Tail regeneration in the Tokay Gecko (Gekko gecko)

Nurhidayat, L.

Chapter 2. Descriptive Analysis of Tail Regeneration in The Tokay Gecko

Abstract

Lizards, including the tokay gecko, possess the remarkable ability to regenerate their tails following autotomy. Understanding the histological structure of the regenerating tail is a fundamental starting point for this thesis and for future research on tail regeneration. It provides crucial insights into the specific cellular and tissue structures involved in the regenerative process, including the blastema and the various regenerating tissues such as muscle, bone, nerves, and blood vessels. We studied seven stages of tail regeneration in the tokay gecko: 4, 8, 16, 20, 24, 28, and 49 days post-autotomy using paraffin histology. The tails were sagittally sectioned and stained with hematoxylin eosin-alcian blue and periodic acid Schiff-alcian blue. In the wound healing phase (4 and 8 dpa), the regenerating tail displayed formation of a wound epithelium and the presence of erythrocytes and leukocytes, at the autotomy site. The blastema formation phase (stages 16 and 20 dpa) was characterized by the formation of undifferentiated blastema tissues. Stages 24 and 28 dpa marked the tissue differentiation phase, characterized by formation of the cartilage tube and segmented skeletal muscles. Finally, the tail maturation phase (stage 49 dpa), exhibiting mature epidermis, skeletal muscles, cartilage tube, and adipose tissue. Interestingly, we saw no evidence of a distal, undifferentiated growth zone. Instead, the distal tissue showed an abundance of blood vessels.

Introduction

Autotomy, or self-amputation of body parts, is a common and effective self-defense mechanism to distract predators. Lizards are an example of animals in which autotomy is possible. It typically occurs in response to predation attempts, allows the lizard to escape (Clause and Capaldi, 2006; Fleming and Bateman, 2012). The autotomy process occurs spontaneously, and there are no visible signs of damage at the site of tail loss, even though the tail is composed of tissues with varying consistencies. Lizards can perform autotomy because they have pre-existing, transversely oriented fracture planes (autotomy planes) in the tail (Delorme et al., 2012; Pratt, 1946). Each fracture plane contains fibroblast-rich connective tissue that connects the dermis to the intermuscular, inter-adipose, and vertebral bone tissue (Bellairs and Bryant, 1985). If autotomy occurs outside the fracture plane, regeneration will be hindered, and may even cease completely (Delorme et al., 2012; Pratt, 1946) .

Lizards have the ability to regenerate their tails after autotomy (Alibardi, 2015; Simpson, 1970). The main differences between the original tail and the regenerated tail lie in the vertebral structure and the spinal cord. Regenerated tails are primarily supported by a longitudinal tube-shaped structure composed of cartilage which replaces the series of vertebrae seen in the original tail (Alibardi, 2014; Gilbert et al., 2013; Lozito and Tuan, 2015). In regenerated tails, the spinal cord is replaced by an ependymal tube lining the cartilage tube, and consisting of ependymal cells, glial cells, and nerve fibers without neuronal cell bodies (Gilbert and Vickaryous, 2018). The ependymal tube consists of cells that originated in the central canal of the original spinal cord (Gilbert and Vickaryous, 2018).

The regeneration of the tail can be divided into four phases (Alibardi, 2010; Lozito and Tuan, 2016): wound healing, blastema formation, differentiation and maturation. In the wound healing phase, stump tissue is broken down and mesenchymal stem cells accumulate underneath the wound epidermis (Lozito and Tuan, 2017). Eventually, the wound is re-epithelialized, forming a thickened epithelium known as the wound epithelium or wound epidermis (Alibardi, 2019). Ependymal cells are organized into the ependymal tube, which is regenerated from the spinal cord. Afterwards, the aggregation of cells, released during the breakdown of the stump tissues, proliferates, and swell beneath the wound epithelium forming the blastema. Blastema formation is followed by rapid tail growth, and the blastema begins to differentiate into tissues including muscle and cartilage (Lozito and Tuan, 2017). A cartilage tube in the regenerated tail takes the place of the vertebrae in the original tail. This tube may originate from stem cells in the intervertebral cartilage (Londono et al., 2017) and almost completely encloses the ependyma. During the elongation phase, the cartilage tube grows, and

muscles elongate and thicken. Lastly, in the maturation phase the regenerated tail reaches maximum length and the tissue is completely differentiated (Alibardi, 2014).

Understanding the histological structure of the regenerating tail is crucial for further research on tail regeneration (Alibardi, 2010; Alibardi, 2014). Histological analysis provides crucial insights into the specific cellular and tissue structures involved in the regenerative process, including the blastema and various regenerating tissues such as muscle, bone, nerves, and blood vessels (Alibardi, 2014; Gilbert et al., 2013; Lozito and Tuan, 2016). Moreover, it allows for the observation of temporal and spatial dynamics during regeneration, revealing how different cell types interact and contribute to tissue renewal (Gilbert et al., 2013; McLean and Vickaryous, 2011). By characterizing the different cell types present, researchers can better understand their roles in regeneration, from stem/progenitor cells to immune cells and differentiated tissues (Alibardi, 2010; Jacyniak et al., 2017). Additionally, histological study facilitates the assessment of tissue quality and functionality, enabling comparisons between regenerated and uninjured tissues (Alibardi, 2010). Considering these factors, this chapter aims to characterize the histological structure of seven stages of tail regeneration in the tokay gecko. This will establish a foundation for the further studies of tokay gecko tail regeneration in the following chapters of this thesis.

Materials 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 sample 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 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.

Histology

The regenerating tail blastema samples were rinsed in RNAse-free PBS and fixed in cold 4% pFA in PBS. The fixed samples were then decalcified by soaking them in Osteosoft® (Sigma-Aldrich) for 3–5 days. The decalcified samples were then dehydrated with a graded concentration series of ethanols (70%, 80%, 90%, 100%) for 2 × 30 min each. The samples were cleared 3 x 1 h (Neo-Clear®, Merck-Millipore). They were infiltrated 2 × 1 h molten paraffin (62 °C) and 1 × 8–18 h. The samples we finally embedded with fresh molten paraffin and solidified at room temperature. Longitudinal sections (6-8 µm thickness) were cut using a microtome. Prior to staining, the sections were deparaffinized with xylene (3 × 5 min) followed by rehydration through graded ethanols (90%, 80%, 70% for 1 min each) and demi-water (2 × 1 min).

For hematoxylin, eosin and alcian blue (HE-AB) staining, the sections were stained in 1% alcian blue 8GX in 2.5% acetic acid for 10–15 min, then washed under running tap water (5 min). Alcian blue gives a blue stain to the acidic polysaccharides and glycosaminoglycans in the extracellular matrix of hyaline cartilage. Next, the sections were stained with Mayer's hematoxylin and eosin according to standard protocols (Bancroft, 2008).

Periodic acid Schiff and alcian blue (PAS-AB) staining was started by staining the sections with alcian blue (10–15 min), 1% alcian blue (8GX) in 2.5% acetic acid and washed under running tap water (5 min). The sections were then treated with 1%

periodic acid (5 min) and washed with demi-water. Next, the sections were stained with Schiff's reagent (10min) and washed in running tap water (5 min). To stain the nuclei, the sections were subsequently stained with hematoxylin (1 min) and washed in running tap water (5 min).

After the staining steps, the sections were washed in demi-water (1 min) and dehydrated with graded ethanols (70%, 80%, 90% for 1 min each and 100% 3 × 1min) followed by xylene (3 × 5 min). Finally, the sections were coverslipped using Eukitt® (Sigma-Aldrich; St. Louis, USA) and left overnight at 37 °C.

Results

Macroscopic appearance

Tail regeneration in the tokay gecko began with the formation of a scab, which appeared 4–8 days post-autotomy (dpa) regenerating tail (Figure 2-1B–C). This scab started to disappear at 12 dpa (Figure 2-1D), and by 16 dpa, it had completely disappeared, and the blastema became dome-shaped (Figure 2-1E). Subsequently, this dome-shaped structure elongated and cone-like (Figure 2-1F–H). The scalation (pattern of scales) of the regenerating tail first began to become visible at 28 dpa, at which stage the scales had a uniform brownish-gray pigment (Figure 2-1H). At 58 dpa, the multiple dark patches of pigment were visible (Figure 2-1K)and by 12 weeks post-autotomy (wpa) the pigmentation pattern of the regenerated tail was quite similar to the original tail (Figure 2-1L). However, despite the similarities in color and scale patterns, the regenerated tail could still be distinguished from the original tail by the absence of the 'segments' that mark the skin of the original tail (Figure 2-1L). These segments presumably represent the site of the fracture planes because autotomy always happens in this location (Luthfi Nurhidayat, unpublished observations).

