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Abstract

The ability to regenerate a fully patterned limb exists in salamanders, while post-metamorphic frogs can only regenerate a hypomorphic spike, which is an unbranched, unsegmented cartilage covered with skin tissues. Upon limb amputation, both axolotls and the African clawed frogs form a blastema, which is composed of proliferative progenitor cells accumulating at the amputation plane.

So far, the cellular and molecular mechanisms that are responsible for the differences between the blastema cells in both species are not completely understood. To address this question, we performed regeneration time courses of both axolotl and frogs where we systematically compared the molecular and cellular behaviours of the limb connective tissue (CT) cells, which is known to play key roles in axolotl limb regeneration. We first confirmed that, as in the case of axolotls, a frog blastema is mainly composed of the descendants of limb CT cells by generating and characterizing *Prrx1:CreER* transgenic frog lines which allowed the labelling and tracing of the limb CT cells. Single cell RNA-seq analysis over the regeneration time course and heterochronic transplantation assay into limb buds showed that axolotl CT cells dedifferentiated and were able to integrate into multiple lineages of the limb buds, including cartilages. On the other hand, frog CT cells only partially dedifferentiated and did not acquire the potency to integrate into the cartilage of the developing limb buds. The result of the transplantation assay indicated the existence of cell intrinsic limitations of frog CT cells. Further analysis on the single cell RNA-seq dataset revealed that the chondrogenic pathway of the spike cartilage was molecularly distinct from the case during limb development.

Overall, my work showed that dedifferentiation of the limb CT cells correlated with the regeneration capability of the organisms and strongly suggests that the cell intrinsic limitations are the key hindrance to complete regeneration.

Zusammenfassung

Salamander sind in der Lage, vollständig strukturierte Gliedmaßen zu regenerieren, während postmetamorphe Frösche nur einen hypomorphen Stumpf regenerieren können. Bei einem hypomorphen Stumpf handelt es sich um einen von Hautgewebe bedeckten, unverzweigten und nicht segmentierten Knorpel. Bei der Amputation von Gliedmaßen bilden sowohl Axolotl als auch afrikanische Krallenfrösche ein Blastema, das aus proliferierenden Vorläuferzellen besteht, die sich an der Amputationsebene ansammeln. Bis jetzt sind die zellulären und molekularen Mechanismen, die für die Unterschiede zwischen den Blastema-Zellen beider Arten verantwortlich sind, noch nicht vollständig entschlüsselt worden.

Um dieser Frage nachzugehen, haben wir Regenerationszeitverläufe von Axolotl und Fröschen durchgeführt und dabei systematisch das molekulare und zelluläre Verhalten der Bindegewebszellen der Gliedmaßen verglichen, die bekanntermaßen eine Schlüsselrolle bei der Regeneration der Axolotl-Gliedmaßen spielen. Wir konnten zunächst bestätigen, dass, so wie beim Axolotl, ein Froschblastem hauptsächlich aus den Nachkommen von Gliedmaßen-Bindegewebszellen besteht, indem wir transgene Froschlinien mit *Prrx1:CreER* erzeugten und charakterisierten, welche die Markierung und Verfolgung der Gliedmaßen-Bindegewebszellen ermöglichten. Einzelzell-RNA-Sequenzierungsanalysen über den Zeitverlauf der Regeneration und heterochrone Transplantationsstudien in Gliedmaßenknospen zeigten, dass Axolotl-Bindegewebszellen dedifferenzierten und in der Lage waren, sich in mehrere Linien der Gliedmaßenknospen, einschließlich Knorpel, zu integrieren. Die Bindegewebszellen des Frosches hingegen dedifferenzierten sich nur teilweise und hatten nicht die Fähigkeit, sich in den Knorpel der sich entwickelnden Gliedmaßenknospen zu integrieren. Das Ergebnis der Transplantationsstudien deutet auf zelleigene Beschränkungen der Frosch-Bindegewebszellen hin. Die weitere Analyse der Einzelzell-RNA-Seq-Daten zeigten, dass sich die chondrogenen Vorgänge des Stumpf-Knorpels molekular von denen während der Gliedmaßenentwicklung unterscheiden.

Insgesamt hat meine Arbeit gezeigt, dass die Dedifferenzierung der Bindegewebszellen der Gliedmaßen mit der Regenerationsfähigkeit der Organismen korreliert und stark darauf hindeutet, dass die zelleigenen Grenzen das Haupthindernis für eine vollständige Regeneration sind.

I. Introduction

I.1 Overview

The ability to regenerate missing body parts varies between species. For example, salamanders, such as axolotls (*Ambystoma mexicanum*), can regenerate brain (Kirsche & Kirsche, 1964), spinal cord (David et al., 1963), jaw (Vieira & McCusker, 2018), heart (Flink, 2002) and appendages (Spallanzani, 1768), while mammals possess rather limited regenerative abilities. Given that many genetic programs during development are common in different species, it is appealing to assume that mammals have the genetic components of regeneration. By understanding how some species regenerate better than others, we might be able to improve regeneration outcomes by re-wiring the genetic programs in animals that have poor regenerative capacities. To achieve this goal, I argue that it is important 1) to understand the mechanisms of successful regeneration animal models, 2) to compare those mechanisms with the less- or non-regenerative animal models, and 3) to test whether we could improve regeneration by translating the knowledge gained from the previous points. These three points not only are interesting topics of basic research to understand how nature works, but also have important medical implications to improve our wellbeing.

One of the most fascinating regeneration processes in vertebrates is the regeneration of salamander limbs. To regenerate a limb, multiple tissue types are recruited and organized in a way that the regenerate is morphologically identical to the original one. The regenerating limb recognizes the amputation level and only regrows the missing part of the limb (Goss, 1969). This process occurs regardless the size of the animal. The difference in the linear dimension between regenerating a small limb and a large limb can be at least up to 10-fold (Brookes & Kumar, 2005). The size of the animal also affects the time required to regenerate, which can span between weeks to months. So far, the ability to regenerate a fully patterned limb seems to be unique to salamanders, as the same type of amputation in mice and chick only leads to the wound closure without tissue outgrowth (Masaki & Ide, 2007; Satoh et al., 2010).

On the other hand, the other major branch of class Amphibia, anuran amphibians, or collectively called frogs, possesses a different dynamic of regenerative capacity from the case in salamanders. Unlike salamanders which can regenerate their limbs throughout their lifetime, frogs can only regenerate a fully patterned limb at the very early stages of limb development (i.e., before developmental stage 51). This ability is then rapidly lost between stage 52 and stage 55 (Dent, 1962). From stage 56 onward, it is considered as patterning-incompetent. A post-metamorphic frog, also called a froglet (i.e., developmental stage 66) can only regenerate a spike, a structure that is mainly composed of an unbranched and unsegmented cartilage rod covered with skin tissues (Dent, 1962; Endo et al., 2000). The ontogenic decrease of regeneration capacity was also observed in different frog species (Agarwal & Niazi, 1980; Fry, 1966; Hayashi et al., 2015), suggesting that the formation of spike is evolutionarily conserved among the anuran amphibians.

What are the differences between the limb regeneration of a froglet and a salamander? What cause the ontogenic decrease of the regenerative ability of frog limbs? Such questions have motivated decades of research, many of which will be discussed in the following sections. The close phylogenetic positions and the contrasting regeneration outcomes between salamanders and frogs have made them attractive models to uncover evolutionarily conserved and diverged mechanisms of limb regeneration. Due to the technological limitations, however, most of the studies on the subject focused on individual species with a resolution at the tissue level. Two recent technological advances have greatly improved our understanding of the molecular and cellular mechanisms of salamander limb regeneration. One was the advent of single cell technologies that allows us to profile thousands of cells simultaneously during axolotl limb regeneration (Gerber et al., 2018; Klein et al., 2015; Macosko et al., 2015). The other was the development of transgenic salamanders that enables the genetic labeling and tracing of limb cells during regeneration (Gerber et al., 2018; Khattak et al., 2013; Tanaka et al., 2016). Importantly, transgenic methods to trace cell lineages have been successfully demonstrated and provided profound insights in the tail regeneration of tadpoles (Gargioli & Slack, 2004; Lin et al., 2007; Ryffel et al., 2003). This new wave of technological advances open the possibilities of lineage tracing in frog limbs and cross-species comparison between salamander and frog limb cells at both molecular and cellular levels. It directly inspired my Ph.D. work.

