Tail regeneration in the Tokay Gecko (Gekko gecko)

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Chapter 7. Summary, Discussion and Perspective

Summary

Regeneration is the ability of an organism to restore damaged or lost tissue, without the production of scar tissue, and with the newly formed tissue able to replace the function of the lost tissue (Pirotte et al., 2016; Poss, 2010). Regeneration is important as one of the survival strategies used by animals to deal with injuries (Bely and Nyberg, 2010; Gurtner et al., 2008). Regeneration goes beyond wound healing or repair, in which the lost tissue is simply covered over by epithelium or replaced by scar tissue (as in mammals, for example). Regeneration can take place at various level of biological organization, from a cell to a whole body, and can initiated at a wide range of stages in an animal's lifecycle (Bely and Nyberg, 2010). It always involves cell proliferation and differentiation after injury (Ricci and Srivastava, 2018; Tanaka and Reddien, 2011). Therefore, it relies on a population of stem cells (Ricci and Srivastava, 2018; Tanaka and Reddien, 2011). The availability of stem cells in different animals may explain the widely differing regenerative capacity in different species.

Regeneration in the vertebrates is more limited compared to that of the invertebrates. In humans and mice, for example, the capacity for true regeneration is limited to the fingertips, provided (in humans) that the nail bed is preserved (Gang and Lenghi, 1982; Muneoka and Dawson, 2021; Narayanan, 2015; Tang et al., 2014). The liver in humans and other mammals can restore lost parts, but there are some researchers who describe this as physiological size regulation rather than true regeneration (Michalopoulos, 2007; Wang et al., 2019b). However, other vertebrates show impressive regeneration capacity. These include teleost such as the zebrafish (*Danio rerio*), lissamphibians such as salamanders (Urodeles) and squamates such as lizards, including geckos (Li et al., 2015). Those animals are commonly used as models for regeneration studies as they can provide insights not available from mammals which have such limited regeneration capacities (Kurup and Ramachandran, 2011; Song et al., 2010).

Lizards are well-known for the phenomenon of tail autotomy whereby the tail breaks off at pre-existing fracture planes when grabbed by a predator. Autotomy is intended to distract a predator because the broken-off tail fragment remains twitching on the ground for some time (Bae et al., 2014). Autotomy in lizards is generally accompanied by an ability to regenerate the lost piece of tail.

Tail regeneration in lizards result in the development of a new, complex structure (the replacement tail) from a group of proliferating cells on the tail stump called the

blastema. It is this blastema formation and differentiation, among other things, that make the lizard a useful model for studying tissue regeneration (Bely and Nyberg, 2010; Hill et al., 2012; Lozito and Tuan, 2015; Russell et al., 2015).

In this thesis, we use the tokay gecko (*Gekko gecko*) as a model species. The tokay gecko is a lizard, a member of the suborder Lacertilia, family Gekkonidae whose members are found from Northeast India to Southern China and throughout Southeast Asia (Das, 2015). The total body length (TL; snout to tip of tail) of the tokay gecko is around 35 cm and the snout-vent length (SVL) is around 18.5 cm (Cobos and Higham, 2022); it is the second largest living gecko after the New Caledonian gecko (*Rhacodactylus leachianus*) (Kongbuntad et al., 2016).

In **Chapter 1**, We have summarized the current literature about regeneration, especially in the vertebrates, and the importance of studying regeneration in different species. Moreover, we have explored in more depth tail regeneration in lizards. As amniotes, lizards are closer to mammals (including humans) than are the urodeles and teleost model species.

In **Chapter 2**, We have described the tissue structure of seven stages of the adult tokay gecko regenerating tail. We found that the process of tail regeneration in the tokay gecko, histologically, involves a series of well-defined phases that mirror those observed in other lizards, albeit with species-specific variations in timing and complexity. Additionally, we found that the blastema in the regenerating tail of the tokay gecko is heterogeneous even when it was still in pre-blastema tissue underneath the wound epithelium at 8 dpa (days post-autotomy). Finally, we find no histological evidence of an apical growth zone as seen in urodeles.

In **Chapter 3**, We describe a distinctive molecular signature at each stage of tail regeneration in the tokay gecko. There was early upregulation of immune-related genes in the wound healing phase, followed by the upregulation of a subset developmental genes in the blastema phase and upregulation of genes related with skeletal muscle maturation and functional development at differentiation phase. Moreover, we also found that transcriptome profile of regenerating tail, with respect to developmental patterning genes, is different compared to that of embryonic tail.

