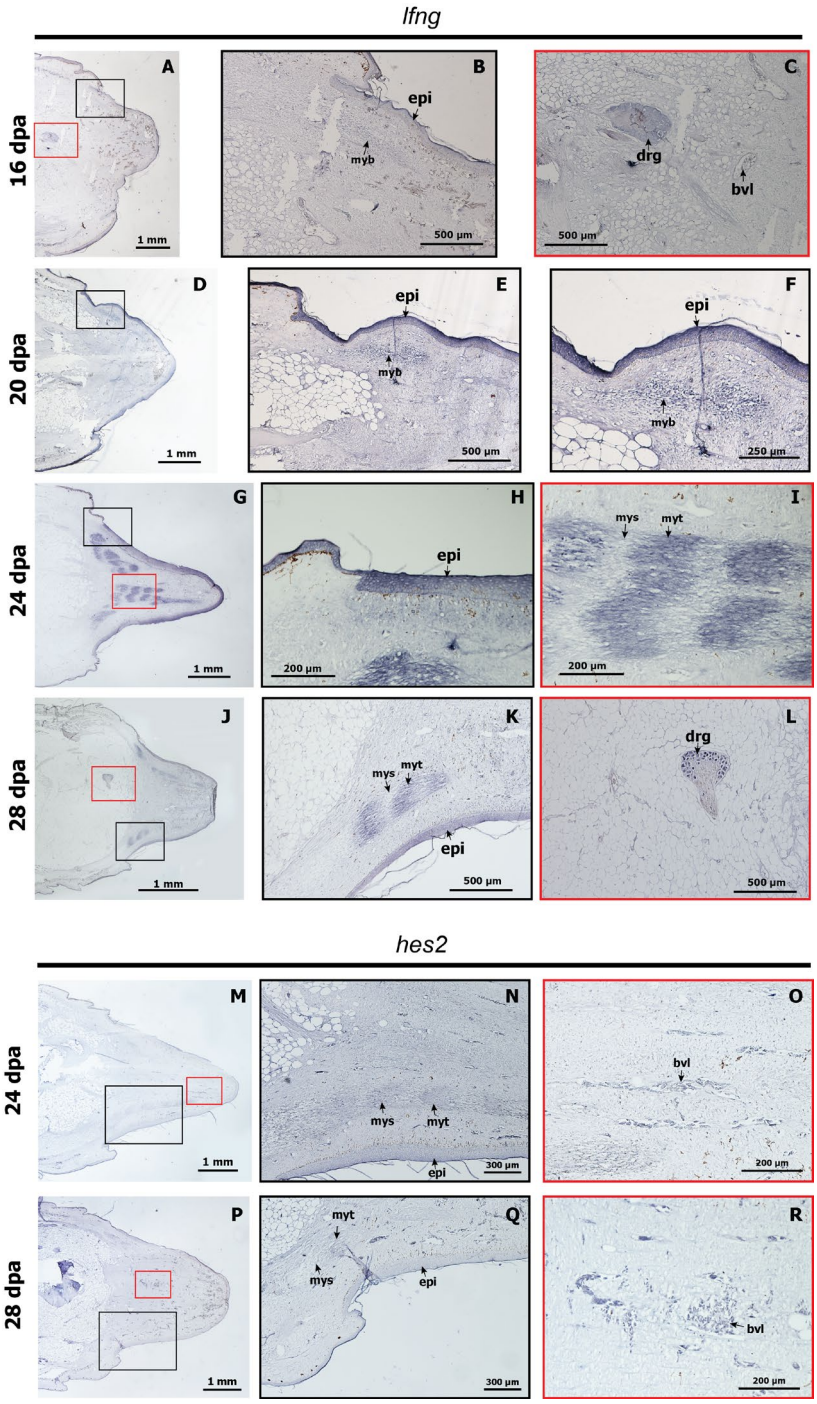

Figure 5-9. **Expression of *Ifng* and *hes2* in the regenerating tail**. *Key*: bvl, blood vessel; drg, dorsal root ganglion; myt, myotube; mys, myoseptum.