In the following sections, I am going to first discuss how the successful limb regeneration happens in salamanders.

I.2 Salamander limb regeneration

I.2.1 Limb regeneration through the formation of a blastema

The regeneration of a salamander limb can be roughly divided into few major events (Stocum, 2017). In brief, when a salamander limb is amputated, the epidermal cells near the cutting site migrate and cover the wound within several hours, forming a single layer of epidermal cells called the wound epidermis (WE). In the following days, the mesenchyme tissue close to the amputation plane undergoes histolysis, and proliferative cells start to emerge. Over time, the WE thickens to become the apical epithelial cap (AEC), and the mass of proliferative mesenchymal cells underneath the AEC forms a cone-shaped structure called the blastema. As the regeneration progresses, the blastema establishes the main axes to restore the missing skeletal pattern. The new, patterned limb will continue to grow until it reaches the same size as the unamputated limb on the contralateral side. Although the term blastema sometimes includes AEC in the literature, it strictly refers to the mesenchyme population in this study to avoid confusion.

To rebuild a limb pattern, the salamander limb blastema and AEC utilize the genetic machineries similar to those used during limb development. Specifically, genes that are essential for the limb development and are absent in the mature limb, are upregulated during regeneration. Such examples include *fgf-2*, *fgf-8*, *wnt-5*, and *sp9* (Christensen et al., 2002;

Ghosh et al., 2008; Han et al., 2001; Mullen et al., 1996; Satoh et al., 2008). Moreover, genes that are important for the formation of primary limb axes during development are expressed in the cognate locations of a blastema. For example, *meis1/2*, *hoxa11* and *hoxa13*, which signify the proximal-distal axis, are expressed in an upper limb blastema (Kragl et al., 2009; Mercader et al., 2005; Nacu et al., 2013; Roensch et al., 2013). *Sonic hedgehog (shh)*, a marker of posterior limb during development, is expressed in the posterior side of blastema (Imokawa & Yoshizato, 1997; Torok et al., 1999). A dorsal limb determinant gene, *lmx-1b*, is also dorsally expressed during regeneration (Shimokawa et al., 2013). These candidate-based analyses suggest that limb patterning in regeneration and development use similar mechanisms. Therefore, salamander limb regeneration has been suggested as a re-development (Nacu & Tanaka, 2011).

I.2.2 The past and the future of blastema cells

To study the cellular properties of a blastema, it is important to determine where the blastema cells come from and what they can become in the new limb. Histological analysis in the early 20th century observed that cartilage, muscle, connective tissues and Schwann cells contributed to the limb blastema (Chalkley, 1954; Hay, 1958, 1959; Thornton, 1938). When these morphologically distinct cell types entered the blastema, they became round-shape and morphologically homogeneous. It was therefore much discussed whether blastema cells are composed of different cell populations or belonged to a single cell type (Steen, 1970). A transplantation assay showed that the isotope-labeled muscle gave rise to the chondrocytes in the regenerated limb of axolotls, but the author pointed out that the cell origin of chondrocytes was uncertain because the donor muscle was a mixture of cell types (Steen, 1968). On the other hand, axolotl cells from the transplanted dermis can become chondrocytes, but not muscle (Muneoka et al., 1986). The question was revisited by a series of lineage tracing assays harnessing modern genetic labeling techniques. When specifically labeled by grafting fluorescent-positive embryonic tissue layers, different tissue types of an axolotl limb contributed to their own lineages in the regenerated limb. For example, the muscle lineage only regenerated muscles and the connective tissue lineage only regenerated connective tissue, and so on (Kragl et al., 2009). Lineage tracing using a transgenic axolotl that allows the genetic labeling of limb connective tissue cells confirmed that axolotl limb connective tissue cells retained their lineage identities during regeneration (Gerber et al., 2018). These results implied that an axolotl limb blastema contains progenitor cells of different lineages, and is therefore not homogeneous. A similar result was also found in the Japanese fire-belly newt (*Cynops pyrrhogaster*) (Tanaka et al., 2016). In contrast, the *in vitro* cultured, clonal expanded muscle progenitor cells were able to become chondrocytes during limb regeneration in the red-spotted newt (*Notophthalmus viridescens*) (Morrison et al., 2010). It remains to be determined whether the difference is a species-specific feature or a result of different experimental methods.

I.2.3 Cell type contribution to the salamander blastema

I.2.3.1 Epidermis, nerve, and muscle tissues

Traditionally, research on the blastema formation focuses on the epidermal and nerve tissues. This is partly because they are easy to manipulate and are essential for blastema formation. The regeneration did not occur if the wound was covered by a flap of normal skin. (Mescher, 1976). Denervation or the daily removal of WE/AEC both prevented the blastema formation (Butler & Schotte, 1941; Thornton, 1957). Interestingly, attaching an AEC at the preaxial wound of a blastema induced an accessory limb (Thornton, 1960; Thornton & Thornton, 1965), suggesting that the AEC can direct the outgrowth of blastema. Deviating nerve bundles to the lateral wound site induced the proliferation of local mesenchyme cells and forms a bump at the wound site (Endo et al., 2004). However, the bump eventually regressed, suggesting that the deviated nerve was not able to maintain the outgrowth. Because removing either the AEC or the nerve tissue will prevent the blastema formation, it remains unclear whether they have direct impact on limb patterning.

A significant portion of a salamander mature limb is composed of muscle tissue, but its impact on the regeneration seems to be mainly within the muscle lineage itself. In the Iberian ribbed newts (*Pleurodeles waltl*), impairing myogenesis by knocking out *pax3* gene resulted in a complete loss of muscle tissues in the limb but did not affect the overall skeletal pattern (Elewa et al., 2017). Remarkably, the muscle-less limb formed a blastema upon amputation and regenerated a muscle-less limb with a correct limb pattern, indicating that the loss of muscle tissues did not affect the overall progression of limb regeneration. A similar result was also observed in axolotls (Nowoshilow et al., 2018), suggesting an evolutionarily conserved role of muscle tissue.

I.2.3.2 Connective tissue

Connective tissue (CT) cells play several key roles during limb regeneration. First, axolotl CT cells are the main constituent of the limb blastema. When limb regeneration was inhibited by X-ray irradiation, grafting an unirradiated axolotl skin, which contains the dermis and the epidermis, regenerated a limb on the host axolotl (Dunis & Namenwirth, 1977). Based on the observations that the epidermis did not contribute to the blastema, and that the dermis contained abundant CT cells, it was suggested that the axolotl dermal CT cells, also called fibroblasts, were the main contributor to the regenerate (Muneoka et al., 1986). The cells derived from the grafted dermis generated a variety of CT cell populations in the regenerated limb, including cartilage, perichondrium, dermis and joint connective tissue cells, showing their plasticity (Dunis & Namenwirth, 1977). Immunostaining against a CT-specific marker, *Prrx1*, also showed that around 70% of the limb blastema cells are CT cells (Gerber et al., 2018). Second, limb CT cells are suggested to possess the so-called positional identity (PI) based on their physical position of the limb, which serves as the reference to rebuild the missing limb pattern. Rotating the skin cuff before amputation resulted in the formation of supernumerary limbs (Carlson, 1975), supposedly due to the PI mismatch between the skin

and the underlying tissues. Tissue grafting experiments and immunostaining further demonstrated that axolotl limb CT cells recognized their PIs, expressing the corresponding patterning genes and moved accordingly in the limb blastema (Nacu et al., 2013). Third, single cell RNA-seq analysis revealed that the blastemal CT populations expressed the patterning genes, such as *meis2*, *hoxa11*, *hoxa13* and *shh* (Gerber et al., 2018), suggesting that the genetic network to establish a patterned limb is at least in part encoded in the blastemal CT cells. Although perturbing the expression of those axes-determining genes at a CT-specific level has not been reported, a global inhibition of *shh* signaling pathway by cyclopamine treatment during the early phase of axolotl limb regeneration resulted in a loss of digit pattern in the regenerated limb (Stopper & Wagner, 2007). These three points indicates that axolotl limb CT cells are the key cell type of limb regeneration.