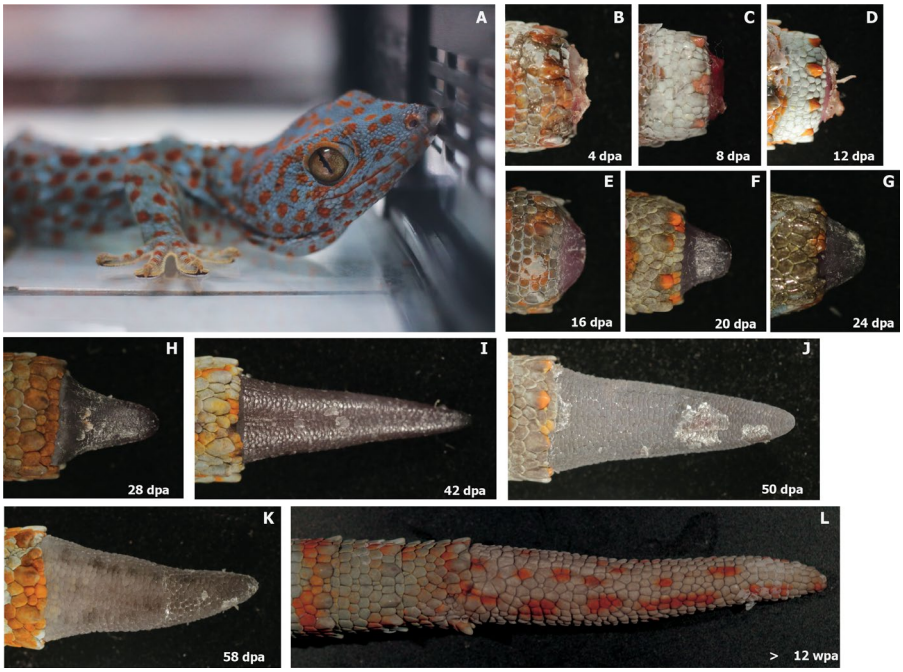

Figure 2-1. **Macroscopic appearance of tail regeneration in the tokay gecko (*Gekko gecko*)**. (A) tokay gecko morphology. (B, C) Regenerating tail undergo wound healing phase marked by scab formation. (D) The scab started to exfoliate. (E – H) The regenerating tail appears in dome-shaped and grows elongated as the progression of the regeneration stages. (I , J) the regenerating tail shows clear scales pattern. (K) Pigmentation patterns start to visible in the regenerating tail. (L) The pigmentation pattern of the regenerating tail is similar with that of the original tail but can still be distinguishable. Note that the original tail looks morphologically segmented.

Wound healing phase

Histology confirms that the wound healing phase involves the formation of a scab covering the wound or autotomy site (Figure 2-2 – Figure 2-5). At 4 dpa, a thinner layer of epithelial cells begins to form under the scab, covering the tissues in the tail stump (Figure 2-2 A). This wound epithelium shows a reduction in its number layers, from multiple layers near the tail stump epidermis (Figure 2-2B) to 1–2 layers of cells in the middle area of the wound site (Figure 2-2D). Notably, populations of blood cells, including both erythrocytes and leukocytes, were observed, particularly in the central region of the wound site (Figure 2-2C). At this stage, thin connective tissue started to form under the wound epithelium. This connective tissue has a matrix with acidic and neutral mucin (Figure 2-3).

At 8 dpa, the scab was still present (Figure 2-4A). The wound epithelium comprised multiple layers of epithelial cells but remained thinner than the normal

epidermis of the tail stump (Figure 2-4B, D). Notably, there was tissue starting to form between the epidermis and the tail stump tissues (Figure 2-4D). This tissue which we envisage as a 'pre-blastema', displayed a heterogeneous composition, consisting of connective tissue, blood vessels, and various cell populations that cannot be characterized using histology. The connective tissue in this area also has a matrix with acidic and neutral mucin scattered through the area (Figure 2-5). Moreover, red blood cells (erythrocytes) and white blood cells (leukocytes) were still noticeable in multiple regions, particularly within the central area of the wound site (Figure 2-4C). We also found that this central area has not been covered by wound epithelium and is still connected to the scab (Figure 2-4C). We did not find any histological evidence of a structure (such as condensed, undifferentiated mesenchyme) resembling an apical growth zone.

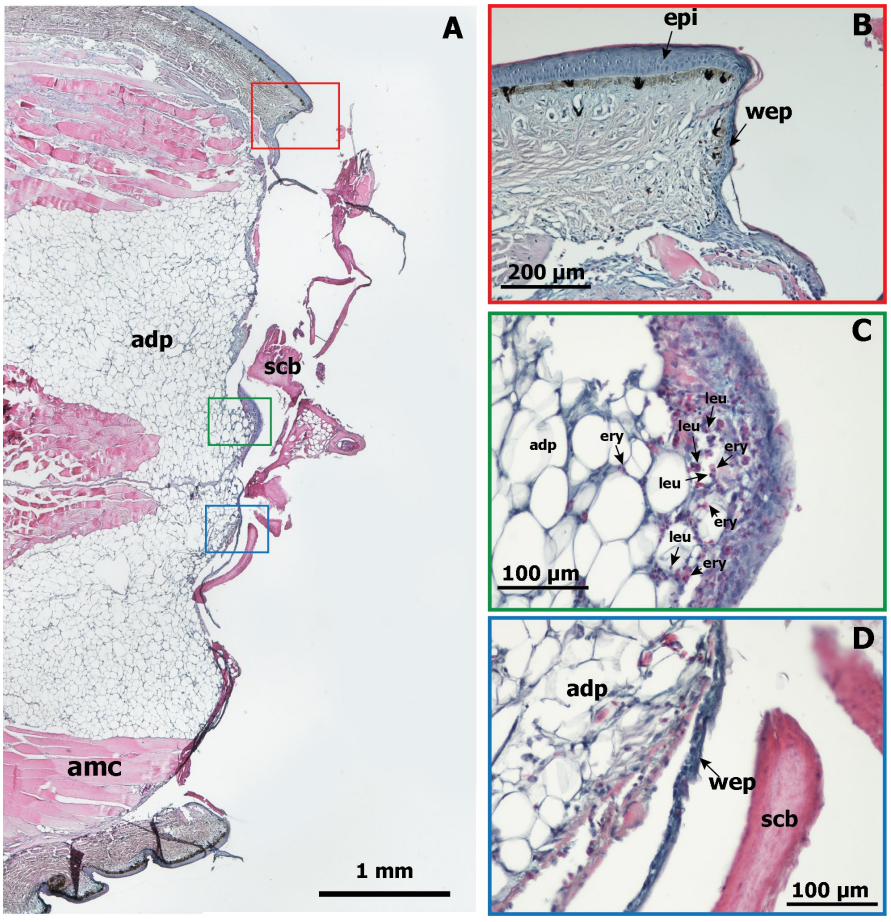

Figure 2-2. **Scab and wound epithelium formations in the wound site of the 4 dpa regenerating tail of the tokay gecko**. (A) Sagittal section of 4 dpa regenerating tail of the tokay gecko showed the scab covering the wound site. (B) Epithelium transition from epidermis to wound epithelium. (C) Blood cell populations (erythrocytes and leukocytes) in the central area of the wound sites. (D) Thin layer of wound epithelial is formed under the scab. Hematoxylin-Eosin Alcian Blue Staining. *Key:* adp, adipose tissue; amc, adult muscle cells; ery, erythrocytes; epi, epidermis; leu, leukocytes; scb, scabs; wep, wound epithelium.