Posterior HOXC genes show temporally colinear expression during regeneration

To examine the expression of the posterior HOXC genes (*hoxc10–13*), we used *in situ* hybridization on tissue sections. We found that these genes are all expressed in the developing skeletal muscles and the wound epithelium of the regenerating tail (Figure 5-10). However, these genes differed in the timing of their expression. Thus, at 16 dpa, weak expression of *hoxc10* was observed in the wound epithelium, and in scattered mesenchymal cells, which we believe to be putative muscle progenitor cells (myoblasts) because of their expression of myod1 (see previous section above) and their peripheral location in the proximal region of the blastema (Figure 5-10A, Figure S 5-1). There was no expression of *hoxc11–13* genes at this stage (Figure 5-10E, I, M).

At 20 dpa, we observed expression of *hoxc10–12* in both the wound epithelium, and in the myotubes of developing muscles, but not the myosepta, thereby forming a metameric pattern (Figure 5-10B, F, and J). *Hoxc13* was not expressed at this stage (Figure 5-10N). At 24 dpa, all posterior HOXC genes are expressed in the wound epithelium and in newly-formed skeletal myotubes with myosepta (Figure 5-10C, G, K, O). *Hoxc13* expression at this stage was relatively weak (Figure 5-10O). At 28 dpa, only *hoxc12* and *hoxc13* expression can still be observed in the wound epithelium and regenerating muscles (Figure 5-10L, P). However, at this stage, *hoxc10*, *hoxc11* and *hox13* are no longer expressed (Figure 5-10D, H).

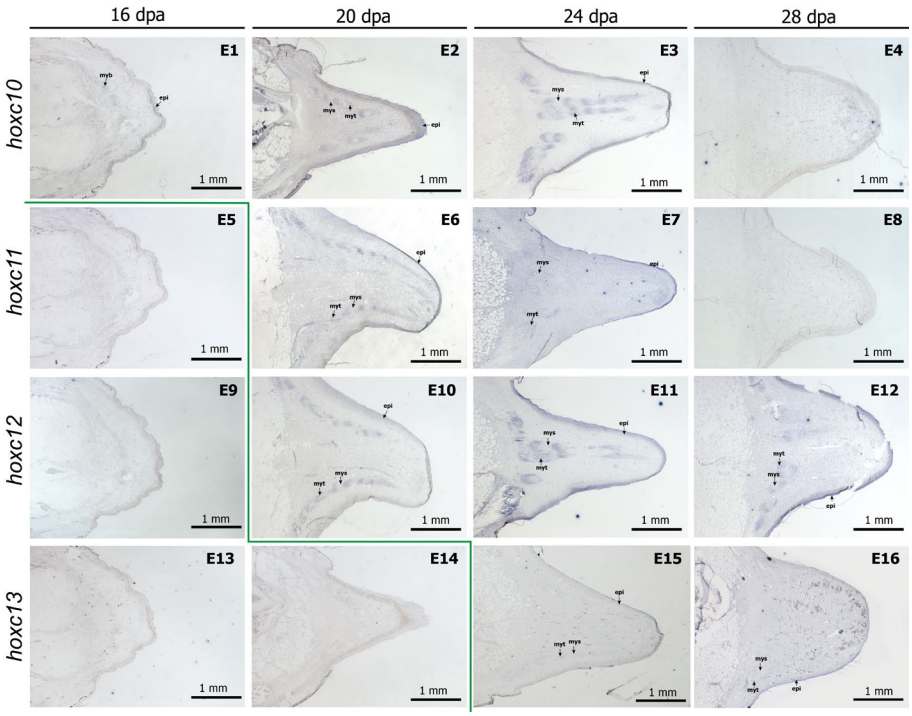

Figure 5-10. **Temporal collinearity in expression of caudal HOXC genes during tail regeneration**. *In situ* hybridization on tissue sections. For high magnification views of these sections, see Figure S 5-1. *Key*: myb, myoblast; myt, myotube; mys, myoseptum; epi, epidermis.