In **Chapter 4**, We show, using single cell mRNA sequencing, that the regeneration blastema, and the embryonic tailbud, in the tokay gecko have different cell clusters. In addition, by using bioinformatics approach, we also find that the terminal states cell clusters in the regenerated tail blastema predominantly develop from within their own lineage. This is different compared to the embryonic tailbud in which the precursor cell population is predominantly pluripotent stem cells.

In **Chapter 5**, We focused particularly on genes involved in axonogenesis, chondrogenesis, myogenesis and segmentation. We examine the candidate genes identified with bulk transcriptomics and single-cell mRNA sequencing for spatial expression in the tissue section of regenerating tail using *in situ* hybridization. The findings presented in this chapter suggest that the regeneration of the tokay gecko tail may involves the early activation of a stem cell population, likely consisting of satellite cells and fibroblasts, that retain positional memory from the original tail. This positional memory facilitates the regeneration of an appropriate tail replacement, without establishing *de novo* patterning, as observed in normal development.

In **Chapter 6**, we have provided a sufficient genome assembly and annotation of the tokay gecko to be used as a genome reference for a diverse range of biological research using the tokay gecko as a model organism, especially studies focusing on tissue regeneration.

In summary, our study suggests that tail regeneration in the tokay gecko utilizes distinct mechanisms compared to the blastema-based regeneration observed in salamanders and teleost fish. Furthermore, regeneration in this gecko is not simply a reactivation of developmental processes. Despite the having similar stages of regeneration to the axolotl and zebrafish (wound healing, blastema formation, differentiation, and maturation), tokay gecko tail regeneration differs in terms of blastema cell recruitment. Thus, it appears to be more reliant on the activation of lineage-restricted resident stem cells within the uninjured tail, rather than on generating stem cells through the dedifferentiation of mature tissues. This may explain why the regenerating tokay gecko tail re-activates only a subset of developmental genes — and even that is at a relatively late stage of the regenerative process (16 dpa), not earlier, as would be expected if the blastema were a developmental field. tokay gecko tail regeneration also appears to leverage the existing positional memory of the resident stem cells to rebuild the lost structure.

Our finding of a lineage-restricted, stem-cell based regeneration mechanism in reptiles could have translational applications in human regenerative medicine. Interestingly, some phenomena in mammalian regeneration, such as the regeneration of mouse digit tips (Johnson et al., 2020; Lehoczky et al., 2011) and deer antlers (Qin et al., 2023; Wang et al., 2019a), have also been found to depend on the activation of lineage-restricted stem cells. However, mammals have certain limitations as research models for regeneration, when compared with the tokay gecko. For example, in the mouse digit model, regeneration is restricted to a very limited area, and only occurs if the digit is amputated distal to the last interphalangeal joint (Borgens, 1982). And the deer antler model would be impractical in terms of expense, and only involves the

regeneration of bone tissue (Qin et al., 2023; Wang et al., 2019a). Therefore, the gecko becomes an attractive model for studying a type of regeneration, that we believe is shared with humans and other mammals, that is based on lineage-restricted resident stem cells.

Future investigations should functionally examine the molecular pathways, signaling cascades, and cellular dynamics governing the activation and differentiation of resident stem cell populations responsible for tokay gecko tail regeneration. This can be complemented by lineage-tracing studies to definitively identify and characterize the resident stem cells involved, confirming their role and determining their activation during regeneration. Additionally, further study of the mechanisms underlying positional memory in tokay gecko resident stem cells would provide valuable insights into how positional memory directs the regeneration process. These insights could potentially be leveraged to develop bioengineering techniques, such as supportive scaffolds, that can guide and facilitate regeneration, thereby translating research findings into medical applications.

Discussion and Perspective

Taken together, these findings support the concept of two major pathways of regeneration in vertebrates (Table 7-1). One is exemplified by caudal fin regeneration in zebrafish, and limb regeneration in axolotls (Cox, 1969; Hutchins et al., 2014). In this type of regeneration, we suggest, the blastemas are relatively small — of the order of 1 mm. This, according to Wolpert, is the size of a classic developmental field, such as an embryonic limb bud or tailbud, in which positional values are specified *de novo* (Wolpert, 1969). In this type of regeneration, and in a classical developmental field, development gene expression is early, distal and occurs in a small area. For example, in zebrafish tail regeneration there is an apical growth zone in which there is a distal-to-proximal gradient in differentiation (Pfefferli and Jazwinska, 2015), very much like a developmental field such as the progress zone of the embryonic limb bud (Tabin and Wolpert, 2007).