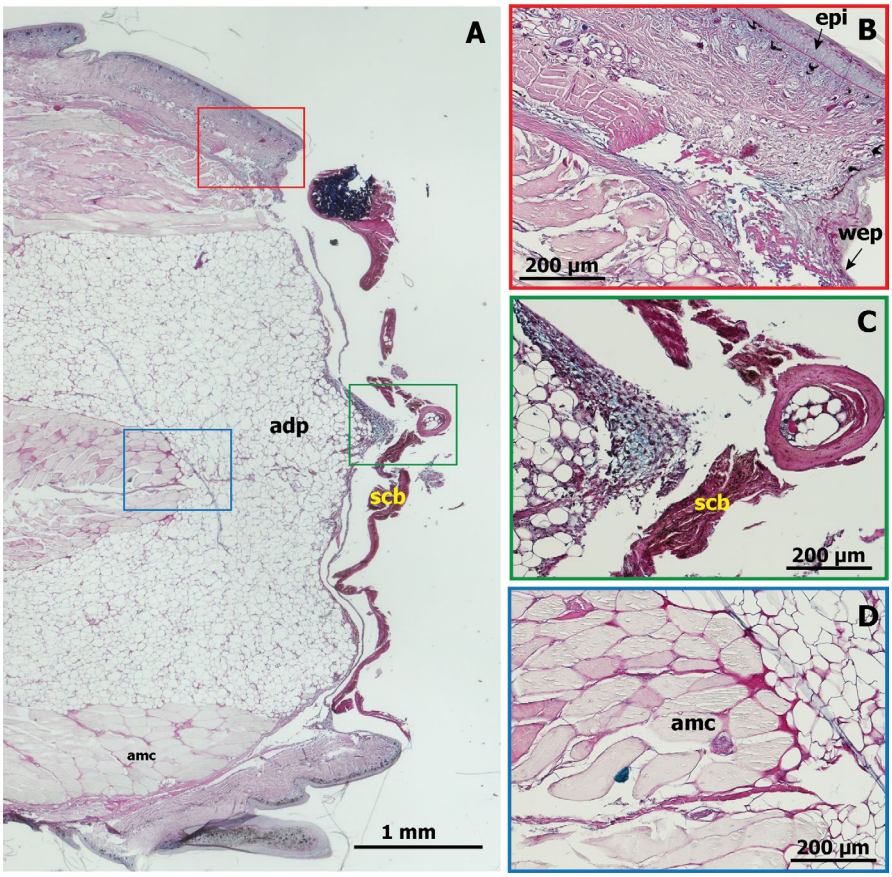

Figure 2-3. **Distribution of neutral mucin (red) and acidic mucin (blue) in the 4 dpa regenerating tail of the tokay gecko**. (A) The original tail tissue generally contains neutral mucin. (B) the subepidermal tissue of original tail is dominated by neutral mucin while the tissue under wound epithelium contains both neutral and acidic mucin. (C) The neutral mucin and acidic mucin are both visible in the central area of the wound site. (D) The adult muscle tissue in the original tail, especially muscle fascia, dominated by neutral mucin. Periodic acid-Schiff alcian blue staining. *Key:* adp, adipose tissue; amc, adult muscle cells; epi, epidermis; scb, scabs; wep, wound epithelium.

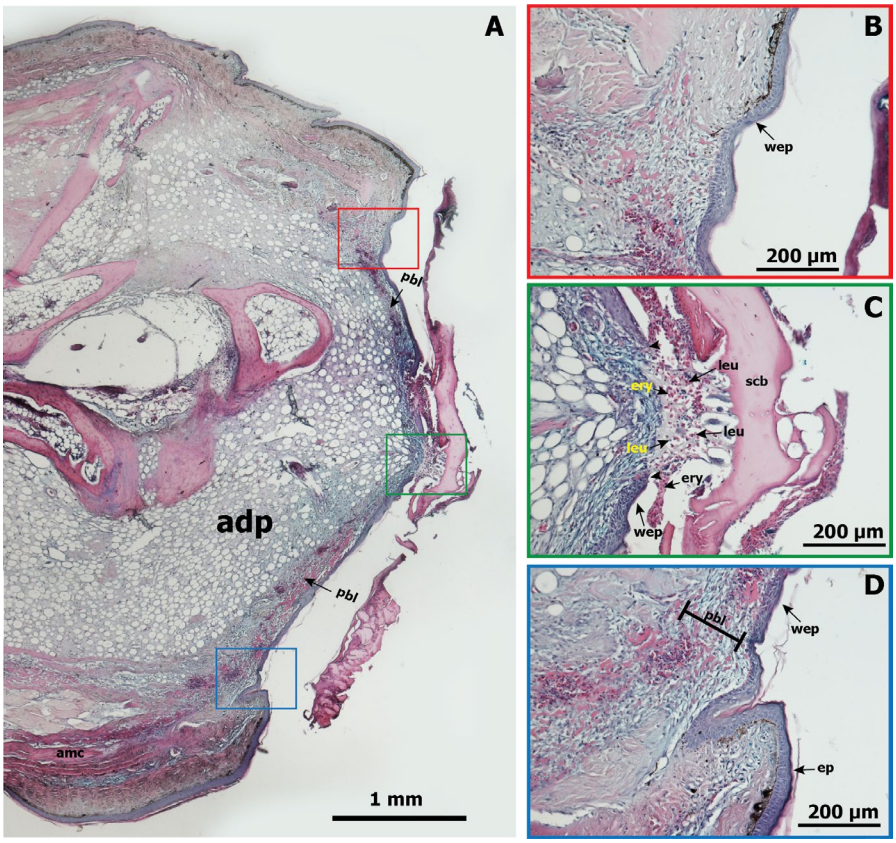

Figure 2-4. **Pre-blastema tissue start to infiltrate equally in the area between the wound epithelium and the tail stump tissues at 8 dpa regenerating tail of the tokay gecko.** (A) The pre-blastema tissue infiltrate from the periphery to the central area of the wound site. (B) The wound epithelium is now multilayered. (C) Blood cell populations (erythrocytes and leukocytes) are still prominent in the central area of the wound sites. Note that this central area has not been covered by wound epithelium. (D) The pre-blastema tissue looks heterogenous. Hematoxylin eosin alcian blue staining. *Key:* adp, adipose tissue; amc, adult muscle cells; ery, erythrocytes; epi, epidermis; leu, leukocytes; pbl, pre-blastema; scb, scabs; wep, wound epithelium.

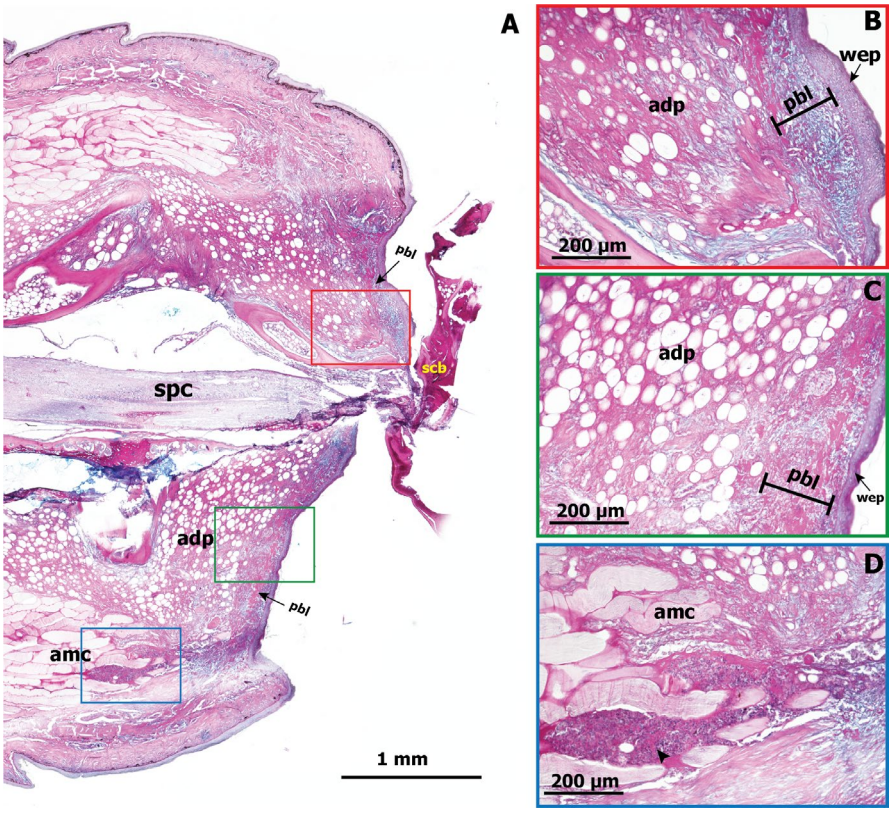

Figure 2-5. **Distribution of neutral mucin (red) and acidic mucin (blue) in the 8 dpa regenerating tail of the tokay gecko**. (A) The pre-blastema tissue matrix shows acidic and neutral mucin. (B,C) The scattered distribution of acidic and neutral mucin in the dorsal (B) and ventral (C) parts of pre-blastema tissue. (D) the adult muscle tissue is dominated by neutral mucin. Periodic acid-Schiff alcian blue staining. *Key:* adp, adipose tissue; amc, adult muscle cells; pbl, pre-blastema; scb, scabs; spc, spinal cord; wep, wound epithelium.