Historically speaking, CT cells is a collective term for multiple cell types, including dermal fibroblasts, interstitial fibroblasts, chondrocytes, perichondria, pericytes and so on. The term interstitial fibroblast refers to the non-pericyte CT cells residing in between dermis and bones. Of all the CT cell types, the interstitial and dermal fibroblasts are the two largest populations as observed by histological analysis and in the single cell RNA-seq experiments (Gerber et al., 2018). Importantly, CT subtypes differentially contribute to the regenerated limb. Tissue grafting experiments where the triploid dermis or the triploid cartilage was transplanted to a diploid limb before amputation showed that dermal cells contributed up to 43% of the blastema cells, while cartilage contributed only 2% (Muneoka et al., 1986). A study combining brainbow cell tracking and live imaging techniques during axolotl digit tip regeneration observed that, while cartilage cells barely move to the blastema, dermal fibroblasts, perichondria, and pericytes are the main contributor to the digit tip blastema (Currie et al., 2016). Of the three CT cell types, only dermal CT cells displayed multipotency, i.e., the abilities to become multiple CT cell types in the regenerate, including chondrocytes. Similar result was observed by grafting experiments. When skin tissues containing genetically labeled dermal fibroblasts were transplanted to a wildtype host limb and were traced during regeneration, the labeled dermal fibroblasts contributed to multiple CT cell types, including dermis, interstitial cells, tendons, and cartilage cells. On the other hand, transplanted bone tissue only contributed to the local cartilage segment. Single cell RNA-seq analysis also showed trajectories of interstitial cells to multiple lineages (Gerber et al., 2018). These results established that the dermal and interstitial fibroblast populations are the main sources of blastema cells. Overall, these results point to the direction that dermal and interstitial CT cells are the center of blastema formation and limb patterning.

1.2.4 Dedifferentiation v.s. stem cells

Two possible routes have been suggested to explain how a cell in the mature limb to become the progenitor cells in the blastema. It is either through the dedifferentiation of the mature cells, or through the release of resident stem cells. Dedifferentiation describes the process in which the cells revert from a differentiated state to a progenitor state of the same lineage (Tata et al., 2013; Yao & Wang, 2020).

Historically, the blastema formation is commonly associated with the term dedifferentiation due to the observation of histolysis near the amputation plane prior to the emergence of proliferative blastema cells. This definition was exemplified by the observation that the multinucleated myotubes broke down into mononucleated cells in the limb blastema of spotted salamander (*Amblystoma punctatum*) (Thornton, 1938). Electron microscopy confirmed that the muscle cells budding from myotubes gradually lost their intracellular myofibril, ER and Golgi organizations, a sign of acquiring the progenitor state (Hay, 1959). A series of studies on the myotubes of red spotted newt (*N. viridescens*) showed that *in vitro* cultured multinucleated myotubes broke into mononucleated cells and contributed to the new muscle tissues after being transplanted into a blastema (Kumar et al., 2000; Lo et al., 1993).

To unambiguously determine whether the blastema cells are from the dedifferentiated limb cells, from the resident stem cells, or both, one has to trace the differentiated cells and the precursor cells of the same lineage during regeneration. Such strategy was reported in the case of tail muscle regeneration in *X. laevis* and the limb muscle regeneration in axolotls and newts. Genetic lineage tracing experiments showed that the muscle of the regenerated tail was not from the existing tail muscle, but from the Pax7⁺ satellite cells (Gargioli & Slack, 2004; Ryffel et al., 2003). In the case of limb muscle regeneration of axolotls and the red-spotted newt, the result showed that the axolotl limb muscles are exclusively regenerated from the resident Pax7⁺ muscle satellite cells, while the newt multinucleated muscle fibers can dedifferentiate and give rise to new muscles (Fei et al., 2017; Sandoval-Guzman et al., 2014).

Of all the CT subtypes, dedifferentiation during regeneration was better described in the cartilage tissue due to its distinct morphology. Histological analysis based on light microscopy showed histolysis on the cartilage and the release of cartilage nuclei near the amputation plane (Butler, 1933). On the other hand, the dedifferentiation of CT cells in the soft tissues, such as the dermal and interstitial fibroblasts, was more difficult to determine with traditional histology and microscopy methods. This is because soft CT cells lack distinct tissue structures and intermingle with other tissue types. For example, it is not possible to distinguish interstitial fibroblasts from residing muscle satellite cells by light microscopy. A similar problem exists when examining the histolysis in the dermis. The release of cells from the dermis was described but whether the mobilized cells were resident stem cells or dedifferentiated cells could not be determined. Therefore, the dedifferentiation of soft CT cells was more often assumed and was not much discussed until recently.

The lack of distinct morphology implies that the dedifferentiation process of CT cells has to be defined at the molecular level. A seminal work was done by Gerber, Murawala, Knapp et al to establish a transgenic axolotl line that conditionally labeled limb CT cells and allowed tracing and single cell profiling of limb CT cell lineages during limb regeneration (Gerber et al., 2018). Single cell RNA-seq analysis showed that soft CT cells rapidly downregulated the expression of their subtype-specific markers during the first few days of regeneration, a prominent sign of dedifferentiation. Strikingly, soft CT cell subtypes

dedifferentiated to a common CT progenitor type in the blastema regardless of their origins, and these progenitors continued to turn on genes that are expressed in early embryonic limb buds. The result suggested that the CT cells were able to fully dedifferentiate from mature CT transcriptomic state into an embryonic CT state. The same analysis also found no evidence of pre-existing CT progenitor cell pool in the mature limb, suggesting that dedifferentiation is the sole mode to generate blastemal CT cells.

I.3 Frog limb regeneration

I.3.1 A froglet limb blastema becomes a spike

At the macroscopic level, the froglet limb regeneration follows a similar progression as the case in the salamander up to the blastema formation phase (Suzuki et al., 2006). Although a froglet blastema can grow to a comparable length as the uncut limb at the contralateral side, it only becomes a spike instead of a patterned limb (Endo et al., 2000). This lack of skeletal pattern correlates with the inability to properly express genes that regulate the formation of major limb axes. For example, *hoxa11* and *hoxa13* were not expressed in the separate regions of froglet limb blastema (Ohgo et al., 2010). *shh* was not expressed at all during froglet limb regeneration (Endo et al., 2000; Endo et al., 1997; Ohgo et al., 2010), likely due to an epigenetic inactivation of a *shh* enhancer sequence during metamorphosis (Yakushiji et al., 2007). These results suggested that the primary limb axes were not correctly specified in a froglet blastema.

However, the mis-expression of patterning genes cannot fully explain the inability to form a patterned limb in froglets. While the activity of *shh* signaling pathway is required for limb patterning during development in frogs (Stopper et al., 2016), pharmaceutically activating the *shh* pathway during froglet limb regeneration did not restore the limb pattern (Yakushiji et al., 2009). It remains unclear whether the ineffective treatment was due to the suboptimal timing and dosage, or due to the global activation of *shh* pathway that created an incorrect condition for limb patterning. In addition, it could also be that missing *shh* is not the only reason. Studies have shown a drastic decrease of *fgf-8* and *fgf-10* expression in the AEC and the blastema, respectively, during metamorphosis (Aztekin et al., 2021; Yokoyama et al., 2000). It suggests that the cause of spike formation is multifactorial.

I.3.2 Cell type contribution to the froglet spike

Like in axolotls, the nerve tissue of a froglet is also important for the blastema formation as complete denervation stops the regeneration process (McLaughlin & Liversage, 1986). Partial denervation resulted in a proportionate decrease of the blastema size (Liversage et al., 1987), suggesting a positive correlation between the extent of innervation and the blastema outgrowth. Histological analysis on the nerve tissue in the stump showed that the number of nerve fibers per unit wound area was lower in the froglet limb compared to the case in the regeneration-competent limb buds (Van Stone, 1955). Strikingly, increasing the nerve supply by surgically deviating the sciatic nerves from the froglet leg to its ipsilateral,

amputated arm resulted in the branching of the spike (Kurabuchi, 1992; Mitogawa et al., 2018; Singer, 1954). Although the branching looks no way like normal digits and the underlying mechanism remains elusive, the phenotype is generally considered as an improvement of regeneration. The result suggests that, at least in the case of anuran amphibians, nerve tissue not only promotes blastema outgrowth, but also participates in pattern formation to certain extent.

Muscle tissue is not present in a spike (Dent, 1962). Ectopically injecting muscle progenitors into the blastema lead to the formation of muscle fibers at the injection site but did not change the overall spike morphology (Satoh et al., 2005). This result resonates the finding in the salamander studies that muscle tissue plays passive roles in limb regeneration and do not have obvious impact on the limb patterning.