The other pathway of regeneration in vertebrates is quite different and is exemplified by tail regeneration in geckos (and other lizards). The regeneration blastema in lizards is much larger that a classical developmental field or regeneration blastema of zebrafish caudal fin (Pfefferli and Jazwinska, 2015) and axolotl limb (Gerber et al., 2018; Kragl et al., 2009). Developmental gene expression and proliferating cells in this system are ‘distributed’ through the blastema (Hutchins et al., 2014), and not restricted to a small, apical growth zone. However, in the tokay gecko, we find no such gradient of expression. Furthermore, we only find expression of developmental patterning genes in regenerating cartilage, muscle and skin. Moreover, the developmental genes are

activated relatively late in tokay gecko tail regeneration. We examined the blastema for evidence of a growth zone but found only *col1a2*$^{+}$ connective tissue cells under the wound epithelium, a finding inconsistent with a growth zone.

We suggest that the large size of the regeneration blastema in the tokay gecko tail might be related to the relatively large body size of this species (see above) compared to other lizards in which regeneration has been studied (e.g. *Anolis carolinensis* (Xu et al., 2020) , *Eublepharis macularius* (Delorme et al., 2012), *Lepidodactylus lugubris* (Vonk et al., 2023), *Hemidactylus frenatus* (Nagumantri et al., 2021), and *Podarcis muralis* (Alibardi, 2017)). Furthermore, we suggest that there is no *de novo* specification of positional information in the blastema of the tokay gecko. Rather, regeneration depends on pre-existing positional values. This hypothesis is supported by HOXC gene expression in the regenerating tail of the tokay gecko. HOX genes are transcription factors that play a key role in embryonic axial patterning (Di-Poi et al., 2010; Kawanishi et al., 2003; van den Akker et al., 2001) and, potentially, in adult tissue regeneration (Gersch et al., 2005; Rux and Wellik, 2017).

Table 7-1. **The tokay geckos and other lizards show a pattern of regeneration that is quite different from a classical developmental field**. Note that the blastemas of the regenerating caudal fin, and the regenerating limbs in the axolotl, are similar in properties and size to developmental fields of embryos. By contrast, the regenerating tail blastema of geckos and other lizards is quite different from a developmental field.

model system	width of bud or blastema *	main site of pattern specification	gradient of cell and tissue differentiation	stage** of expression of patterning genes	Ref.
Tokay gecko adult tail regeneration	3–5 mm	distributed	distributed	middle-late	this study; (Hutchins et al., 2014)
Tokay gecko embryo tailbud	200 µm	distal only	distal to proximal	early	this study
Zebrafish adult caudal fin regeneration ***	150–200 µm	distal only	distal to proximal	early	(Thummel et al., 2007)
Zebrafish tailbud	125 µm	distal only	distal to proximal	early	(Kimmel et al., 1995)
Axolotl adult limb regeneration	300 µm	distal only	distal to proximal	early	(Gerber et al., 2018)
Axolotl embryo limb bud (Stage 40)	150 µm	distal only	distal to proximal	early	(Bordzilovskaya et al., 1989)

* Our interpretation based on the scale indicated in Figures within the manuscript.

** relative to cell differentiation and tissue maturation events

*** We assume that the caudal fin has not a single large blastema, but multiple small blastemas, one at the tip of each ray.

Our data show that HOX gene expression exhibits temporal collinearity in the regenerating gecko tail, primarily at mid-stages of regeneration, and primarily in

myoblasts. This contrasts with the regenerating axolotl limb, where HOX expression may not be temporally or spatially collinear (Mallo and Alonso, 2013; Woltering et al., 2009). These observations suggests that HOX expression plays a role in gecko tail regeneration. One possible function is that the late-stage expression of *hoxc13* signals the end of pattern formation during tail regeneration, similar to its role in normal tail development (Aires et al., 2019). Another potential function is that HOX expression provides positional cues that guide the regeneration process, as seen in other vertebrate's models where only the appropriate missing parts are regenerated. For instance in axolotl limb regeneration (Otsuki and Tanaka, 2022; Pescitelli and Stocum, 1980) and zebrafish caudal fin regeneration (Isabella et al., 2021; Uemoto et al., 2020) only the lost parts are replaced.