Discussion

In this chapter, we examined the expression of genes involved in axonogenesis, chondrogenesis, myogenesis, segmentation, and tissue patterning during tokay gecko tail regeneration. The results revealed unique gene expression patterns associated with the histogenesis of the regenerating tail. Furthermore, the findings indicated the activation of stem cells residing within the tissues of the original tail, which we suggest utilizing their existing positional memory to guide the regeneration process.

As we have found in Chapter 3 with our transcriptomics study, the regenerating tail of the tokay gecko seems to implement an atypical pattern of gene regulation for tissue development. For example, in this chapter, we find that only three out of eight examined axonogenesis-related genes were expressed in the regenerating gecko tail. These three genes (*unc5a*, *nefh*, and *notch3*) were all expressed in differentiating myotubes at 28 dpa. It is likely that this expression corresponds to the formation of motor end plates because it is known from studies on tail regeneration in other lizard species that the myomeres become innervated by axons that grow into the blastema

from the uninjured stump (Alibardi, 2021; Tokuyama et al., 2018). *Unc5a* orchestrates axonal repulsion, outgrowth, and branching initiated by Netrin-1 (Tokuyama et al., 2018). It is possible that *unc5a* expression in the regenerating myotubes activates axonal branching as growth cones reach the myotubes, and that it also influences the site of formation of neuromuscular junctions (Tokuyama et al., 2018). *Nefh* encodes the neurofilament heavy chain subunit (Elder et al., 1998). In our study, *nefh* is expressed in myoblasts at 20 dpa, and in differentiated myotubes at 28 dpa. On the other hand, we find expression of *notch3*, known for inhibiting the differentiation of neural stem cells, in muscle progenitors at 28 dpa. This indicates a probable role for *notch3* in maintaining the development of regenerating muscle tissue, consistent with prior findings (Kitamoto and Hanaoka, 2010).

In addition, we also found robust expression of *nefh* and another axonogenesis-related gene *gap43* in the dorsal root ganglia (DRGs) of the uninjured part of the tail. This supports the notion (Alibardi, 2021; Lozito and Tuan, 2017) that the uninjured DRGs are the source of axons that innervate the regenerating tail blastema. DRGs proximal to the autotomy plane are known to exhibit hypertrophy and heightened amino acid uptake following autotomy (Geuna et al., 1998). Consistent with this, increased *gap43* expression in DRGs has been observed in rats following nerve injury (Li et al., 2015), and GAP43 protein was detected in the regenerating lizard tail (Alibardi, 2021). Our data also implicate the involvement of axonal pathfinding receptor genes, particularly *unc5a*, in sensory axonogenesis, especially in axons elongated from DRGs of 28-dpa gecko regenerating tails. Our finding of robust expression of *unc5a* in DRGs is consistent with its role in the axonogenesis of sensory neurons during lizard tail regeneration (Alibardi, 2021).

In Chapter 3, we found that the GO term 'cartilage development' was enriched at 16 dpa. However, in this chapter, we find that early cartilage histogenesis did not begin until 20 dpa, when the *sox9*$^{+}$ pre-cartilage precursor of the cartilage tube was first seen. This precursor had the form of a pre-cartilage condensation. In general, pre-cartilage condensations consist of chondroprogenitor cells which differentiate into chondroblasts, secrete extracellular matrix and ultimately develop into mature cartilage cells or chondrocytes (Song and Park, 2020). These processes culminate in the formation of the first hyaline cartilage in the proximal part of the tail by 49 dpa. The cartilage tube continues to differentiate after the skin and muscles have matured, consistent with findings in other lizard species (Alibardi, 2014; McLean and Vickaryous, 2011).