The majority of froglet spike cells are cartilage cells, suggesting that the CT cell population in the stump contributes to the blastema. However, whether the cells originate from dermal, interstitial, or bone remains largely unexplored. Grafting experiments in froglets were uncommon due to their strong immune rejection developing at the early metamorphosis stages (Horton, 1969). It is also unclear whether the mature CT cells dedifferentiate, or the resident CT precursors proliferate, to become the blastema cells. Interestingly, studies on the frog limb bud and the frog blastema have suggested that frog limb cells also use a patterning mechanism similar to the position identities of axolotl limb CT cells. When the distal part of a developing limb bud was rotated 180 degrees, the formation of a supernumerary limb was observed (Endo et al., 1997). A supernumerary limb was induced when a reaggregated cell pellet of limb bud mesenchymal cells was transplanted into a limb bud stump and covered with the limb bud epidermis (Yokoyama et al., 1998). Interestingly, when the same experimental setup was done using a cell pellet from the mesenchyme of a regeneration-incompetent stage, a limb with fewer digits was formed (Yokoyama et al., 1998). It is unclear whether the decrease of digit number was due to a loss of PI during development, or due to the other inhibitory mechanisms yet to be discovered. Overall, although studies solely on the limb CT population has not been done, the evidence we have so far strongly suggests that the property of frog limb CT cells are changed during development.

1.3.3 The patterning ability is an intrinsic property of blastema

An important work done by Sessions and Bryant in 1988 showed that the patterning ability is locally encoded in the frog limbs. When a froglet limb was amputated after being grafted onto an embryonic limb stump, it regenerated a spike. On the other hand, an embryonic limb was able to regenerate a patterned limb after being grafted onto a froglet stump (Sessions 1988). These results showed that the patterning ability of donor tissues was not affected by the environmental cues, such as the neurotrophic or circulatory factors. Interestingly, when an axolotl limb skin was replaced by froglet limb skin, the frog epidermis was able to sustain the blastema formation consisting of axolotl mesenchyme cells (Carlson, 1982). It was unclear whether the axolotl blastema could pattern with the frog epidermis because the frog epidermis was outgrown by the axolotl epidermis before the patterning

started. Nevertheless, it suggested that the froglet epidermis was functionally equivalent to its axolotl counterparts. Interestingly, covering the patterning-incompetent limb mesenchyme with a limb bud skin did not improve limb patterning during regeneration. Conversely, covering the limb bud mesenchyme with a skin from a patterning-incompetent stage did not impair limb patterning (Yokoyama et al., 2000). This result indicates that the ability to regenerate a patterned limb is encoded in the mesenchyme, i.e., in the blastema. To which extent this intrinsic feature can be attributed to CT cells remains to be investigated.

1.3.4 Improving froglet limb regeneration by ectopic factors

Several treatments of ectopic factors have been used to improve the regeneration outcome to various extent. Implanting Fgf-10-soaked beads resulted in the formation of multiple digits of the stage 56 tadpole limb stump, which already lost its patterning ability (Yokoyama et al., 2001). The presence of ectopic Fgf-10 upregulated the expression of endogenous *fgf-10*, *msx-1*, and *hoxa13* in the mesenchyme which were absent without the treatment, suggesting that such treatment induce the regenerative potency of mesenchyme cells. Another study showed that multiple cartilage branches were induced by co-electroporating *fgf-2*, *fgf-8*, *shh* and *bmp4* to the froglet blastema, although the electroporation efficiency and the electroporated cells was not determined (Mitogawa et al., 2018).

A more sophisticated experimental design improved regeneration at the froglet stage (Lin et al., 2013). It first showed that tissue grafting was possible using the immune-compromised hosts generated by thymus removal at early tadpole stages. A fibrin patch was developed to encase and attach the dissociated bcat* limb bud cells, together with multiple factors (Fgf-10, Shh, and thymolysin b), to a froglet stump. The bcat* limb bud cells were from transgenic tadpoles that expressed a constitutively active form of b-catenin proteins induced by a heat shock treatment. This grafting setup induced the proliferation and reduced the cell death of stump cells and dramatically improved the regeneration outcome, which contained multiple separated digits with bone segments. Remarkably, the host froglet cells, which normally only formed a spike, participated in the regenerated limb. This result suggests that the froglet limb cells were able to respond to the extrinsic factors to form a complex limb pattern.

A striking improvement of froglet limb regeneration were recently reported with a single progesterone treatment (Herrera-Rincon et al., 2018). The progesterone was prepared in a hydrogel-based “bioreactor” that attached to the wound site immediately after the limb amputation for 24 hours. This one-time treatment resulted in the bifurcation of spike observed five months after amputation. The same group further demonstrated that combinatorial treatment of five small compounds (1,4-DPCA, brain-derived neurotropic factor, growth hormone, resolvin D5, and retinoic acid) through the bioreactor resulted in a regenerated tissue with complex structure (Murugan et al., 2022). Although the regenerate did not look like a normal limb, the multi-drug treatment led to a lengthened cartilage and partially restored the sensorimotor system. These results demonstrate that froglet blastema

cells can respond to external signals, and that even a 24-hour treatment at day one could have significant impact on the regeneration outcome. It encourages the idea that one day we might be able to induce the perfect limb regeneration by applying a correct panel of factors.

Despite such progress in the recent year, none of the treatments resulted in a perfect limb regeneration in frogs, and the underlying mechanisms of each treatment are often unclear. It is partly because there are too many moving parts in the system, making the interpretation of results very challenging. Could it be that a factor only improved the regenerative ability of a cell type but worsened the ability of the others, so that the overall effect cancelled out? It is impossible to tell under the current tissue-scale resolution. The complexity is further amplified when comparing the results across species.

I.4 Objectives of my PhD study

The goal of my PhD study was to study mechanisms of limb regeneration by systematic comparing the limb regeneration process of axolotls (*A. mexicanum*) and frogs (*X. laevis*) at the cellular and molecular levels. Given that both species form a blastema upon limb amputation but regenerate very different structures, and that axolotl limb CT cells play key roles in limb regeneration, I hypothesize that the key differences may lie within the frog CT cells. Therefore, I focused on the limb CT cells, and ask:

- 1) What is the source of CT cells in a frog limb blastema and how is it different from the case in axolotls?
- 2) What are the gene expression profiles of frog limb CT cells during regeneration, and how is it different from the case in axolotls?
- 3) Are frog limb CT cells capable of regenerating a patterned limb if the environment is permissive?

To answer those questions, I established transgenic *Xenopus* lines to lineage trace the frog limb CT cells, performed scRNA-seq analysis to uncover the genetic programs during limb regeneration, and developed a universal cell transplantation assay to test the regenerative potency of blastema cells. The work resulted in two research papers published on international journals (Chapter 2.1 and 2.2).

II. Publication & Manuscript

II.1 Inducible and Tissue-Specific Cell Labelling in *Cre-ER^{T2}* Transgenic *Xenopus* Lines

One of the main limitations facing the limb regenerative field in the context of the African clawed frogs (*Xenopus laevis*) was the lack of genetic tools to properly trace and manipulate specific cell populations. Thus, one of our first endeavors was to adapt the *Cre-ER^{T2}/LoxP* system which we used to label and lineage trace frog limb connective tissue (CT) cells for downstream analysis. We first generated stable transgenic frog lines with a pan-expression of *ERCreER* driven by the *CAGGs* promoter and showed that muscle, dermal, and epidermal cells were conditionally labeled by the 4-hydroxytamoxifen (4OHT) treatment. We found that the presence of *ERCreER* sequence significantly lowered the transgenesis efficiency and that the timing of 4OHT treatment was crucial to label limb cells. Using the same principles, we generated stable transgenic frog lines with the *CreER* cassette driven by the mouse *Prrx1* enhancer and successfully labeled frog limb CT cells in an inducible manner. Histological analysis showed that the *Prrx1:CreER* lines also labeled cells in other tissues, such as brain, heart, and tail dermis. Overall, this study showed the utilization of the *Cre-ER^{T2}*-based lineage tracing system in *X. laevis* and provided a detailed characterization of the *Prrx1:CreER* lines as a resource for future studies.

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Contributions:	TY.L. and S.H. generated transgenic <i>Xenopus laevis</i> lines with assistance from P.M. and Y.TS. TY.L. designed and performed the experiments. TY.L. and E.M.T. analyzed data and wrote the manuscript.