Blastema formation phase

The blastema formation phase can be distinguished at **16 dpa**. At this time, the regenerating tail consists of cells and tissues in the early stages of differentiation (Figure 2-6A) as well as connective tissue. The early differentiating cells included dorsal and ventral clusters aggregates of myoblast-like cells (Figure 2-6B). We still could not evidence of any mesenchymal cells organized into a growth zone in the distal part of blastema. Instead, this distal region consisted mainly of connective tissues containing many blood vessels (Figure 2-6C). The wound epithelium at this stage already showed a mature epidermal structure similar to the epidermis of the tail stump but was weakly keratinized and unpigmented (Figure 2-6D). In addition, nerve fibers can be seen at this stage, possibly originating from the dorsal root ganglia in the uninjured tail.

We found that blastema tissue had atypical distribution of acidic mucin and neutral mucin (Figure 2-7A). Acidic mucin dominated the dorsal and ventral areas of the regenerating tail (Figure 2-7B, D), while neutral mucin was concentrated in the central area (Figure 2-7C). Moreover, we also found that the connective tissue of the uninjured tail muscle extends into the regenerating tail showing the similar characteristics with neutral mucinous connective tissue (Figure 2-6A, Figure 2-7A, D).

At **20 dpa** the regenerating tail showed a similar structure to that of 16 dpa (Figure 2-8). The main differences that can be observed at 20 dpa are that the myoblast population is more clearly visible and cartilage condensation begins to occur in the central area of the tail (Figure 2-8A & D). Moreover, pre-cartilage condensations are visible in the proximal part of the tail (Figure 2-8B). The blood vessels also appear to be more concentrated in the distal part of the regenerating tail at this stage (Figure 2-8C).

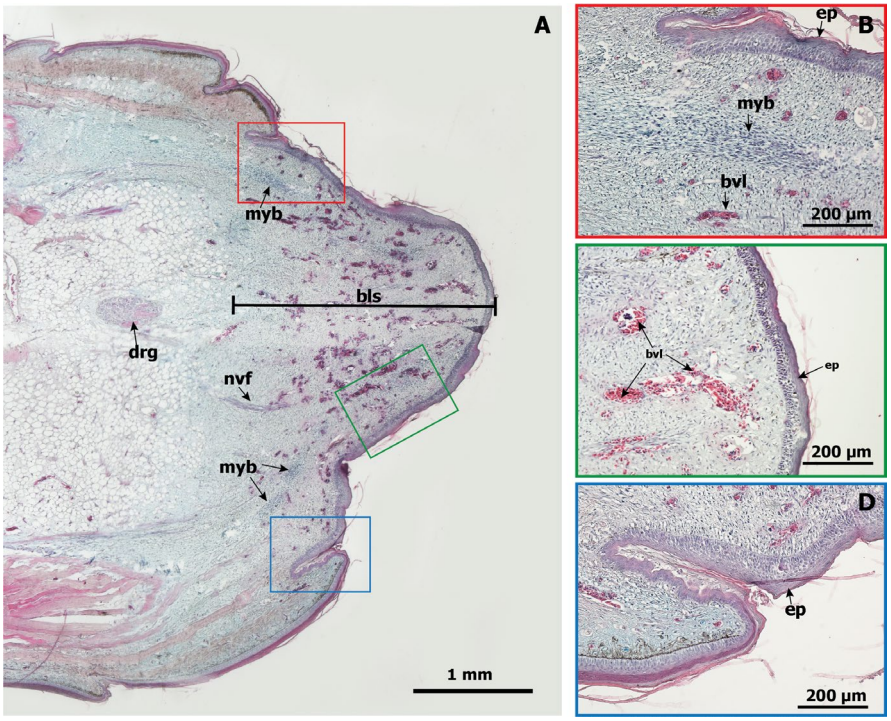

Figure 2-6. **The blastema consist of heterogenous tissue structure at 16 dpa regenerating tail of the tokay gecko**. (A) the heterogenous tissues structure within the blastema, including myoblast, nerve fibers, and blood vessels. (B) the myoblasts aggregate in the dorsal area. (C) The distal region of blastema consisted mainly of connective tissues and blood vessels. (D) The wound epithelium has transformed into more mature epidermal structure, but without pigmentation. Hematoxylin eosin alcian blue staining. *Key*: bls, blastema; bvl, blood vessel; drg, dorsal root ganglia; ep, epidermis; myb, myoblast; nvf, nerve fiber.

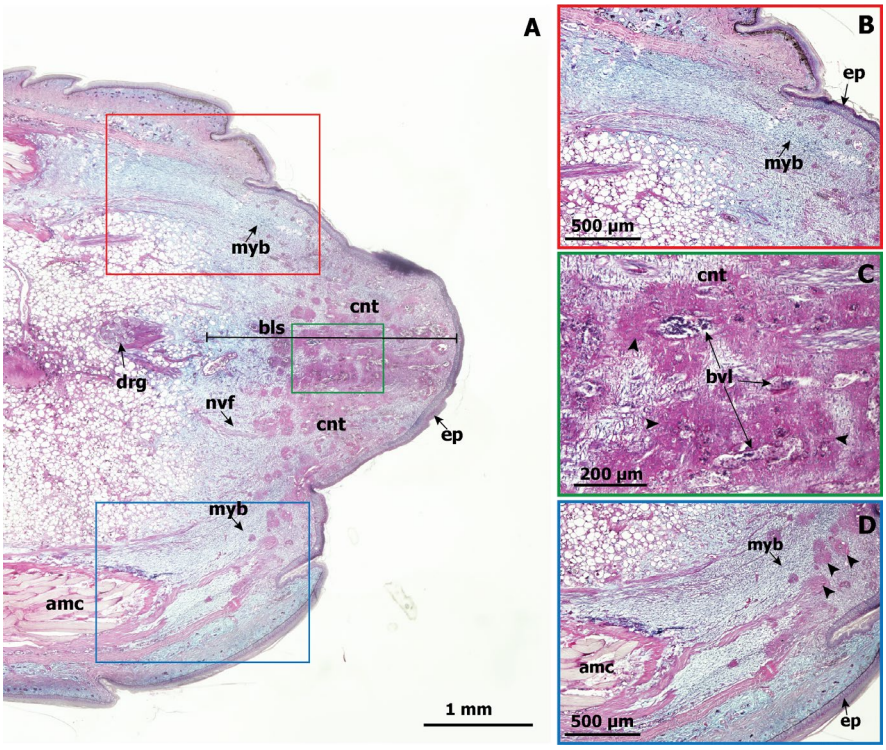

Figure 2-7. **Atypical distribution of neutral mucin (red) and acidic mucin (blue) in the regeneration blastema of the tokay gecko at 16 dpa.** (A) An overview of the mucin distribution in the regeneration blastema. (B,D) Acidic mucin dominates the dorsal (B) and ventral (D) areas of the regeneration blastema. (C) Neutral mucin is concentrated in the central area of the regeneration blastema. Periodic acid-Schiff alcian blue staining. *Key:* amc, adult muscle cells; bls, blastema; bvl, blood vessel; cnt, connective tissue; drg, dorsal root ganglia; ep, epidermis; myb, myoblast; nvf, nerve fiber.