Sox9 is expressed not only in the pre-cartilage condensations at 20 dpa but is also strongly expressed in the cartilage tube at 49 dpa. This is consistent with a role of *sox9* in chondroblast differentiation, a process that may be co-activated by *sox5* and *sox6* (Kozhemyakina et al., 2015). *Sox9* may also inhibit bone differentiation (Dy et al., 2012) which is interesting because the cartilage tube is still un-ossified at oldest stage we

examined (49 dpa). *Bmp3* is also thought to suppress osteoblast differentiation and inhibit ossification (Daluiski et al., 2001; Kokabu et al., 2011) and was also expressed at 49 dpa. *Wnt3* was expressed in the distal part of the regenerating cartilage tube, consistent with a known role in chondrocyte differentiation (Green et al., 2015). *Wnt5a* was expressed in the pre-cartilage condensations and in myoblasts, consistent with its multiple roles in lizard tail regeneration (Hutchins et al., 2014). Our findings align with studies emphasizing the importance of Wnt pathways in tissue patterning and differentiation during development and regeneration (Klaus and Birchmeier, 2008; Nusse and Clevers, 2017).

We found that the reactivation of resident stem cells may underlie myogenesis during tail regeneration, as it does in axolotl limb regeneration (Sandoval-Guzman et al., 2014). The maturation of skeletal muscle becomes apparent at 24 dpa with the appearance of myotubes expressing *myog, tnc* and *ckm*. There are two existing hypotheses of myogenesis in tetrapod tail and limb regeneration. One postulates the dedifferentiation of mature skeletal muscle fibers in the uninjured stump tissue (Echeverri et al., 2001; Slack, 2006). The other postulates the activation of resident stem cells (Rodrigues et al., 2012; Sandoval-Guzmán et al., 2014; Slack, 2006). For example, limb regeneration in newts requires the dedifferentiation of myotubes, but myogenesis during limb regeneration in axolotls relies on the activation of resident stem cells (Sandoval-Guzman et al., 2014).

The results show that the expression of the muscle lineage markers *pax3*, *pax7* and *myod1* began at 8 dpa. It is possible, therefore, that myogenesis during gecko tail regeneration involves an early activation of satellite cells (muscle resident stem cells). Our reasoning is that *pax7* and *myod1* are co-expressed during satellite cell activation (Zammit et al., 2006). Based on previous studies (Alibardi, 2014; Wosczyna and Rando, 2018), and on our data in this chapter, it is likely that cells that express *pax3*, *pax7* and *myod1* will aggregate and differentiate into mononuclear myoblasts at 16 dpa. The early expression of *pax3* and *pax7* is also seen in the regenerating tail blastema of the green anole lizard *Anolis carolinensis* (Hutchins et al., 2014) and Schlegel's Japanese Gecko, *Gekko japonicus* (Liu et al., 2021).

The fact that the skeletal muscle in the regenerated lizard is arranged into segments (bundles of myotubes separated by myosepta) raises the question of whether regeneration involves a re-activation of embryonic segmentation genes. Such genes could included *lfng* and *hes7* which are necessary for somitogenesis in the embryo (Bessho et al., 2001; Kageyama et al., 2012). Our results suggest that only *lfng* is expressed in the muscle lineage of the regenerating gecko tail. *Lfng* is a modulator of the Notch signaling pathway (Mugisha et al., 2022), and this pathway is recognized for its function in promoting the self-renewal of satellite cells through direct modulation of *pax7* (Wen et al., 2012).

Among the Hes genes, only *hes2* was expressed in gecko tail regenerates, in the skeletal muscle and haemopoietic lineages. And even *hes2* is unlikely to be involved in muscle segmentation because we only find it expressed at 24 dpa, after the muscles have already segmented. Our suggestion, of a lack of Hes gene involvement in muscle segmentation in the regenerating gecko tail, is consistent with a study on axolotl tail regeneration (Wouter et al., 2024). That study found evidence that *lfng*$^{+}$ multipotent stem cells (which they called 'asomitic' stem cells) give rise to segmented structures during regeneration without the involvement of *hes7* (Wouter et al., 2024). We suggest that muscle segmentation in the regenerating tokay gecko tail relies on positional memory in satellite cells, which 'remember' the segmental pattern of the original muscle. This hypothesis is supported by a study of mouse cell lines, showing that satellite cells have positional memory regulated by *hoxa10* (Yoshioka et al., 2021). In summary, there is evidence supporting our resident stem-cell hypothesis in muscle regeneration in the tokay gecko tail.

