Tail regeneration in the Tokay Gecko (Gekko gecko)

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Chapter 1. General Introduction and Thesis Outline

Abstract

Regeneration is the ability of an organism to grow a new tissue or organ that can act as a functional replacement for lost or damaged tissue. Invertebrates, such as *Hydra* sp. and Planarians, show a remarkable regenerative capacity. Regeneration in the vertebrates is more limited, but some taxa (zebrafish, salamanders, frogs, and lizards) show impressive regeneration capacity. Lizards (Scincidae, Gekkonidae, Lacertidae) are common animal models in regeneration studies and are able to fully regrow their tail after accidental amputation or self-amputation (autotomy). Tail regeneration in lizards includes formation of a multipotent cell mass (blastema)and its differentiation into a regenerated tail which shows small differences from the original tail. Regeneration has some similarities with embryonic development, but these similarities are not well understood. The aim of this thesis is to make a comprehensive characterization of the cellular and molecular mechanisms underlying tail development and regeneration in the tokay gecko (*Gekko gecko*). We used histology, bulk transcriptomics, single-cell sequencing, *in situ* hybridization and genome sequencing. We compare multiple stages of tail regeneration with the embryonic tail bud. This will provide insight into shared and unique gene regulatory pathways involved in both regeneration and normal development. The studies reported here, together with other research on lizard tail regeneration, have potential applications in regenerative medicine.

Regeneration

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 function the same as the original tissue (Pirotte et al., 2016; Poss, 2010). Regeneration goes beyond wound healing or repair, in which the lost tissue is simply covered over by epithelium or replaced by scar tissue. Regeneration can take place at various level of biological organization, from a cell to a whole body, and can be induced by a wide-variety of events in the 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 species may explain the widely differing regenerative capacity in different animals.

Regeneration biology has the potential for translational applications in human regenerative medicine. This is a field that offers potential for treatments of traumatic injuries, heart disease, degenerative diseases, cancers and other diseases for which treatment options are limited (Ahmed et al., 2014; Oviedo and Beane, 2010). One example of a translational application is in the treatment of degenerative bone and cartilage diseases such as osteoarthritis. This disease is suffered by about 10% of people over 60 years of age (Tiku and Sabaawy, 2015).

Cartilage is an important connective tissue mostly found in the ear, nose, and joints, and it can be damaged due to injury, such as the common sporting injuries, or by diseases. Human cartilage lacks self-healing capacity and so, much attention has been given recently to potential regenerative therapies (Zhang et al., 2016). In this context, it is interesting to note that lizards are able to regenerate a cartilage tube in their tail. This makes them an interesting animal model for engineering cartilage tissue for osteoarthritis treatment (Lozito and Tuan, 2016). In the more distant future, it may even be possible to replace a whole limb using regenerative biology (Gilbert et al., 2015).

Evolutionary aspects of regeneration

Regeneration is important as a one of the survival strategies used by animals to deal with injuries (Bely and Nyberg, 2010; Gurtner et al., 2008). Furthermore, regeneration is used routinely by animals such as the cnidarian *Hydra* sp. and planarians such as *Schmidtea mediterranea* and *Dugesia japonica*. These animals show a remarkable regenerative capacity, not only at the tissue level but also at the whole-body level (Ivankovic et al., 2019; Vogg et al., 2019). The regenerative capacity in both *Hydra* and planarians is supported by an abundant stem cell population.

Hydra possess three variant stem cells populations to deal with body-loss. They are two kinds of unipotent epithelial stem cells, the epidermal (eESCs) and gastrodermal (gESCs) stem cells; and one type of multipotent interstitial stem cell (ISC) (Vogg et al., 2019). These three stem cell populations each have specialized roles in proliferation and differentiation to generate new tissues or organs. By contrast, planarians have numerous adult stem cells scattered all over the body, corresponding to a single stem-cell type called a neoblast (Ivankovic et al., 2019; Ricci and Srivastava, 2018; Wagner et al., 2011). Neoblasts play an important role in whole body regeneration (Ivankovic et al., 2019; Sánchez Alvarado, 2006; Wagner et al., 2011).