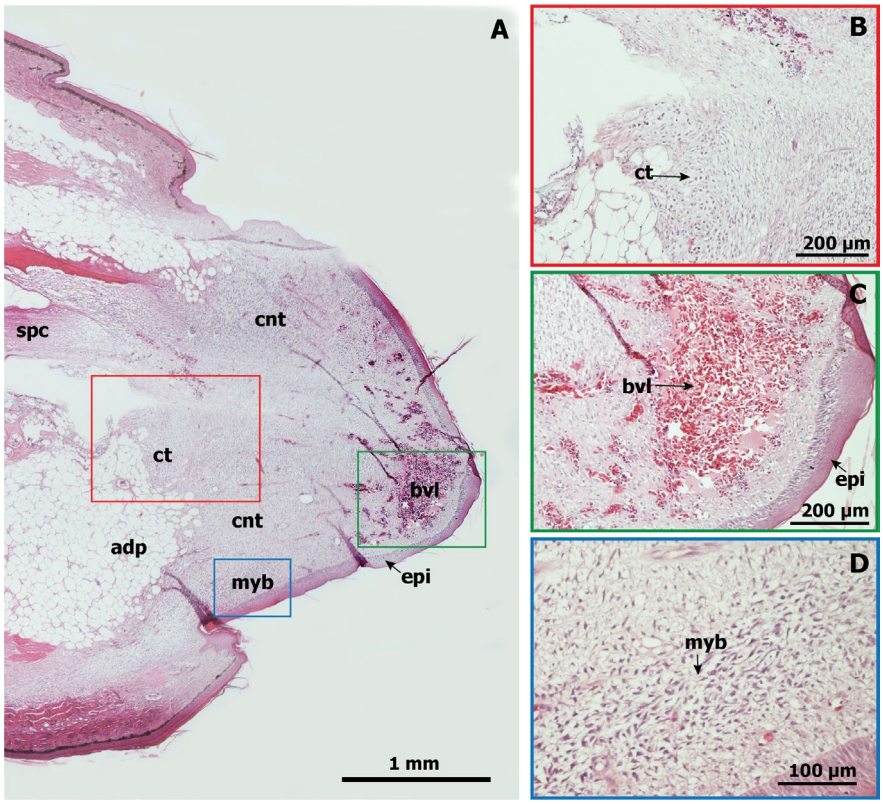

Figure 2-8. **The regeneration blastema of the tokay gecko at 20 dpa is quite similar with that of 16 dpa.** (A) the heterogenous tissues structure within the blastema, including myoblast, nerve fibers, and blood vessels. (B) pre-cartilage condensations are visible in the proximal part of the tail. (C) The blood vessels are more concentrated in the distal region of the blastema. (D) the myoblasts aggregate in the ventral area. Hematoxylin Eosin Staining. *Key:* adp, adipose tissue; amc, adult muscle cells; bls, blastema; bvl, blood vessel; cnt, connective tissue; ct, cartilage tube; ep, epidermis; myb, myoblast; nvf, nerve fiber; spc, spinal cord.

Tissue differentiation phase

The tissue differentiation phase becomes apparent at **24 dpa** in the regenerating tail (Figure 2-9). At this stage, the cartilage tube is distinctly visible from the proximal to distal parts of the regenerating tail (Figure 2-9A). Notably, the proximal segment of the cartilage tube is attached to the vertebra in the uninjured tail (Figure 2-9B), exhibits a more mature chondrification compared to the distal part. Concurrently, the regenerating muscle displays well-defined myotubes and myosepta, indicating muscle segmentation (Figure 2-9C). Although the former wound epidermis has developed into an epidermis that is thickened basally, has increased pigmentation, but no distinguishable dermis layer yet (Figure 2-9C). Additionally, blood vessels remain prominently scattered throughout the regenerating tail, with larger vessels observable

in the ventral region, likely originating from the ventral aorta of the tail stump (Figure 2-9).

We found more evenly distributed mucin in the connective tissue of the regenerating tail at this stage, predominantly comprising neutral mucin (Figure 2-10). However, the cartilage tube is characterized by acid mucin, distinguishing it from neighboring connective tissue (Figure 2-10B&D). Furthermore, muscle tissue also exhibits acid mucin (Figure 2-10C).

By **28 dpa**, signs of ongoing tissue differentiation persist (Figure 2-11). Myotubes were lengthened, and myosepta shortened, compared to the 24 dpa regenerating tail (Figure 2-11B). Epidermal pigmentation intensifies further (Figure 2-11D). Overall, the structural composition of the 28 dpa regenerating tail is similar with that observed at 24 dpa (Figure 2-12).

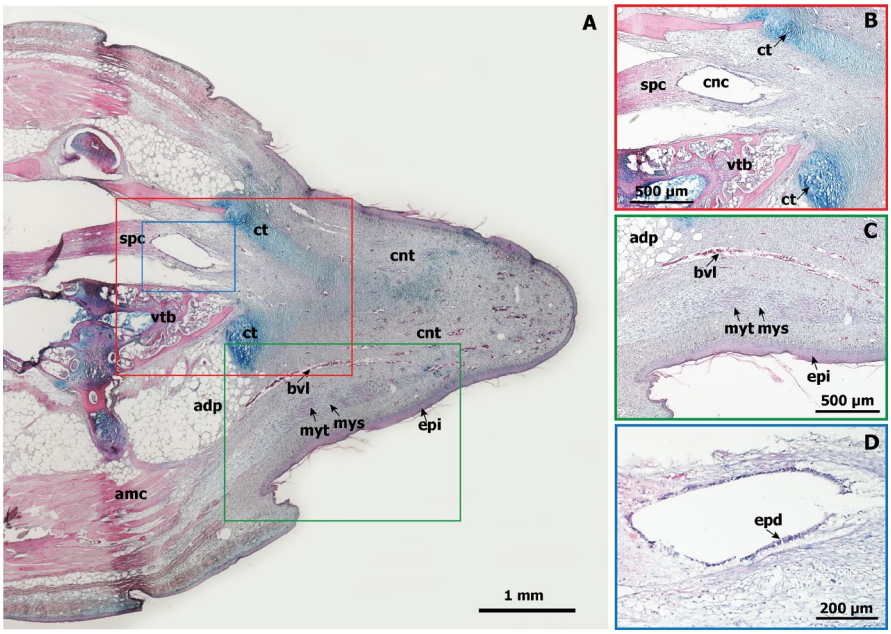

Figure 2-9. **The tissue differentiation become evidence at 24 dpa regenerating tail of the tokay gecko.** (A) the cartilage tube and muscles structure is distinctly visible in the regenerating tail. (B) The attachment of the proximal part of cartilage tube with the vertebra of uninjured tail. (C) Skeletal muscle segmentation can be clearly observed in the regenerating tail. (D) The central canal of spinal cord consisted of ependymal cell layer. Hematoxylin Eosin Alcian Blue Staining. *Key:* adp, adipose tissue; amc, adult muscle cells; bvl, blood vessel; ct, cartilage tube; cnc, canalis centralis; cnt, connective tissue; ep, epidermis; epd, ependymal cells; mys, myoseptum; myt, myotubes; spc, spinal cord; vtb, vertebra.

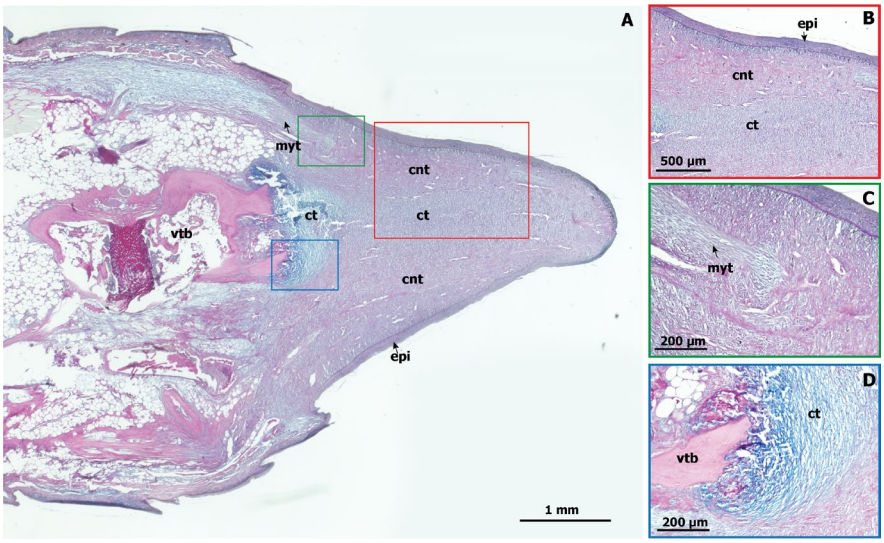

Figure 2-10. **The mucin in the connective tissue of the regenerating tail at 24 dpa is more distributed.** (A) An overview of the mucin distribution in the regenerating tail. (B, D) The cartilage tube is characterized by acid mucin (blue) and can be distinguished from the neighboring connective tissue containing neutral mucin (red). (C) muscle tissue demonstrates acid mucin. Periodic acid-Schiff alcian blue staining. *Key:* bvl, blood vessel; ct, cartilage tube; cnt, connective tissue; ep, epidermis; mys, myoseptum; myt, myotubes; spc, spinal cord; vtb, vertebra.