In relation to the concept of positional memory, we also found interesting results on posterior HOXC gene expression. These genes are member of the HOX family that may encode anatomical positional memory or cellular identity (Steens and Klein, 2022; Yoshioka et al., 2021). During embryonic development, HOX gene expression is both spatially and temporally collinear (Mallo and Alonso, 2013; Woltering et al., 2009), meaning that HOX genes become transcriptionally active along the body axis, and over developmental time, in a sequence reflecting their position along the chromosome. However, our results indicate that the posterior HOXC genes (*hoxc10–13*) show temporal collinearity, but not spatial collinearity, in the regenerating tail.

Hox genes are known to be important in regeneration. In caudal fin regeneration in the zebrafish, for example, *hoxc13a* and *hoxc13b* are expressed early and distally in the blastema (Thummel et al., 2007). It would be interesting to know whether HOXC expression patterns in the regenerating tail are determined by existing positional information in the intact tail, before it was shed. This could arise, for example, if there is differential expression of hox genes along the normal tail. It would also explain why the regenerating gecko tail replaces a similar number of muscle segments that were present before the tail was amputated. This is also the case in the axolotl limb, which regenerates only those limb parts appropriate to the proximodistal level of the amputation plane (Pescitelli and Stocum, 1980). A similar situation is seen with the zebrafish caudal fin regeneration in which only the appropriate missing parts are replaced (Uemoto et al., 2020).

In mammals, at least some HOX genes are expressed in the adult, often in the same position-specific pattern or 'hox code' that they showed during embryonic development (Hubert and Wellik, 2023; Leucht et al., 2008; Rux et al., 2016). Furthermore, fibroblasts from adult humans (Chang et al., 2002) and skeletal muscle

from adult mice (Yoshioka et al., 2021) have 'positional memory' that reflects their anatomical location. Together, these findings may shed light on tail regeneration in the gecko. Thus, it is possible that resident stem cells at the autotomy site may have some kind of positional memory. Consistent with this idea, our data also indicate that resident stem cells might be the main precursor cell population in the regenerating tail. Our single cell analysis (Chapter 4) confirmed this.

These and other data suggest a model in which resident stem cells, in this case satellite cells, at the amputation plane use their positional memory to regenerate an appropriate number of muscle segments. Furthermore, we suggest that resident stem cells do this without generating *de novo* a system of positional information as is the case in normal development (of the embryonic limb bud or tailbud, for example). We suggest this because the gecko regeneration blastema has properties very different from a classical developmental field, in which positional values are specified *de novo*. Finally, we suggest that the late expression of *hoxc13* that we observed here represents the finalization of the patterning process in gecko tail regeneration, since it is known that *hoxc13* is essential for terminating normal tail development in the embryo (Aires et al., 2019).

In summary, this chapter provides evidence that the regeneration of the tail in the tokay gecko involves an early activation of a stem cell population, likely comprising satellite cells and fibroblasts, that retain positional memory from the original tail. This positional memory allows the regeneration of an appropriate tail replacement, without the regenerative blastema establishing a new positional coordinate system *de novo* as seen in normal development.