Regeneration in the vertebrates is more limited compared to that of the invertebrates described above. 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., 2019). Other vertebrates, however, show impressive regeneration capacity. These include teleosts such as the zebrafish (*Danio rerio*), lissamphibia such as salamanders (Urodela), Squamata such as lizards (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).

Tail autotomy and regeneration in lizards

Lizards (including Scincidae, Gekkonidae, Lacertidae, Eublepharidae) are able to shed part of their own tail to help them distract, and escape from, a predator (Bae et al., 2014). This shedding of the tail is called autotomy and is followed by regeneration of the tail. The autotomized tail will continue to twitch for up to 30 minutes due to the event of anaerobic metabolism (Dial and Fitzpatrick, 1983; Higham and Russell, 2010). This will divert the attention of predator, giving the lizard a chance to escape (Daniels, 1983). Autotomy is observed in the majority of lizard families with the exception of only the Chamaeleonidae and Varanidae (Bateman and Fleming, 2009; Higham et al., 2013b). Only lizards that show autotomy show tail regeneration. Many researchers have found that tail autotomy and regeneration make lizards a useful model for studying tissue regeneration (Alibardi, 2010; Bely and Nyberg, 2010; Hill et al., 2012; Lozito and Tuan, 2015; Russell et al., 2015).

Autotomy is a useful anti-predatory strategy that may help the lizard escape. However, there are many fitness costs associated with autotomy, resulting in a trade-off. For example, the normal lizard tail stores more than half of the body's valuable

storage fat (Congdon et al., 1974; Russell et al., 2015). Therefore, autotomy of the tail means the loss of stored energy. However, at least some lizards may return to the place where they lost their tail and eat the autotomized tail as compensation (Sanggaard et al., 2012). Furthermore, loss of the tail temporarily comprises locomotor performance of the lizard (Bateman and Fleming, 2009). Another fitness cost is that lizards who have shed their tail lose social status in their colony (Fox and McCoy, 2000) and may show decreased courtship and mating success (Boozalis et al., 2012; Martin and Salvador, 1993).

Tail autotomy also causes a decrease of somatic growth and reproductive output, leading to a smaller home range and less access to females for males (Fox and McCoy, 2000; Simou et al., 2008). For these reasons, loss of the tail can be maladaptive because the tail has significant functions (Sanggaard et al., 2012; Sheppard and Bellairs, 1972).

Two types of autotomy

Intravertebral autotomy

Intravertebral autotomy is found in most lizard species and involves the presence of preformed breaks (fracture planes) in the postpygal vertebrae. These anatomical modifications of the vertebrae are found mainly in lizard tails (Gilbert et al., 2013; McLean and Vickaryous, 2011). In the tokay gecko (*Gekko gecko*) the fracture planes are situated posterior to the transverse processes of each postpygal vertebra (Rumping and Jayne, 1996).

Fracture planes are effectively pre-existing planes of weakness formed by connective tissue (Cox, 1969; Delorme et al., 2012; Kusumi and Fisher, 2012; Pratt, 1920). Other supporting structures playing important roles in autotomy are the interdigitation arrangement of tail muscles (Sanggaard et al., 2012) and modified structure of the spinal cord and caudal vasculature (Bellairs and Bryant, 1985; Gilbert et al., 2013). Although fracture planes do not extend into the neural canal, in the Italian Wall Lizard (*Podarcis sicula*), the spinal cord at the level of each fracture plane is thinner than in the non-autotomous region (Alibardi, 2009). Another feature plane in lizards is that the caudal artery thickens around each preformed break, this thickening resembling the structure of a sphincter. The caudal veins are equipped with valves situated just in front of each fracture plane (Bellairs and Bryant, 2001). This system of vascular sphincters and valves prevents excessive blood loss after tail amputation (Gilbert et al., 2013; Sanggaard et al., 2012), as does the rapid clotting response post-autotomy (Fernando et al., 2011; Mescher et al., 2015).