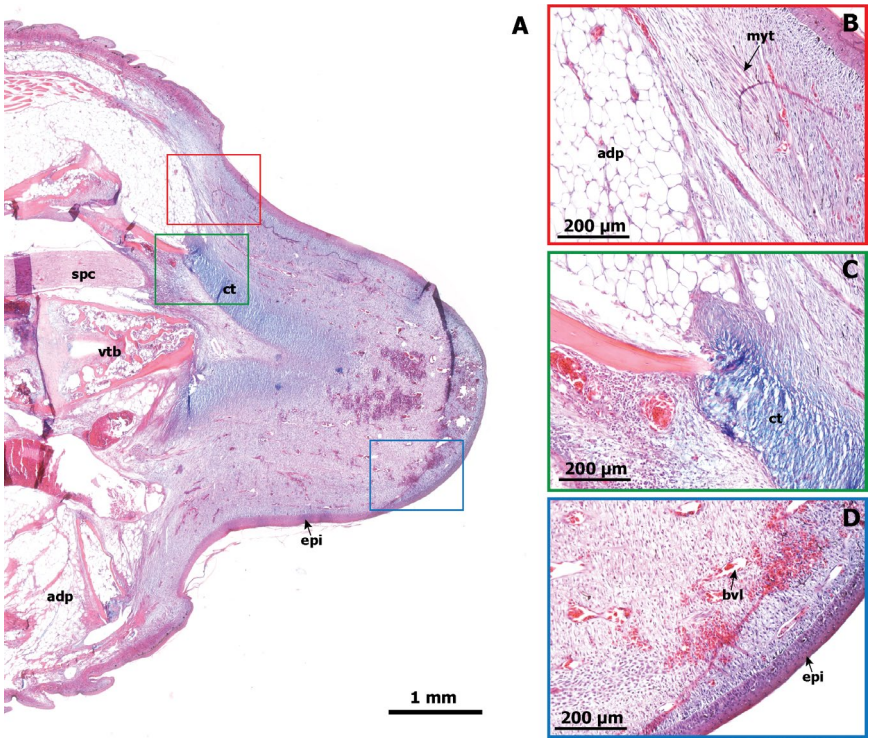

Figure 2-11. **Tissue differentiation is advancing at 28 dpa regenerating tail of the tokay gecko**. (A) The tissue structures of the regenerating tail, such as epidermis, cartilage tube, and skeletal muscle, are more advanced compared to that of 24 dpa. (B) the myotubes are lengthened. (C) The cartilage tube shows clear chondrocytes and extracellular matrix arrangement. (D) the epidermal pigmentation is more visible. Hematoxylin Eosin Alcian Blue Staining. *Key:* adp, adipose tissue; bvl, blood vessel; ct, cartilage tube; ep, epidermis; myt, myotubes; spc, spinal cord; vtb, vertebra.

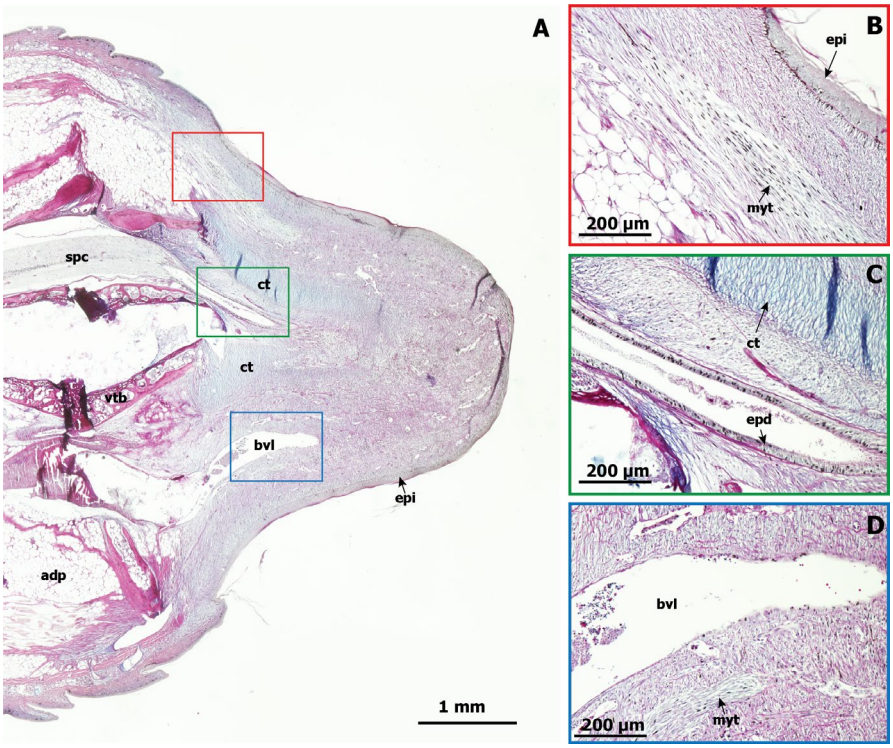

Figure 2-12. **The mucin distribution in 28 dpa regenerating tail is quite similar with that of 24 dpa regenerating tail.** (A) The regenerating tail tissue contain mostly neutral mucin (red), specifically the connective tissue, except the cartilage tube. (B) the neutral mucin start to be identified in the muscle but not as strong as the surrounding connective tissue. (C) Acidic mucin (blue) is still prominent in the cartilage tube. (D) A big blood vessel originating from the ventral aorta of the tail stump is visible in the ventral part of regenerating tail. Periodic acid-Schiff alcian blue staining. Key: adp, adipose tissue; bvl, blood vessel; ct, cartilage tube; cnt, connective tissue; ep, epidermis; epd, ependymal cells; mys, myoseptum; myt, myotubes; spc, spinal cord; vtb, vertebra.

Tail maturation phase

The tail maturation phase becomes evident at **49 dpa** in the regenerating tail and is characterized by a structure that increasingly resembles the original tail (Figure 2-13A). Skin maturation is identified by the development of scales in the epidermis and the distinct presence of the dermis layer (Figure 2-13C). In the regenerating tail muscles, longer myofiber structures and shorter myosepta indicate the maturation of muscle tissue (Figure 2-13C). Additionally, the cartilage tube exhibits a mature hyaline cartilage structure, with a clear arrangement of chondroblasts and chondrocytes (Figure 2-13D). The presence of adipose tissue between the muscles and cartilage tube further indicates the maturity of the tail at this stage (Figure 2-13C).

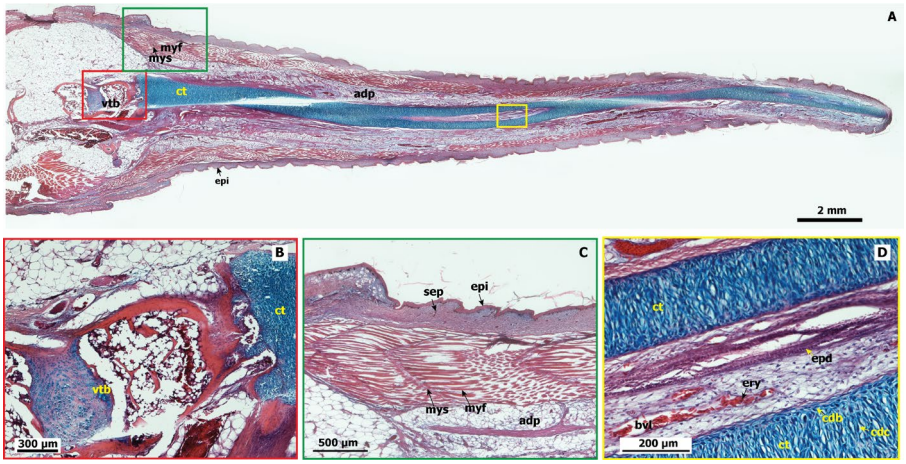

Figure 2-13. **The tissue structures of 49 dpa regenerating tail increasingly resembles the tissue structure in the original tail.** (A) an overview of the mature tissue structure of the regenerating tail indicated by the visible scale development in the skin and adipose tissue underneath the muscle tissue. (B) The attachment of the proximal part of cartilage tube with the vertebra of uninjured tail. (C) mature structure of muscle indicated by longer myofiber with shorter myosepta. The presence of sub-epidermal tissue (dermis) also indicates the maturation of the skin. (D) the clear arrangement of chondroblasts and chondrocytes in the cartilage tube implies the maturation of the hyaline cartilage structure. Hematoxylin Eosin Alcian Blue Staining. *Key:* adp, adipose tissue; bvl, blood vessel; cdb, chondroblast; cdc, chondrocytes; ct, cartilage tube; cnt, connective tissue; ep, epidermis; epd, ependymal cells; ery, erythrocytes; mys, myoseptum; myf, myofibers; sep, sub-epidermal tissue (dermis); vtb, vertebra.