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Supplementary Information

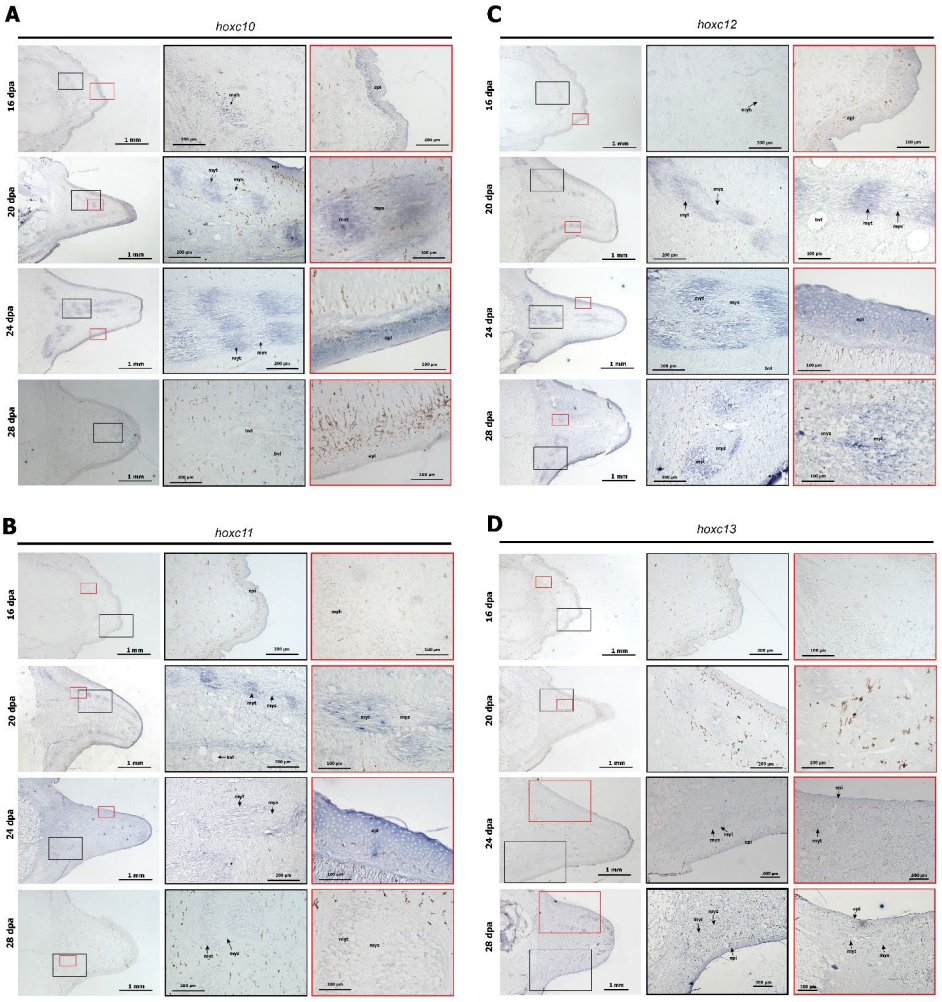

Figure S 5-1. **The expression of posterior HOXC genes in regenerating tail of tokay gecko (*G. gecko*).** (A) Detail expression of *hoxc10* in 16, 20, 24, and 28 dpa of regenerating tail. Key: bvl, blood vessel; ep, epidermis; myb, myoblast; mys, myoseptum; myt, myotubes. (B) Detail expression of *hoxc11* in 16, 20, 24, and 28 dpa of regenerating tail. (C) Detail expression of *hoxc12* in 16, 20, 24, and 28 dpa of regenerating tail. (D) Detail expression of *hoxc13* in 16, 20, 24, and 28 dpa of regenerating tail.