Intervertebral autotomy

Intervertebral autotomy involves intervertebral planes as the site of autotomy (Bateman and Fleming, 2009). In this type of autotomy, there are no pre-existing fracture planes

or other obvious modifications in the normal tail (Bateman and Fleming, 2009). It occurs in fewer species (most agamids and some of iguanids) (Sanggaard et al., 2012).

Tail regeneration processes

The ability of some lizards to regrow their tail following tail autotomy or amputation is known as tail regeneration (Hutchins et al., 2016). The following account applies intra- and intervertebral autotomy. Tail regeneration involves the formation of a blastema, which is a mass of progenitor cells. In the lizards that have been studies, these cells are produced by the dedifferentiation of mature cells at the wound site (Alibardi, 2019; Alibardi and Lovicu, 2010; Daniels et al., 2003). These progenitor cells play an important role in regeneration (Alibardi, 2015). They proliferate and re-differentiate to form new tissues to replace the lost or damaged ones (Bruce, 2007; Narayanan, 2015). Tail regeneration can be divided into four phases (timing is given in parentheses and indicates days post amputation or dpa):

Phase 1. Wound healing (0–10 dpa)

The wound healing phase is initiated by the formation of a highly proliferative wound epithelium within hours of amputation (Echeverri et al., 2001), and the degradation of the stump bone by osteoclasts (Fernando et al., 2011). The wound site will be covered by the wound epithelium, a stratified squamous epithelium with a prominent apical thickening (epithelial cap) that forms within days of autotomy. Without the wound epithelium, regeneration will not take place, suggesting an important role for the epithelium in regeneration (Gilbert et al., 2015). In the wound healing phase of the anole lizard (*Anolis carolinensis*), osteoclasts appear at 2 dpa at the autotomy plane, producing a focal area of bone dissolution. By 4 dpa, a large number of osteoclasts are concentrated in the area between the last remaining caudal vertebra and the newly-formed wound epithelium. This vertebra is extensively degraded, producing a population of chondrocytes destined for the cartilage tube. The chondrocytes are initially flat in shape, and in contact with the bone (Alibardi, 2015; Cox, 1969; Lozito and Tuan, 2015). The osteoclasts then disappear two or three days after the completion of wound healing process (Cox, 1969).

Phase 2. Blastema formation (10–15 dpa)

During regeneration in lizards with intravertebral autotomy, numerous mesenchymal calls are produced in the autotomy plane from the existing dermis, muscle myoseptum, periosteum of the vertebra, adipose tissue and bone marrow. These cells may be the source of the regeneration blastema (Alibardi, 2010; Bellairs and Bryant, 2001) and are believed to re-differentiate into the same cell lineage that they arose from. It is thought that all injured tissues of the tail stump contribute to the formation of the blastema, although the contribution of each tissue is unclear (Alibardi, 2009). As the blastema is

forming, there is a focal degeneration of the spinal cord which leaves only its ependymal lining remaining as a tube-like structure, the so-called ependymal tube. When the blastema has formed underneath the wound epithelium, the ependymal tube grows in the center of the blastema and acts as a guide for the regeneration process (Alibardi, 2009; Alibardi and Miolo, 1990; Gilbert et al., 2015; Shieh and Cheng, 2015).

Phase 3. Tail growth and differentiation (15–25 dpa)

The regenerating tail does not usually elongate during wound healing (phase 1, above) and blastema formation (phase 2 above); however, it will grow rapidly several weeks later (Bellairs and Bryant, 2001). A population of blastemal cells around the ependymal tube forms a pre-cartilaginous aggregation. The cells in this aggregation rapidly differentiate a tissue resembling hyaline cartilage, except that it has little intercellular matrix. This cartilage tissue then quickly grows to surround the newly-formed ependymal tube. By doing so, the two tissues together form the so-called cartilage tube. The cartilage tube isolates the ependymal tube from other tissues, with the exception of blood vessels which reach the ependymal tube through foramina in the tube wall.