Submission Confirmation



Thank you for your revision

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Title Inducible and Tissue-Specific Cell Labelling in Cre-ERT2 Transgenic Xenopus Lines

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1 Title: Inducible and Tissue-Specific Cell Labelling in *Cre-ER^{T2}* Transgenic *Xenopus*

2 Lines

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Abstract

Investigating cell lineage requires genetic tools that label cells in a temporal and tissue-specific manner. The bacteriophage-derived *Cre-ER^{T2}/loxP* system has been developed as a genetic tool for lineage tracing in many organisms. We recently reported a stable transgenic *Xenopus* line with a *Cre-ER^{T2}/loxP* system driven by the mouse *Prrx1* (*mPrrx1*) enhancer to trace limb fibroblasts during the regeneration process (*Prrx1:CreER* line, Lin et al., 2021). Here we describe the detailed technological development and characterization of such line. Transgenic lines carrying a *CAG* promoter-driven *Cre-ER^{T2}/loxP* system showed conditional labeling of muscle, epidermal and interstitial cells both in the tadpole tail and the froglet leg upon 4-hydroxytamoxifen (4OHT) treatment. We further improved the labeling efficiency in the *Prrx1:CreER* lines from 12.0% to 32.9% using the optimized 4OHT treatment regime. Careful histological examination showed that *Prrx1:CreER* lines also sparsely labeled cells in the brain, spinal cord, head dermis, and fibroblasts in the tail. This work provides the first demonstration of conditional, tissue-specific cell labeling with *Cre-ER^{T2}/loxP* system in stable transgenic *Xenopus* lines.

Introduction

The ability to label cells and unambiguously trace their progenies provide valuable insights on the cellular mechanisms during development and regeneration. To achieve this, specific cell type of interests has to be permanently labelled to allow detection at later time points. In addition, temporal labeling is required to obtain unambiguous lineage tracing results. The latter is especially crucial because many developmental genes are re-activated during regeneration, leading to potential new labeling of cells if the labeling system is not temporally controlled. Another important point to consider is that regeneration studies usually focus on adult tissues, so that such lineage tracing system must be integrated into the genome as a stable transgenic line to enable labeling at the late development and adult stages.

The bacteriophage-derived *Cre-ER^{T2}/loxP* system is the most common lineage tracing system first developed in mammalian cell lines (Feil et al., 1997). The Cre recombinase is fused to the mutated ligand binding domain of the human estrogen receptor (ER^{T2}), which prevents the recombinase from entering the nucleus by binding to a heat shock protein hsp90. Upon binding to 4-hydroxytamoxifen (4OHT), ER^{T2} releases hsp90, allowing the recombinase to enter the nucleus to recombine *loxP* sites on the reporter cassette. This method was further established *in vivo* in mice (Metzger & Chambon, 2001) and has been adopted in multiple organisms, including zebrafish (Hans et al., 2009) and axolotls (Khattak et al., 2013). However, this approach has not been successfully established in *Xenopus laevis* as stable transgenic lines despite its utility in

the developmental research. One attempt to utilize *Cre-ER^{T2}/loxP* system to lineage trace limb muscle cells resulted in very few labeled muscle cells and the line was determined unsuitable for lineage tracing (Rodrigues et al., 2012).

We recently reported a stable transgenic *Xenopus* line that harbors the *Cre-ER^{T2}/loxP* system to investigate the contribution of fibroblasts lineage during *Xenopus* limb regeneration using the mouse *Prrx1* enhancer (*Prrx1:CreER* line, Lin et al., 2021). Subsequent isolation and sequencing of the labeled fibroblasts further revealed the molecular progression of fibroblasts during limb regeneration. Here we present the entire development of our transgenic *Xenopus* lines harnessing the *Cre-ER^{T2}/loxP*-based lineage tracing method. We first report the proof-of-principle *ER^{T2}-Cre-ER^{T2}* transgenic lines driven by the *CAG* promoter (referred to as *CAGGs:ERCreER* in this study). Despite having a lower transgenic efficiency, *CAGGs:ERCreER* lines showed conditional labeling of tadpole tail when treated with 4OHT at onset of metamorphosis. Notably, leg tissues were labeled only with the post-metamorphic, but not the pre-metamorphic 4OHT treatment, indicating that the treatment timing was crucial for optimal cell labeling. We then further determined the optimal 4OHT treatment regime for our *Prrx1:CreER* lines and improved the labeling efficiency in the limb bud from 12.0% to 32.9%. Detailed immunohistochemistry characterization showed that the new 4OHT treatment regime labeled limb bud mesenchyme cells, as well as some cells in the brain, heart, tail dermis, and spinal cord to a lesser extent. Importantly, single *ER^{T2} CAGGs* lines (*CAGGs:CreER*) showed 4OHT-independent labeling, while double *ER^{T2} Prrx1* lines (*Prrx1:ERCreER*) showed very low labeling efficiency. We hope that our

- 81 discovery could serve as a reference for the *Xenopus* community and benefit the future
- 82 development of lineage tracing techniques in *Xenopus laevis*.

83

Results

Conditional labeling of tail tissues in F0 *Xenopus laevis* carrying an *ER^{T2}-Cre-ER^{T2}/loxP* system driven by the *CAG* promoter

To investigate whether 4OHT-induced cell labeling could be robustly achieved in tadpole stages, we performed REMI transgenesis using both *Cre-ER^{T2}* and *loxP* reporter plasmids (Figure 1A) (See Materials and Methods for details). The *CAG* promoter was used to drive the pan-expression of the *ER^{T2}-Cre-ER^{T2}* cassette (*CAGGs:TFPnls-T2A-ER^{T2}-Cre-ER^{T2}*, abbreviated as the *CAGGs:ERCreER* plasmid), which included a *TFP* with a nucleus localization signal (nls) sequence as a screening marker. The *loxP* reporter plasmid was designed to have a floxed stop codon plus a triple *SV40 polyA* sequence, with a *mCherry* sequence at the second position (*CAGGs:loxP-Stop-3xSV40pA-loxP-mCherry*, abbreviated as the *CAGGs:Stop-lp-Cherry*). The *CAGGs:ERCreER* and *CAGGs:Stop-lp-Cherry* plasmids were mixed and used for transgenesis. The surviving F0 embryos were kept until stage 49 (Nieuwkoop & Faber, 1956) when we screened for the nuclear TFP expression in the tail (Figure 1B). A low efficiency (TFP-positive over total surviving embryos) was observed. From four batches of REMI experiments, 8 TFP-positive tadpoles out of 172 survived embryos were obtained (Figure 1A). The average efficiency of the experiments using the *CAGGs:ERCreER* plasmid (4.9%) was significantly lower compared to the experiments using the *CAGGs:TFPnls* plasmid (71.8%) (Figure 1C). The low transgenesis efficiency is likely due to the increased cassette length, as we observed a negative correlation

between transgenic efficiencies and the length of DNA cassettes (Supplementary Figure S1A). Despite having such a low transgenic efficiency, an induced mCherry signal was observed throughout the tail of all the TFP-positive tadpoles five days after an overnight 4OHT treatment at stage 49 (Figure 1D). This result confirms that conditional labeling induced by 4OHT can be achieved at the tadpole stages.

Conditional labelling in F1 *CAGGs:ERCreER* transgenic *Xenopus* lines

To determine if the convertibility of the reporter cassette could be inherited, three of the eight *CAGGs:ERCreER* founders were raised until sexual maturity and were mated with the F1 *loxP* reporter lines carrying *CAGGs:loxP-EGFP-loxP-mCherry* cassettes (hereafter abbreviated as *CAGGs:lp-Cherry*) (Lin et al., 2021). Because we were not able to distinguish GFP-single positive and GFP/TFP-double positive animals by fluorescent signals, we treated all the GFP positive embryos with 4OHT at stage 49 and screened for embryos that express mCherry (Figure 2A). As expected, we observed a 4OHT-induced mCherry expression in the tail tissues of the F1 (Figure 2B). The *CAGGs:ERCreER/CAGGs:lp-mCherry* double positive F1 animals were identified by genotyping and used for the subsequent immunohistochemistry (IHC) analysis (Supplementary Figure S1B). Notably, all the *CreER*-positive animals contained *CAGGs:Stop-lp-Cherry* cassettes, suggesting that the mCherry signal might come from both *CAGGs:Stop-lp-Cherry* and *CAGGs:lp-Cherry* cassettes (Supplementary Figure S1B). Immunohistochemistry (IHC) analysis of the tail tissue showed that labeled cells

were found in the epidermis in the skin region, muscle bundles and interstitial cells (Figure 2C and 2D). In a parallel experiment where we established transgenic lines with the single *ER^{T2}* version to control the Cre activity (*CAGGs:CreER*), we observed a 4OHT-independent mCherry expression in the tail (Supplementary Figure S1C). This result is consistent with a previous finding showing that single *ER^{T2}* was not able to block Cre activity when the cassette was driven by the *CMV* promoter (Werdien et al., 2001).