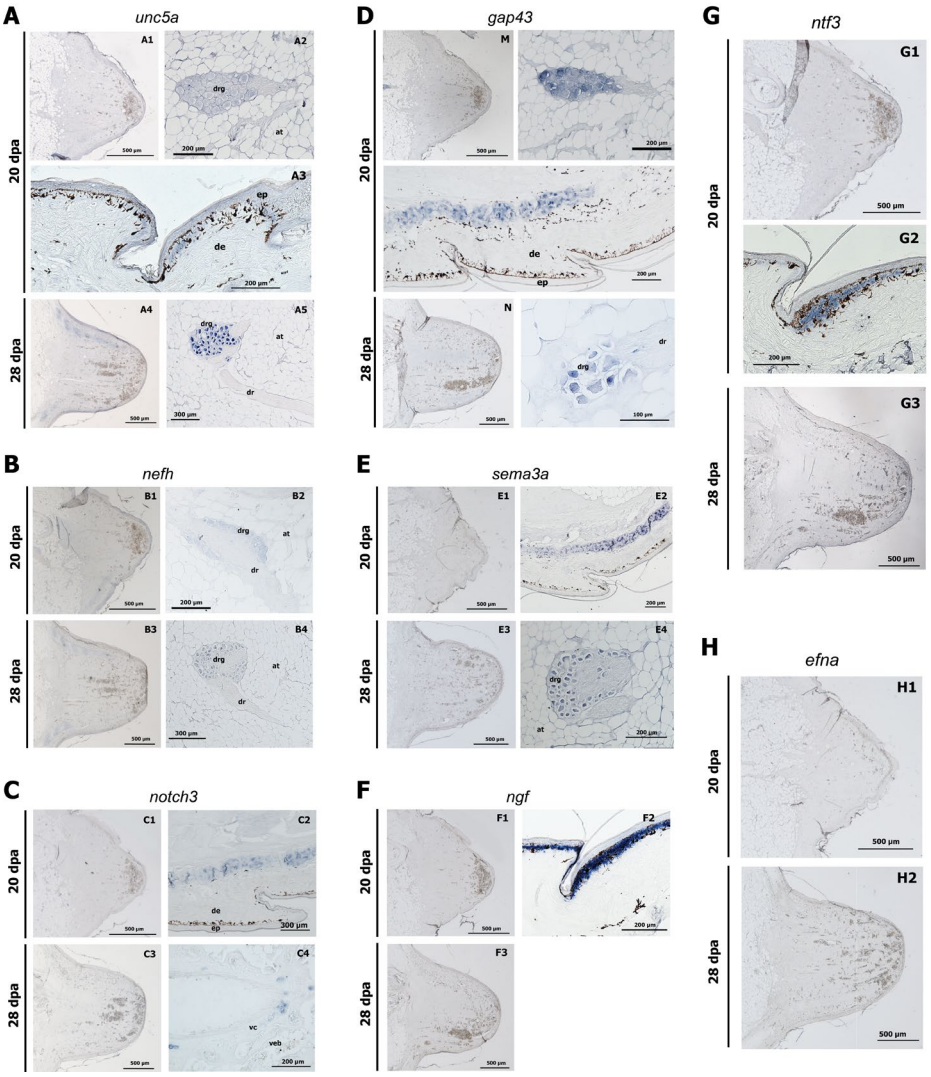

Figure S 5-2. **The expression of axonogenesis genes in the regenerating tail of tokay gecko (G. *gecko*).** (A) Detail expression of *unc5a* in the regenerating tail. Key: at, adipose tissue; bvl, blood vessel; de, dermis; dr, dorsal root nerve; drg, dorsal root ganglia; ep, epidermis. (B) Detail expression of *nefh* in the regenerating tail**.** (C) Detail expression of notch3 in the regenerating tail. (D) Detail expression of gap43 in the regenerating tail. (E) Detail expression of *sema3a* in the regenerating tail. (F) Detail expression of *ngf* in the regenerating tail. (G) Detail expression of *ntf3* in the regenerating tail. (H) Detail expression of *efna* in the regenerating tail.