Discussion

The tokay gecko is one of many lizard species showing caudal autotomy, whereby it can shed its tail to distract the attention of predators in order to escape (Rumping and Jayne, 1996). Generally, tail regeneration in this Gecko shows similarities to that in other lizards, such as the green anole, *Anolis carolinensis* (Cox, 1969), and the leopard gecko, *Eublepharis macularius* (McLean and Vickaryous, 2011).

In the tokay gecko, the wound healing phase starts with the formation of a scab at 4–8 days post-autotomy (dpa), which gradually disappears by 16 dpa. This scab serves as a temporary barrier protecting the wound site while the wound epithelium forms underneath. In axolotl and zebrafish regeneration, the formation of the scab is accompanied by the migration of epithelial cells and the recruitment of blood cells to the injury site, and these two processes facilitate tissue repair (Mescher et al., 2015). Moreover, the formation of the wound epidermis is crucial for subsequent regenerative processes, providing a protective barrier and a source of signaling molecules that promote regeneration (Mescher et al., 2015).

Tail regeneration in other lizards that show autotomy, including the green anole (Cox, 1969), the Common Wall Lizard, *Podarcis muralis* (Alibardi and Toni, 2005) and leopard gecko (McLean and Vickaryous, 2011), also shows scab formation followed by re-epithelialization. This re-epithelialization creates a conducive environment for blastema formation (Alibardi, 2010). The timeframe of the wound healing phase in the tokay gecko (4–8 d) is comparable with that in other lizards, namely: 5–7 d in the leopard gecko (McLean and Vickaryous, 2011), 7–9 d in the green anole (Cox, 1969), and 5-7 d in the Common Wall Lizard (Alibardi and Toni, 2005).

The presence of a thin layer of connective tissue under the wound epithelium, that stains for mucins, is consistent with the remodeling of the extracellular matrix during the wound healing phase. This matrix remodeling is essential for providing structural support and facilitating cell migration during tissue repair (Olczyk et al., 2014; Provenzano et al., 2005). One of the common early features of regeneration in the tokay gecko and other lizards is the inflammatory response. This can be seen from the presence of blood cells (including erythrocytes and leukocytes), both inside and outside blood vessel lumina, at the wound site. The recruitment of immune cells, especially macrophages, to the wound site plays a essential role regeneration by modulating the regenerative environment, promoting tissue repair and preventing infection (Godwin and Rosenthal, 2014; Londono et al., 2020; Mescher et al., 2015).

As we have shown in this chapter, blastema formation in the tokay gecko begins around 16 dpa, marked by a dome-shaped structure that undergoes elongation and differentiation. Blastema formation is a critical regenerative phase observed across multiple vertebrates. In the axolotl (*Ambystoma mexicanum*), for example, the blastema comprises dedifferentiated cells that proliferate and eventually differentiate into various tissues, guided by complex signaling pathways (Kragl et al., 2009).

However, the blastema in the regenerating tail of the tokay gecko is heterogeneous, even when there was only pre-blastema tissue underneath the wound epithelium at 8 dpa (This chapter). Another striking feature of the tokay gecko blastema is its rich vascularization at blastema formation stages, particularly in the distal part of the blastema. (Hutchins et al., 2014) also noted the rich vascularization of the tail regeneration blastema in the green anole lizard and contrasted this with the largely avascular regeneration blastema of the axolotl and zebrafish. In Chapter 3, we will show that this pre-blastema tissue expressed markers for connective tissue and muscle lineages. Therefore, this pre-blastema is heterogeneous, and the fact that it contains connective tissue, makes it unlike the typical progress zone of the limb or other developmental fields. Studies on the green anole and leopard gecko have also shown that the blastema contains a heterogeneous population of cells, including progenitors for cartilage, muscle, and epidermis (Londono et al., 2017; Lozito and Tuan, 2017; McLean and Vickaryous, 2011).

The distribution of acidic and neutral mucin within the blastema tissue indicates dynamic extracellular matrix (ECM) remodeling during blastema formation. In the tokay gecko, the distribution of acidic and neutral mucins within the blastema indicates the presence of a complex ECM that supports diverse cellular functions. Acidic mucins may facilitate cell proliferation and migration, while neutral mucins provide structural support and contribute to ECM stability (Godwin et al., 2014). Hyaluronic acid, a critical component of the extracellular matrix (ECM), plays a key role in tissue regeneration and wound healing, with high molecular weight HA showing anti-inflammatory properties and low molecular weight HA being proinflammatory (Litwiniuk et al., 2016). HA contributes to regeneration blastema formation in zebrafish (Ouyang et al., 2017) and lizards (Alibardi, 2018).

The tissue differentiation phase in the tokay gecko tail, marked by the formation of a cartilage tube, well-defined muscle structures, blood vessel development, and increased epidermal pigmentation, parallels similar phases in other regenerating species. The presence of well-defined myotubes and myosepta in the tokay gecko regenerating tail indicates successful muscle differentiation, essential for restoring tail functionality. Studies in green anole and leopard gecko reveal similar stages of differentiation, with cartilage and muscle tissues becoming distinct by 14–21 dpa (Cox, 1969; McLean and Vickaryous, 2011). However, the green anole and leopard gecko demonstrate a faster timeline for differentiation, likely due to differences in species-specific regenerative capacities.

The role of blood vessels in supporting tissue differentiation is highlighted by studies on zebrafish caudal fin regeneration (Bayliss et al., 2006) and axolotl tail regeneration (Ritenour and Dickie, 2017), where angiogenesis is critical for supplying nutrients and oxygen to the regenerating tissues, promoting cell survival and differentiation. The balanced distribution of mucins in the connective tissue of the regenerating tail of the tokay gecko further underscores the importance of the extracellular matrix in giving structural support and assisting cell signaling during regeneration (Godwin et al., 2014).

The maturation phase in the regenerating tokay gecko tail becomes evident at 49 dpa, it exhibits morphological similarities to the original tail, especially in the pattern of scales and skin pigment. These observations are consistent with previous studies on the green anole (Fisher et al., 2012) and leopard gecko (McLean and Vickaryous, 2011) that also regenerate producing similar scaling and pigmentation patterns as the original tail. In all three species, the boundary between the original and regenerated tail is visible because the scalation and pigment patterns are not identical with the original.

The tail maturation phase in the tokay gecko is characterized by the development of scales, mature muscle fibers, and well-defined cartilage tissue (in the cartilage tube).

The histological appearance of these tissues closely resembles those of the original tail, indicating the successful regeneration of complex tissue architectures. In both the tokay gecko and the green anole, although the maturation of the regenerated tail involves the formation of scales and pigmentation patterns similar to the original, the distinctive segmentation marks of the original tail (this Chapter and (Ritzman et al., 2012) are lacking in the regenerated tail (Lozito and Tuan, 2016; Lozito and Tuan, 2017). The presence of adipose tissue in the tokay gecko regenerated tail indicates the restoration of a fully functional and integrated tissue environment. Adipose tissue is a common structure in the tail of autotomous lizards suggesting the importance of the fat layer for autotomy (Sheppard and Bellairs, 1972).

In summary, we have shown in this chapter that the process of tail regeneration in the tokay gecko involves a series of well-defined phases that mirror those observed in other lizards, even though with species-specific variations in timing and complexity.