The analysis of our single-cell mRNA sequencing and *in situ* hybridization data suggests that the tokay gecko regeneration blastema is derived from existing resident stem cells. For instance, early expression of the myogenic markers *pax3*, *pax7*, and *myod1* indicates an early activation of satellite cells in the regeneration blastema, and their possible contribution to myogenesis. While the Notch signaling modulator *lfng* is expressed in tokay gecko tail regeneration, the Hes genes associated with embryonic segmentation, such as *hes7*, do not appear to be expressed. This observation is consistent with evidence from axolotl tail regeneration that *lfng+* multipotent 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 blastema may rely on positional memory in satellite cells, which 'remember' the segmentation of the original muscle. This suggestion is supported by a study of mouse cell lines, showing the satellite cells have positional memory regulated by *hoxa10* (Yoshioka et al., 2021).

The details of cartilage patterning in the gecko tail also support the idea that there is no *de novo* patterning in the regenerating gecko tail, but rather a reliance on existing positional information. Our starting point for this argument is that the regenerated cartilage tube in the tokay gecko tail is unsegmented, whereas the skeletal muscle is segmented. This is quite different to axolotl tail regeneration, where *de novo* patterning generates both segmented vertebrae and segmented muscles (Wouter et al., 2024). The lack of segmentation of the cartilage tube in gecko might be caused by the absence of *pax1* expression. In embryonic tail development, *Pax1* is needed to mediate SHH signaling by the notochord, which induces sclerotome development (Wallin et al., 1994) and is also essential for further development of the vertebral column (Koseki et al., 1993). Of these two genes, only *Shh* and not *Pax1* is expressed in the ependymal lining of the cartilage tube in the regenerating tokay gecko tail (Lozito and Tuan, 2015). The fact that the regenerated muscles are segmented but the cartilage tube is not, suggests that their regeneration is uncoupled. Nonetheless, we suggest that both muscle and cartilage regeneration are both guided by Wnt signaling genes (*wnt3* and

wnt5a) because we find that both are expressed in cartilage and muscle in the regenerating tail.

Given these differences between the gecko tail regeneration blastema, and other developmental systems, it appears that tail regeneration in the lizards is an evolutionarily derived process not reported in other vertebrates. Our proposed model is that pattern formation in the blastema is based on the existing positional values of existing resident stem cell populations. This model is consistent with the finding that the cartilage tube in the regenerating lizard tail is derived from stromal cells resident in the vertebral periosteum (Lozito and Tuan, 2016); and with the finding that regenerating muscle might derive from stromal cells in muscle of the tail stump (Londono et al., 2017).

Studies on regeneration in other vertebrates have often been difficult to translate to human medicine because it apparently requires the reactivation of developmental-scale processes as well as pluripotent stem cells. The latter are lacking in adult humans. This thesis work shows that regeneration may take place in the tokay gecko using resident stem cells, and these cells are present in adult human tissues. Therefore, further translation studies in mammalian regeneration could be informed by the single-cell analyses and other data reported here. Another potential translational application of our data might be in cancer research. It has already been shown that regeneration shares many pathways with cancer (Alibardi, 2019; Greco et al., 2023). In this study, we also find several pathways involved in cancer are enriched in the regenerating tokay gecko tail, especially early stages (4 dpa). The potential links between regeneration and cancer are enigmatic and worth exploring in future studies.

This thesis has several limitations. It did not involve functional studies to experimentally validate the roles of the identified genes and pathways in the context of tokay gecko tail regeneration. While the bioinformatic analyses and *in situ* hybridization provided critical insights into spatial gene expression patterns, they did not establish direct causal relationships. Furthermore, the research did not involve lineage tracing (e.g. by cell labeling experiments), which could have tracked the origins, fates, and differentiation trajectories of individual cell populations throughout regeneration. Consequently, the correspondence between specific gene expression patterns and cell behaviors or tissue formation during regeneration remains unclear. To overcome these limitations, future studies could incorporate functional approaches, such as gene knockdown or overexpression experiments, in combination with lineage tracing techniques. This integrated approach would help validate the roles of key genes and provide a more comprehensive understanding of the cellular dynamics and molecular mechanisms underlying tail regeneration in geckos, ultimately strengthening the conclusions drawn from this thesis. Additionally, further investigation of the mechanisms governing positional memory in tokay gecko resident stem cells would yield valuable insights into how positional memory directs the regeneration process.

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