Despite the induction of mCherry signal in the tail after the 4OHT administration at stage 49, the same animals did not have apparent mCherry expression in the limb buds and legs (Supplementary Figure S2A). Even with three consecutive pulses of 4OHT treatment before stage 49, we were not able to label limb bud cells in the *CAGGs:ERCreER* lines (data not shown). This was not due to a lack of loxP reporter in the limb as we detected GFP expression in the limb tissues (Supplementary Figure S2B) and were able to label limb cells using a different promoter (see below). We suspect that the activity of *CAG* promoter was low in the stage 49 limb bud so that the Cre activity was below the conversion threshold. Due to the lack of TFP antibody, we could not verify the TFP expression in the early limb buds. In contrast, we were able to induce mCherry expression when treating the siblings with 4OHT at stage 66 (froglet stage) (Figure 2E). IHC analysis of the leg samples showed that mCherry signal was mostly, if not all, in the muscle tissue (Figure 2F). Overall, this result demonstrates a proof-of-principle example that a temporal control with the *Cre-ER^{T2}/loxP* system could be achieved in stable transgenic *Xenopus laevis*.

Establishing *Prrx1:CreER* transgenic lines

Next, we investigated whether we could achieve tissue-specific labeling using the *Cre-ER^{T2}/loxP* system in stable transgenic *Xenopus* lines. Our goal was to label and trace limb connective tissue cells during limb regeneration in *Xenopus* (Lin et al., 2021). To do so, we used the mouse *Prrx1* (*mPrrx1*) enhancer to drive the expression of the *Cre-ER^{T2}* cassette. The *mPrrx1* enhancer was shown to be active in the limb mesenchyme of multiple species, including *Xenopus laevis* (Gerber et al., 2018; Logan et al., 2002; Martin & Olson, 2000; Suzuki et al., 2007). Specifically, a previous work has established that *mPrrx1* enhancer was able to drive connective tissue-specific *Cre-ER^{T2}* expression in axolotls (Gerber et al., 2018). It is worth noting that here we describe the single *ER^{T2}-Cre* (*Cre-ER^{T2}*) version instead of the double *ER^{T2}* one used above (See below and Discussion).

When we performed transgenesis with the *Prrx1:CreER* plasmid, we observed very faint TFP expression in the early limb bud to an extent that we could not efficiently distinguish TFP-positive embryos from the wildtype (data not shown). To circumvent this problem, two additional adjustments were made. First, we optimized the *TFPnl5-T2A-CreER^{T2}* sequence based on the codon usage preference of *Xenopus laevis* to boost the translation of the *Cre-ER^{T2}* protein. Second, a γ -crystallin promoter-driven *mCherry* cassette was added in a reversed orientation upstream to the *mPrrx1* enhancer as a

second screening marker (Figure 3A). The *Crystallin:mCherry;mPrrx1:xen(TFPnls-T2A-CreER^{T2})* plasmid (hereafter abbreviated as *Prrx1:CreER*) were used to generated transgenic founders.

Eleven lens-Cherry-positive F0 animals were raised until sexual maturity and mated with the *CAGGs:lp-Cherry* reporter lines. When treating the lens-mCherry/trunk-GFP double positive F1s with 4OHT at stage 49, we observed a 4OHT-dependent mCherry expression in the limb bud in embryos from 9 out of the 11 founders. Out of the 9 founders, we identified 1 founder (*Prrx1:CreER-F0-#2*) that had a robust mCherry expression in the limb bud but not in the tail (Supplementary Figure S3A). The F1 transgenic lines derived from the *Prrx1:CreER-F0-#2* founder were further characterized and described below.

Conditional labeling of limb connective tissue cells

To find the effective labeling timing, we first performed an overnight 4OHT treatment at either stage 47 (st47T-1x), stage 52 (st52T-1x) or stage 66 (st66T-1x) and assessed the mCherry expression at the froglet stage (Supplementary Figure S3B). An overnight DMSO treatment at stage 47 (st47D-1x) was done as a negative control for the st47T-1x samples. The mCherry expression was observed in st47T-1x, but not in st47D-1x, suggesting that the conversion was specific to 4OHT treatment (Supplementary Figure

S3C upper panels). The mCherry expression was strong in the st47T-1x samples and drastically weaker in the st52T-1x and the st66T-1x samples, indicating a narrow window in the early development for effective 4OHT-induced cell labeling (Supplementary Figure S3C).

To find the optimal cell labeling strategy, we performed three 4OHT treatment regimens during early development and determined the conversion efficiency by the amount mCherry expression in the stage 51 limb buds (Figures 3A and 3B). Like a previously published result (Lin et al., 2021), three overnight 4OHT treatments starting from 9-day post-fertilization (dpf) (9d-3x) labeled $12\% \pm 1.2\%$ of the limb bud mesenchymal cells, compared to 0.0% in the control limb buds (Untreated). Six times of 4OHT treatments starting from 15 dpf (15d-6x) increased the efficiency to $29.9\% \pm 2.7\%$. The efficiency was further increased to $32.9\% \pm 2.7\%$ when embryos were treated 13 times with 4OHT starting from 7 dpf (7d-13x) (Figures 3C and 3D). The more-than-doubled amount of 4OHT treatment only labeled 3% more cells, suggesting that 15-31 dpf was the critical time window to label limb cells.

Upon examining the tadpoles after the 4OHT treatment, we observed apparent 4OHT-induced mCherry signal in the limb bud and in the brain region (Supplementary Figure S4A). The mCherry signal remained in the leg throughout metamorphosis and was most obvious in the joint regions (Figure 3E). This pattern was reminiscent to the labeling pattern of a previously reported *Prrxl1:CreER* axolotl line (Gerber et al., 2018). IHC analysis showed that Cherry-positive cells were found in the dermal, interstitial and

cartilage regions as previously reported (Figure 3F) (Lin et al., 2021). It is worth noting that the 4OHT treatments were at the early embryonic stages, and the IHC analysis was done after metamorphosis. Therefore, the labeled cells may turn down *prrx1* expression when they differentiated into different cell types. An example is the Prrx1-negative/Cherry-positive cartilage cells showed in Figure 3F. We did not find labeled cells in the epidermis (Figure 3C and 3F).

Unexpectedly, some leg muscle cells were labeled in many animals upon inspection in the froglet stage (stage 66). The pattern of the labeled muscle cells varies not only between animals, but also between left and right legs (Supplementary Figure S4B). It remains unclear whether it was due to an artifact of the mouse *Prrx1* enhancer activity, a sporadic fusion of limb connective tissue cells with muscle cells, or a reflection of the endogenous *Prrx1* activity in the frog leg muscle tissue. Interestingly, two recent studies confirmed the existence of a connective tissue/muscle dual identity cell population at the muscle tendon junction in both mice and chicken (Esteves de Lima et al., 2021; Yaseen et al., 2021). It is likely that our *Prrx1:CreER* lines labeled such population in *Xenopus* legs. To be on the safe side, leg samples that have extensive muscle labeling were excluded from downstream analysis, such as the single cell RNA-seq and cell transplantation assays in Lin et al. 2021.

It is worth noting that we also established transgenic lines that carries the double *ER^{T2}* version of the *Cre* cassette (*mPrrx1:TFPnlS-T2A-ER^{T2}-Cre-ER^{T2}*). However, we did not characterize these lines further because they labeled fewer cells (Supplementary Figure

S4C). Overall, we successfully established *Prrxl1:CreER* transgenic lines that allows temporal control of tissue-specific labeling.