Pre-muscular condensations, originating from clusters of myoblasts, are formed surrounding the cartilage tube (Alibardi, 2009). These myoblasts will differentiate into myotubes (Simpson and Cox, 1967). Connective tissue will subsequently develop between the forming muscle bundles and the cartilage tube. It consists of a heterogeneous population of cells consisting of fibroblasts, melanocytes, nerve fibers and small adipocytes in The adipocytes increase in size a few days later as they accumulate lipids. Cell proliferation, mostly in the axial tissues, determines the rate of tail growth (Alibardi, 2009). The end result is a regenerated tail with an axial skeleton in the form of a hyaline cartilage tube instead of segmented caudal vertebrae.

Phase 4. Tail maturation (25-60 dpa)

Tail maturation is reached when the tail epidermis becomes completely covered with keratinized, pigmented scales (Alibardi, 2010). These scales are smaller than the original scales on the tail, and are arranged in a simpler scalation pattern (Alibardi, 2009). This stage is usually reached at 25–60 dpa (Fisher et al., 2012; McLean and Vickaryous, 2011). In the tail maturation phase, differentiation continues in the more distal part of the regenerated tail. A small remnant of blastemal cells is still found beneath the wound epithelium at the tip of the new tail. Overall, tail regeneration in lizards yields a new tail which shows small differences from the original tail, namely: (1) the axial skeleton of the regenerated tail is replaced by a cartilage tube, which also resulting on no true tendons in the muscle; (2) the spinal cord in the regenerated tail is formed only from meninges and the ependymal cells lining the central canal; and (3) the size and pattern of scales covering the regenerated tail is different (Higham et al., 2013a).

We will now consider the embryonic development of the tail, because the pathways of adult regeneration are often compared with those in embryonic development (at least in some amphibians: (Gerber et al., 2018; Muneoka and Bryant, 1982).

Embryonic development of the tail

The embryonic development of the tail is a useful reference for understanding tail regeneration because both processes involve the production of patterned tissues from precursor cells. A comparison of normal embryonic tail development with adult tail regeneration may provide insight into shared regulatory pathways involved in both processes; or into pathways unique to one process. In this thesis, we are particularly interested in examining these issues in the tokay gecko.

The vertebrates tail develops from an embryonic structure called the tailbud which consists of mesenchymal cells covered by an ectodermal cap (Griffith et al., 1992). The tailbud continues the morphogenesis of axial structures (neural tube, somites etc.), a process that took place earlier in the head and trunk (Aires et al., 2018; Beck, 2015; Griffith et al., 1992; Handrigan, 2003). Tail bud development involves secondary neurulation and somite formation by the tailbud mesenchyme (Beck, 2015; Handrigan, 2003). This is unique since tailbud mesenchyme give rise to both ectodermal and mesodermal tissue. Secondary neurulation in the tailbud is started by mesenchymal cell condensation to form a solid cord which subsequently undergoes hollow neural tube formation (Beck, 2015; Griffith et al., 1992; Handrigan, 2003). Meanwhile, somite formation in the tailbud involves similar mechanism to that of trunk somites and develop from caudal pre-somitic mesoderm (PSM) cells (Beck, 2015; Handrigan, 2003).

Tailbud development involves a genetic network including *gdf11*, *lin28*, and paralog 13 hox genes, especially *hoxc13* (Aires et al., 2019). The somites develop from the tailbud mesenchyme under the influence of cyclical expression of the *hes7* gene (a segmentation gene) which is high in regions expressing high canonical *wnt* and FGF signaling but low in regions expressing retinoic acid (Beck, 2015; Cunningham et al., 2011; Goto et al., 2017; Handrigan, 2003; Takada et al., 1994).