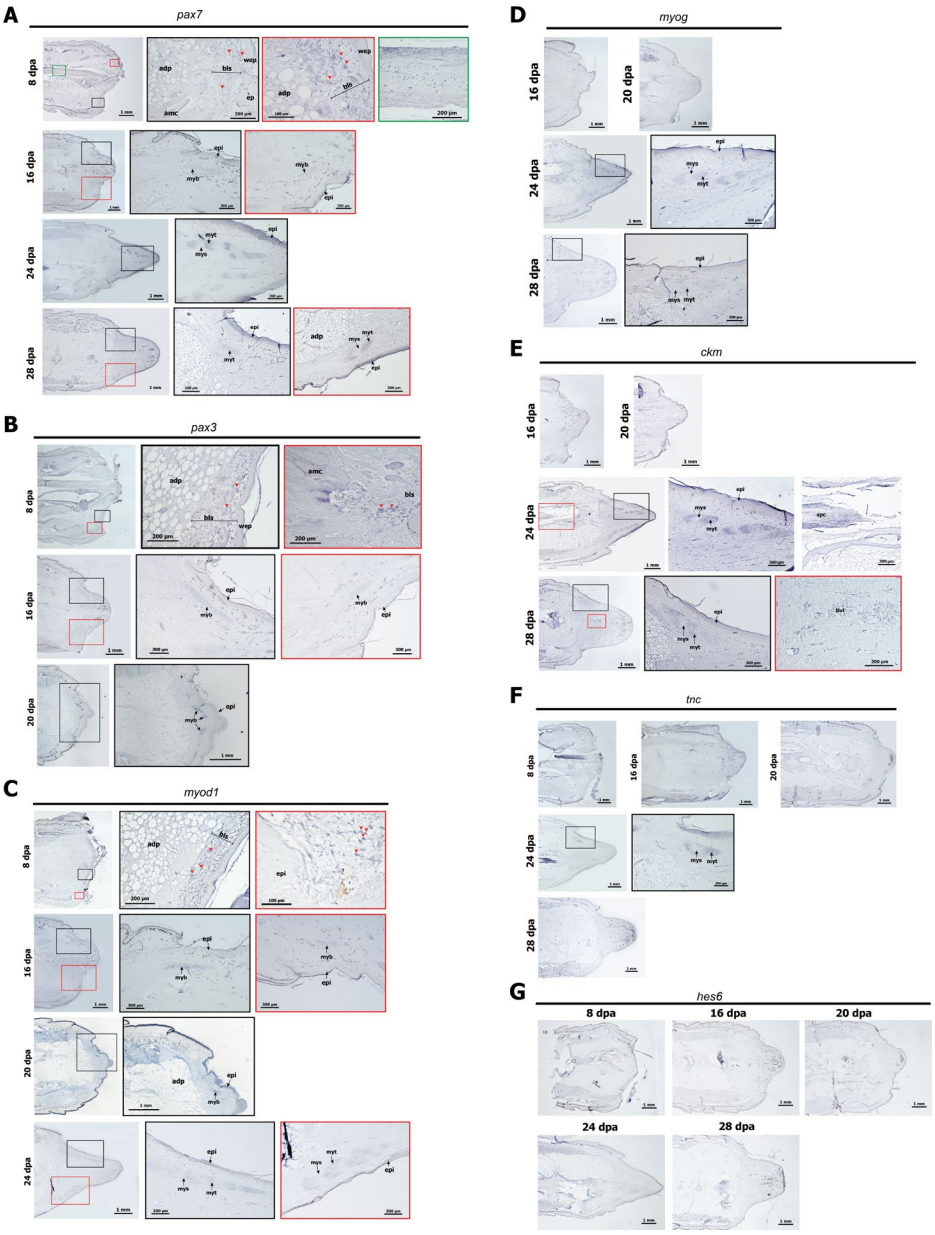

Figure S 5-3. **The expression of muscle genes in regenerating tail of tokay gecko (G. *gecko*)**. (A) Detail expression of *pax7* in regenerating tail. adp, adipose tissue; amc, adult muscle cells; bls, preblastema; ep, epidermis; myb, myoblast; mys, myoseptum; myt, myotubes; wep, wound epithelium. (B) Detail expression of *pax3* in the regenerating tail. (C) Detail expression of *myod1* in the regenerating tail. (D) Detail expression of *myog* in the regenerating tail. (E) Detail expression of *ckm* in the regenerating tail. (F) Detail expression of *tnc* in the regenerating tail. (G) Detail expression of *hes6* in the regenerating tail.