Conditional labeling of other organs in *Prrxl1:CreER* frog lines

In addition to having activity in the limb mesenchyme, the mouse *Prrxl1* enhancer has been shown to have activities in the maxillary process and brain regions (Martin & Olson, 2000). We observed mCherry signals in the brain regions of the 4OHT-treated, stage 50 tadpoles (Figure 4A and Supplementary Figure S4A). IHC analysis showed Cherry expression in the midbrain region (Figure 4B). Specifically, we found Cherry-positive cells in the ventricular layer, where the ependymal cells, or the neural stem cells, resides. Trails of Cherry-positive cells were commonly found stemming from the ventricular cell layer extending outward, suggesting that our *Prrxl1:CreER* lines labeled ependymal cells and their neuronal descendants. Although mCherry expression in other organs was not obvious in the whole mount image (Supplementary Figure S3A), IHC analysis showed Cherry-positive cells in the head and tail dermis (Figures 4C and 4D) and fin mesenchyme (Figure 4E). We also found few Cherry-positive cells in the heart and spinal cord tissues (Supplementary Figures S5A and S5B), although the exact origin of those cells remains to be determined. Importantly, we did not find any Cherry-positive cells apart from the lens in the untreated controls (data not shown), indicating that the mCherry expression is 4OHT-dependent. It also indicated that the Crystallin promoter only drove mCherry expression in the lens. This data suggests that our

Prrx1:CreER lines could be valuable for future studies on those tissues and that *Cre-ER^{T2}/loxP* system could be applied to label cells other than limb fibroblasts.

Discussion

Using the *Cre-ER^{T2}/loxP* system to trace cells is the most common strategy and has been adopted in many species. We recently reported a transgenic *Xenopus* line harnessing such system (Lin et al., 2021), the details of which we described in this study. Here we discuss critical variables that we identified in making such transgenic lineage tracing system in *Xenopus laevis*.

A significant lower transgenic efficiency was observed when generating *Cre-ER^{T2}* transgenic lines, as only 4.9% of the surviving embryos that had detectable TFP signal when using the *CAGGs:ERCreER* plasmid, compared to 71.8% when using the *CAGGs:TFPnl*s plasmid. A possible explanation is that the pan-expression the *ER^{T2}-Cre-ER^{T2}* protein was adverse to early development so that the surviving embryos must either have low copy number of *ER^{T2}-Cre-ER^{T2}*, or silence the integrated *ER^{T2}-Cre-ER^{T2}* cassettes. Consistent with this hypothesis, we observed a low intensity of TFP signals in *CAGGs:ERCreER* animals compared to the case in *CAGGs:TFPnl*s animals (data not

shown). Alternatively, the low efficiency might also be due to the increased size of the Cre cassettes as we observed a negative correlation of transgenic efficiency and the cassette length in general (Supplementary Figure S1A). The underlying mechanism however remains to be investigated.

Choosing a suitable fluorescent marker to facilitate the screening process was crucial to identify positive embryos. We used TFP because it is 1.58-fold brighter than eGFP (Ai & Campbell, 2008) and were able to detect nucleus TFP signal in the tails of *CAGGs:ERCreER* animals. Of the 8 TFP-positive embryos identified, all of them showed robust 4OHT-induced mCherry expression in the tail. On the other hand, we were not able to unambiguously identify TFP signals in the hindlimb bud in the case of *Prrxl:CreER* experiments. This is likely due to the combination of low transgene expression and the auto-fluorescent nature of the *Xenopus* limb bud tissue in the blue and green channels. The addition of the lens marker helped tremendously in that regard. A caveat for this method was that the lens-mCherry expression did not reflect the *in situ* *Cre-ER^{T2}* expression because they were driven by different promoters. This explained the fact that only the progenies from one of the nine *Prrxl:CreER* founders had robust and specific mCherry expression in the limb bud mesenchyme even though they all have mCherry-positive lens. For generating new transgenic lines in the future, one might consider using different fluorescent proteins that have longer emission wavelength to circumvent this issue.

The presence of ER^{T2} controls the distribution of Cre protein between the cytosol and the nuclei in an equilibrium state, and the recombination of chromosome happens likely when the nucleus presence of Cre protein reaches a certain threshold. Therefore, to achieve stringent but robust cell labeling, it is important to consider the balance between the promoter strength and the number of ER^{T2} used in the system. For example, when pairing the single *ER^{T2}* version of *Cre* (*CreER*) with a strong promoter, such as the *CAG* promoter, we observed mCherry expression without 4OHT treatment (Supplementary Figure S1B). The “leaky” conversion of the *CAGGs:CreER* lines was likely due to the over-the-threshold Cre activity in the nuclei resulting from the high copy number of Cre-ER^{T2} protein. In the case of the double *ER^{T2}* version (*ERCreER*), the equilibrium shifts toward the cytosol and the recombination threshold was not reached due to the lower basal level of Cre protein in the nucleus in the homeostatic state. However, the control of double ER^{T2} might be too “tight” when the *Cre-ER^{T2}* cassette is driven by other promoters. For instance, when pairing the *ER^{T2}-Cre-ER^{T2}* cassette with the *mPrrxl* enhancer, we could only label few cells in the limb (Supplementary Figure S4C). Unfortunately, whether to use the single or double *ER^{T2}* version has to be determined empirically at the moment. Investigating the relationship between the recombination threshold and the copy number of Cre protein would help to reduce the time and effort to establish *Cre-ER^{T2}* transgenic *Xenopus* lines in the future.

Our data shows that the timing of 4OHT administration is crucial to label leg cells. Bathing *CAGGs:ERCreER* tadpoles in 4OHT at stage 49 labeled tail tissues, but not leg tissues (Supplementary Figure S2A). However, the leg cells could be labeled when the animals of the same batch were treated with 4OHT at the froglet stage (Figure 2E). On

the other hand, the 4OHT treatment must be before the onset of limb bud development when using the *mPrrxl* enhancer to achieve robust cell labeling in the limb mesenchyme (Supplementary Figure S3C). Therefore, identifying the optimal 4OHT treatment regime for individual promoters is not trivial. A previous attempt to label leg muscle tissue with a split-*Cre* system only labeled tail muscles but not leg muscles (Rodrigues et al., 2012). In that study, one part of the Cre protein was controlled by a heat shock promoter, and the other part by a cardiac actin promoter. We suspect that the reason why the leg muscle was not labeled was because the heat shock activation was done before the onset of the limb bud development (2-4 weeks after birth).

Our work shows that *Cre-ER^{T2}/loxP*-mediated conditional and tissue-specific cell labeling could be established as stable transgenic *Xenopus* lines. In the future, one could consider using the CRISPR/Cas9 method to knock-in the *Cre-ER^{T2}* cassette to the genomic locus of interests. Although whether a single copy of the transgene would be enough to achieve a robust *Cre-ER^{T2}/loxP*-mediated cell labeling in *Xenopus* remains to be determined, such method has been successfully established in axolotls (Fei et al., 2017). The expression pattern of the *Cre-ER^{T2}* cassette would better reflect the expression pattern of the endogenous gene of interest, without the need of a pre-characterized promoter. This feature would open the door to trace cells based on the expression of literally any genes. To sum up, we hope that the present study would serve as a reference and encourage researchers to establish such *Xenopus* lines that are suitable for lineage tracing.

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AUTHOR CONTRIBUTIONS

TY.L. and S.H. generated transgenic *Xenopus laevis* lines with assistance from P.M. and Y.TS. TY.L. designed and performed the experiments. TY.L. and E.M.T. analyzed data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare that there is no conflict of interests.

Materials and Methods

Animal husbandry

Wildtype *Xenopus laevis* were obtained from European Xenopus Resource Center (EXRC), UK and NASCO, USA, and were maintained in CRTD and IMP animal facilities. Transgenic lines were generated in the CRTD facility and maintained in CRTD and IMP facilities, in which the temperature was between 19-20°C. All animal handling procedures were carried out as described in (Sive et al., 2000) and (Lin et al., 2021) and in accordance with local ethics committee guidelines. Animal experiments were performed as approved by the State Authorities Saxony and the Magistrate of Vienna. To obtain embryos from natural mating, a female and a male of chosen genotype were injected with 500 IU and 50 UI of human chorionic gonadotropin (hCG, Sigma-Aldrich, CG10), respectively. The pair were then put together in 7L 20mM NaCl in room temperature overnight. The embryos were collected for the next two days before the mating pair was separated. To obtain frog eggs for transgenesis, wildtype females were primed with 50 IU of pregnant mare serum gonadotropin (PMSG, ProSpec, HOR-272) one week before the injection of 500 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich, CG10) in the evening before egg collection. Depend on the developmental stages, animals were anesthetized in 0.01%-0.003% benzocaine (Sigma-Aldrich, E1501) before inspection under a stereoscope (Olympus SZX16).