Somite-formation and BMP-signaling in the mouse and chicken tailbud requires induction by the ventral ectodermal ridge (VER). The VER is ridge-like, midline thickening on the ventral aspect of the tail bud. In anurans, by contrast, induction is by the ventral blastopore lip (Beck, 2015). Secondary neurulation seems to depend on different genetic pathways in different species. For example, zebrafish secondary neurulation depends on canonical wnt signaling repression; in anurans it relies on *notch/wnt* signaling; and in mice, *tbx6* exerts negative regulation (Beck, 2015). Regulation of *notch* signalling by *lfng* along the neural tube is conserved in all vertebrates, but in mammals and birds there

are *hes*-binding regulatory mechanisms that allow *lfng* cycles within the PSM (Beck, 2015). Other transcription factors and signaling molecules needed for normal tail development include: *T* (*brachyury*) (Herrmann et al., 1990), *tbx6* (Chapman and Papaioannou, 1998), *cdx4* (van Nes et al., 2006), *cyp26a1* (Abu-Abed et al., 2001; Sakai et al., 2001) .

The tokay gecko

In this thesis, we use the tokay gecko (*Gekko gecko*) as a model species. This lizard tokay gecko is a member of the Suborder Lacertilia, Family Gekkonidae whose members are found from South Asia to Southeast Asia (Das, 2015). There are two subspecies of tokay gecko, namely: *G. g. gecko* (Linnaeus, 1758) and *G. g. azhari* (Mertens, 1955). The first is the common tokay gecko found in Southeast Asia, China and India, and the latter is endemic to Bangladesh (Rösler, 2001; Rösler, 2005). The tokay gecko inhabits tropical rain forest; some individuals have entered human habitation and live, and sometimes breed, in houses (Kongbuntad et al., 2016). The tokay gecko feeds mainly on insects (Aowphol et al., 2006).

The Indonesian tokay gecko population exhibits low genetic diversity despite geographic barriers (Kadafi et al., 2024). This contrasts with the higher genetic diversity observed in tokay geckos from mainland Southeast Asia, which suggests biogeographical separation and ecological barriers have played a role. The reduced genetic diversity of the Indonesian tokay gecko population may be attributed to anthropogenic influences their adaptation to human environments (Kadafi et al., 2024).

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 geckos after the New Caledonian gecko (*Rhacodactylus leachianus*) (Kongbuntad et al., 2016). The head of the tokay gecko is large and there is a distinct neck region. The eyes are protruding and large with vertical pupils and a yellow iris, while the ears appear as two small holes on each side of the head. The limbs are well-developed and the digits are equipped on their volar surface with toe pads (setae) enabling them to adhere to, and move fast on, vertical surfaces such as trees, cliffs and walls (Henkel and Schmidt, 1995). The skin is covered by granular scales interspersed with subconical tubercles. The coloration of the dorsal skin is bluish-grey with red or orange spots, while the ventral part is cream-colored, with or without grey or pink spots. The male is usually brighter in color and slightly larger than the female (Das, 2015). For details of the histology of the normal and regenerating tail, see Chapter 2.

Single-cell sequencing

Single-cell sequencing (sc-seq) is a tool to obtain individual cell genome or transcriptome profiles. It is therefore a powerful technology to uncover cellular responses, cell population diversity and, cellular evolutionary connections (Dey et al., 2014; Haque et al., 2017; Tang et al., 2019). This technique eliminates the problem of bulk mRNA sequencing, namely that the tissue sample is homogenized from all of the cells, meaning that all unique, single-cell information is lost (Tang et al., 2019; Wang and Navin, 2015). Bulk transcriptomics remains useful however, because of its much lower cost. Among its applications, single-cell mRNA sequencing (sc-mRNA seq) is able to reveal the heterogeneity of tumor cell populations, providing accurate new diagnostic and treatment options (Lim et al., 2020; Tang et al., 2019).