References

Alibardi, L. (2010). 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.

---- (2014). Histochemical, Biochemical and Cell Biological aspects of tail regeneration in lizard, an amniote model for studies on tissue regeneration. *Progress in Histochemistry and Cytochemistry* **48**, 143-244.

---- (2015). Original and regenerating lizard tail cartilage contain putative resident stem / progenitor cells. *Micron* **78**, 10-18.

---- (2018). Hyaluronate likely contributes to the immunesuppression of the regenerating tail blastema in lizards: Implications for organ regeneration in amniotes. *Acta Zoologica* **99**, 321-330.

---- (2019). Stimulation of regenerative blastema formation in lizards as a model to analyze limb regeneration in amniotes. *Histology and Histopathology* **34**, 1111-1120.

Alibardi, L. and Toni, M. (2005). Wound keratins in the regenerating epidermis of lizard suggest that the wound reaction is similar in the tail and limb. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* **303A**, 845-860.

Bancroft, J. D. G., M. (2008). *Theory and Practice of Histological Techniques*. London: Churchill Livingstone.

Bayliss, P. E., Bellavance, K. L., Whitehead, G. G., Abrams, J. M., Aegerter, S., Robbins, H. S., Cowan, D. B., Keating, M. T., O'Reilly, T., Wood, J. M., et al. (2006). Chemical modulation of receptor signaling inhibits regenerative angiogenesis in adult zebrafish. *Nat Chem Biol* **2**, 265-273.

Bellairs, A. D. and Bryant, S. V. (1985). Autotomy and Regeneration in Reptiles. (ed. C. G. A. F. Billet), pp. 303-410. New York: John Wiley & Sons, Inc.

Clause, A. R. and Capaldi, E. A. (2006). Caudal Autotomy and Regeneration in Lizards. *Journal of experimental zoology* **305A**, 965-973.

Cox, P. G. (1969). Some aspects of tail regeneration in the lizard, Anolis carolinensis. I. A description based on histology and autoradiography. *Journal of Experimental Zoology* **171**, 127-149.

Delorme, S. L., Lungu, I. M. and Vickaryous, M. K. (2012). Scar-Free Wound Healing and Regeneration Following Tail Loss in the Leopard Gecko, Eublepharis macularius. *Anatomical Record* **295**, 1575-1595.

Fisher, R. E., Geiger, L. A., Stroik, L. K., Hutchins, E. D., George, R. M., Denardo, D. F., Kusumi, K., Rawls, J. A. and Wilson-Rawls, J. (2012). A histological comparison of the original and regenerated tail in the green anole, Anolis carolinensis. *Anatomical record (Hoboken, N.J. : 2007)* **295**, 1609-1619.

Fleming, P. A. and Bateman, P. W. (2012). Autotomy, Tail Regeneration and Jumping ability in Cape Dwarf Geckos (Lygodactylus capensis) (Gekkonidae). *African Zoology* **47**, 55-59.

Gilbert, E. A. B., Payne, S. L. and Vickaryous, M. K. (2013). The anatomy and histology of caudal autotomy and regeneration in lizards. *Physiological and Biochemical Zoology* **86**, 631-644.

Gilbert, E. A. B. and Vickaryous, M. K. (2018). Neural stem/progenitor cells are activated during tail regeneration in the leopard gecko (Eublepharis macularius). *Journal of Comparative Neurology* **526**, 285-309.

Godwin, J., Kuraitis, D. and Rosenthal, N. (2014). Extracellular matrix considerations for scar-free repair and regeneration: Insights from regenerative diversity among vertebrates. *The International Journal of Biochemistry & Cell Biology* **56**, 47-55.

Godwin, J. W. and Rosenthal, N. (2014). Scar-free wound healing and regeneration in amphibians: Immunological influences on regenerative success. *Differentiation* **87**, 66-75.

Hutchins, E. D., Markov, G. J., Eckalbar, W. L., George, R. M., King, J. M., Tokuyama, M. A., Geiger, L. A., Emmert, N., Ammar, M. J., Allen, A. N., et al. (2014). Transcriptomic Analysis of Tail Regeneration in the Lizard Anolis carolinensis Reveals Activation of Conserved Vertebrate Developmental and Repair Mechanisms. *PLOS ONE* **9**, e105004.

Jacyniak, K., McDonald, R. P. and Vickaryous, M. K. (2017). Tail regeneration and other phenomena of wound healing and tissue restoration in lizards. *The Journal of Experimental Biology* **220**, 2858-2869.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H. and Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* **460**, 60-65.

Litwiniuk, M., Krejner, A., Speyrer, M. S., Gauto, A. R. and Grzela, T. (2016). Hyaluronic Acid in Inflammation and Tissue Regeneration. *Wounds : a compendium of clinical research and practice* **28 3**, 78-88.

Londono, R., Tighe, S., Milnes, B., DeMoya, C., Quijano, L. M., Hudnall, M. L., Nguyen, J., Tran, E., Badylak, S. and Lozito, T. P. (2020). Single cell sequencing analysis of lizard phagocytic cell populations and their role in tail regeneration. *Journal of Immunology and Regenerative Medicine* **8**, 100029-100029.

Londono, R., Wenzhong, W., Wang, B., Tuan, R. S. and Lozito, T. P. (2017). Cartilage and Muscle Cell Fate and Origins during Lizard Tail Regeneration. *Frontiers in Bioengineering and Biotechnology* **5**, 1-9.

Lozito, T. P. and Tuan, R. S. (2015). Lizard tail regeneration: Regulation of two distinct cartilage regions by Indian hedgehog. *Developmental Biology* **399**, 249-262.

---- (2016). Lizard tail skeletal regeneration combines aspects of fracture healing and blastema-based regeneration. *Development* **143**, 2946-2957.

---- (2017). Lizard tail regeneration as an instructive model of enhanced healing capabilities in an adult amniote. *Connective Tissue Research* **58**, 145-154.

McLean, K. E. and Vickaryous, M. K. (2011). A novel amniote model of epimorphic regeneration: the leopard gecko, Eublepharis macularius. *BMC Developmental Biology* **11**, 50-50.

Mescher, A. L., Neff, A. W. and King, M. W. (2015). Inflammation and immunity in organ regeneration. *Developmental and Comparative Immunology*.

Olczyk, P., Mencner, Ł. and Komosinska-Vassev, K. (2014). The Role of the Extracellular Matrix Components in Cutaneous Wound Healing. *BioMed Research International* **2014**, 747584.

Ouyang, X., Panetta, N. J., Talbott, M. D., Payumo, A. Y., Halluin, C., Longaker, M. T. and Chen, J. K. (2017). Hyaluronic acid synthesis is required for zebrafish tail fin regeneration. *PLoS One* **12**, e0171898.

Pratt, C. W. (1946). The plane of fracture of the caudal vertebrae of certain lacertilians. *Journal of anatomy* **80**, 184-188.

Provenzano, P. P., Alejandro-Osorio, A. L., Valhmu, W. B., Jensen, K. T. and Vanderby, R. (2005). Intrinsic fibroblast-mediated remodeling of damaged collagenous matrices in vivo. *Matrix Biology* **23**, 543-555.

Ritenour, A. M. and Dickie, R. (2017). Inhibition of Vascular Endothelial Growth Factor Receptor Decreases Regenerative Angiogenesis in Axolotls. *Anat Rec (Hoboken)* **300**, 2273-2280.

Ritzman, T. B., Stroik, L. K., Julik, E., Hutchins, E. D., Lasku, E., Denardo, D. F., Wilson-Rawls, J., Rawls, J. A., Kusumi, K. and Fisher, R. E. (2012). The gross anatomy of the original and regenerated tail in the green anole (Anolis carolinensis). *Anat Rec (Hoboken)* **295**, 1596-1608.

Rumping, J. M. and Jayne, B. C. (1996). Muscle activity in autotomized tails of a lizard (Gekko gecko): a naturally occurring spinal preparation. *Journal of comparative physiology. A, Sensory, neural, and behavioral physiology* **179**, 525-538.

Sheppard, L. and Bellairs, A. A. (1972). The mechanism of autotomy in Lacerta. *British Journal of Herpetology* **4**, 276-286.

Simpson, S. B. (1970). Studies on regeneration of the lizard's tail. *Integrative and Comparative Biology* **10**, 157-165.