The sc-mRNA seq workflow starts with the isolation of single cells in suspension by enzyme disaggregation of the tissue. This is followed by mRNA extraction, amplification, library preparation, next-generation sequencing, and, finally, bioinformatical analysis (AlJanahi et al., 2018; Haque et al., 2017). Various protocols and platforms are available and can be applied depending on the type of sample, number of cells required, and transcripts capture per cell (AlJanahi et al., 2018; Dey et al., 2014; Haque et al., 2017; Tang et al., 2019; Wang and Navin, 2015). A typical run might sequence, per sample, 5,000 cells to a depth of 25k transcripts per cell; but the parameters can be varied, and the technology is rapidly advancing.

Sc-mRNA seq is a very promising method for understanding molecular programs within cells during regeneration. For instance, it has already uncovered the role of phagocytic cell populations during lizard tail regeneration (Londono et al., 2020). It has also shown that phagocytes induce the activation of fibroblast genes during lizard blastema formation in response to Hedgehog signaling (Vonk et al., 2023). In addition, sc-mRNA seq has also elucidated the cellular heterogeneity and lineage restriction during mouse digit tip regeneration (Johnson et al., 2020). Finally, Gerber et al. (Gerber et al., 2018) revealed the significant role of connective tissue cells during axolotl limb regeneration using sc-mRNA seq. Those authors were able to explain how connective tissue cells form a patterned limb skeleton which serves as a guide for the other limb tissues. In this thesis, I use sc-mRNA seq on both the embryonic tail and adult regenerated tail to identify the shared and distinctive cell populations associated with tail regeneration.

Aims and outline of the thesis

This thesis investigates the genes and cell populations involved in tail regeneration in the adult tokay gecko (*G. gecko*) and compare them with those in normal embryonic tail

development. Several studies have given rise to the hypothesis that developmental genes are reactivated during tail regeneration. We will test this hypothesis by making a detailed comparison between adult tail regeneration and embryonic tail development.

One possibility is that different genetic programs initiate embryonic tail development and adult tail regeneration. This possibility will be addressed as follows. (1) We will characterize the histological structure of the regenerating tail. (2) We will perform bulk transcriptomic analysis on multiple stages of regenerating tail of tokay gecko and compared the results with the bulk transcriptome of the embryonic tail. This will hopefully reveal a molecular signature for each stage of tail regeneration, as well as revealing differences with embryonic tail development. (3) We will perform sc-mRNA seq of the adult regenerating tail blastema, and the embryonic tailbud, to identify cell populations, their gene expression profiles, and their differentiation direction probability. (4) Once critical candidate genes have been identified, we will study their expression in depth using *in situ* hybridization on tissue sections. (5) Finally, because of the potential of the tokay gecko as a model in regeneration research, we will sequence, assemble, and annotate the genome of this species.

In **Chapter 2,** We establish a foundation for this thesis using paraffin histology to describe the tissue structure of seven stages of the adult tokay gecko regenerating tail.

In **Chapter 3,** We sequence and analyze the bulk transcriptome of the regenerating tail of the tokay gecko and identify a molecular signature of each stage of regeneration. I also compare the transcriptome profile of the regenerating tail with that of the embryonic tail.

In **Chapter 4,** We describe the single-cell mRNA sequencing analysis of regeneration blastema cells and embryonic tail cells. I identify cell populations, their gene expression patterns, and their differentiation direction probability.

In **Chapter 5,** We examine the candidate genes identified with bulk transcriptomics and single-cell mRNA sequencing by means of *in situ* hybridization on tissue sections. This permits a spatial and temporal analysis of gene expression during tail regeneration and allows me to make predictions about their roles in tail regeneration.

In **Chapter 6,** We describe sequencing, assembly and annotation the genome of the tokay gecko. This genome provides a crucial resource for this emerging model species.

Finally**,** in **Chapter 7,** We provide a summary of the thesis, including our new findings on tail regeneration pathways in the tokay gecko. We also summarize how tail regeneration

compares with tail development. We also consider the potentials for translating my findings to regenerative medicine.

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