Plasmid construction

The *CAG* promoter were cloned upstream to the *TFPnls-T2A-ER^{T2}-Cre-ER^{T2}* and the *TFPnls-T2A-Cre-ER^{T2}* cassettes in the same fashion as described in (Gerber et al., 2018). The construction of *Prrx1:CreER* and the *CAGGs:lp-Cherry* plasmids was described in (Lin 2021). In brief, the original *TFPnls-T2A-Cre-ER^{T2}* cassettes in the *mPrrx1:TFPnls-T2A-Cre-ER^{T2}* plasmid was replaced with a codon-optimized version (*xen(TFPnls-T2A-Cre-ERT2)*, GeneArt) with restriction enzymes NheI and EcoRI. Fragments of the *Crystallin:mCherry* cassette (γ -*Crystallin* promoter, *mCherry*, *SV40 poly-A* sequences) was assembled using over-extension PCR before being subcloned into the *mPrrx1:xen(TFPnls-T2A-Cre-ER^{T2})* construct using restriction enzymes ApaI and MluI. The new constructs *Crystallin:mCherry;mPrrx1:xen(TFPnls-T2A-Cre-ERT2)* was termed *Prrx1:CreER* in short and used throughout the current study. All plasmids used in transgenesis were prepared with QIAGEN Maxi kit (Qiagen, 12163).

Transgenesis, screening and 4-Hydroxytamoxifan (4OHT) treatment

All transgenic *Xenopus laevis* lines were established based on the REMI protocol (Kroll & Amaya, 1996) and described in (Lin et al., 2021). Specifically, freshly collected wildtype eggs were dejellied using 2% L-cysteine pH7.8 (Sigma-Aldrich, C126) and aligned on a 2% agarose mold filled with 0.4x MMR/5% ficoll (GE Healthcare, 17-0300-50). To prepare the injection solution, 2 μ L purified plasmids (300-600ng in total) were incubated with 4 μ L wildtype sperm nucleus (4x10⁵ nuclei) for 5 minutes in the room temperature before 18 μ L SDB, 2 μ L egg extract, 2 μ L 100mM MgCl₂, and 1 μ L I-SceI enzyme (NEB, R0694L, 1:10 diluted) were added to the mixture. 15 minutes later, 5 μ L of the new mixture were mixed with 150 μ L SDB buffer and back loaded into a

glass needle with a 40-60mm opening. The needle was connected to an infusion pump (World Precision Instruments, SP100i) with a flow rate 0.6nL/second. Around 5-10nL of the plasmid/nuclei mixture were injected into each egg. The injected eggs were kept in 0.1xMMR in 16°C overnight before transferring to fresh 0.1xMMR. The MMR solution was refreshed daily until day 5 when surviving embryos that developed normally were counted.

To screen the *CAGGs:ERCreER* founders, embryos were screened at stage 49 (around 30 days old) using the nucleus TFP signal. The transgenesis efficiency were calculated as the number of fluorescent marker-positive animals over the number of total surviving animals at day 5. TFP-positive animals were bathed in 1μM 4OHT/0.1xMMR or DMSO/0.1xMMR overnight and were screened for mCherry expression 5-7days later. 3 independent *CAGGs:ERCreER* founders were kept until sexual maturity and their TFP-positive offspring were screened in a similar fashion as described above. The *CAGGs:Stop-lp-Cherry* cassette was not excluded when screening under the microscope. The F1 transgenic lines (*Tg(CAGGs:TFPnl-T2A-ER^{T2}-Cre-ER^{T2})*) are termed *CAGGs:ERCreER* lines in the current study.

To identify *Prrxl:CreER* founders, lens mCherry fluorescent signals were screened 7 days after transgenesis. Eleven founders were obtained, kept and mated with the *CAGGs:lp-Cherry* reporter lines. The animals were screened for lens mCherry and trunk GFP fluorescence signals at day 7. Lens-Cherry/trunk-GFP double positive animals were then bathed in 1μM 4OHT overnight at the specific days and for specific times

illustrated in Figure 3B and the Supplementary Figure S3B. mCherry expression in the hindlimb bud were checked at least one week after the last 4OHT treatment, or at the specific time point indicated in Figure 3B and the Supplementary Figure S3C. The F1 transgenic lines (*Tg(Crystallin:mCherry;mPrrx: xen(TFPnls-T2A-Cre-ER^{T2}))*) are termed *Prrx1:CreER* lines in the current study.

Genotyping

Tadpole tail tissues were collected by tail clipping and lysed for 5-10 minutes in 95 degrees in 100μL 50mM NaOH. After cooling down to room temperature, 10μL of 1M Tris pH8 was added to the solution to neutralize the pH. The crude tissue lysates were diluted 10 times before use as the template input of PCR. The primers used in this study were: Stop-lp-Cherry: forward: 5'-TTCGGCTTCTGGCGTGTGAC-3' (on the *CAG* promoter), reverse: 5'-TTATCCTGCATCGACCTAGACTAG-3' (before the second loxP site); CreER: forward: 5'-CAACGAGTGATGAGGTTCGCAAG-3', reverse: 5'-TTGGTCCAGCCACCAGCTTG-3'; GFP: forward: 5'-GCGACGTAAACGGCCACAAG-3', reverse: 5'-TCGTCCATGCCGAGAGTGATC-3'. The PCR products were separated by electrophoresis and visualized in the ChemiDoc MP image system (Bio-rad, 12003154).

In vivo imaging

To check the developmental stages and the fluorescence signals, an anesthetized animal was loosely wrapped with a piece of kimwipe (Kimtech Science, 7551) and was placed

on 100mm dish. The kimwipe was to keep the animal moist and to hold the animal in a certain position so that the tissue region of interest was facing upward. Ring forceps were used to handle the animal and the kimwipe. The dish was then placed under a dissecting microscope setup (Olympus SZX16 with a Zyla 5.5 sCMOS camera and a X-Cite Xylis Broad Spectrum LED Illumination System as the fluorescence light source). Metamorph (MetaMorph Microscopy Automation and Image Analysis Software (RRID:SCR_002368)) was used as the software to capture the images.

Immunohistochemistry and microscopy

Animals were sacrificed before tissue collection. Tissues of interest were dissected under a dissecting microscope (Olympus SZX10) and fixed overnight in 4% MEMFA (1x MEM (41.86g MOPS, 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.52g EGTA, dissolve in H_2O and fill up to 200mL, pH 7.4), 3.7% formaldehyde (Sigma-Aldrich, 1040031000)) on a rotor in a cold room overnight. Samples were washed three times in PBS and incubated with 30% sucrose overnight in the cold room. An additional overnight decalcification step using 8% EDTA/PBS was added after the fixation step when preparing stage 66 leg samples. After the sucrose treatment, samples were washed 3 times in 1xPBS and embedded as OCT blocks ready for cryosection and further analysis. The OCT blocks were sectioned into 8 mm thickness and collected on loading slides with a cryostat (NX70, Thermo Scientific). Cryosections were dried and stored in -20°C . Before immunostaining, slides were thawed and were stained with primary antibodies against GFP (Rockland, 600-401-215, 1:1000, and MPI-CBG, Dresden, 1:1000), mCherry (Acris Antibodies,

AB0040-200, 1:1000), Prrx1 (Gerber et al., 2018, 1:200), MHC (Developmental Studies Hybridoma Bank, A4.1025, 1:200) and Sox2 (Mchedlishvili 2012, 1:2000). Alexa 488, Alexa 568, Alexa 647 (Invitrogen, 1:500) were used as secondary antibodies. All sections were stained with DAPI (Sigma-Aldrich, D9542, 1:1000) and mounted in Mowiol 4-88/DABCO mounting medium (Sigma-Aldrich, 9002-89-5). Images were taken using a Zeiss Axio Imager Z2 (upright) with a sCMOS camera. The ratio between the number of Cherry-positive cells and all the limb bud mesenchymal cells was calculated as the conversion efficiency. At least 3 sections of each sample were counted using the ZEN Blue software. Statistical analysis was done using Prism 9.0.2 (GraphPad Software).

References

- Ai, H., & Campbell, R. (2008). Teal fluorescent proteins: Characterization of a reversibly photoswitchable variant. *Proc. SPIE.*, 6868:68680D. <https://doi.org/https://doi.org/10.1117/12.761423>
- Esteves de Lima, J., Blavet, C., Bonnin, M. A., Hirsinger, E., Comai, G., Yvernogeu, L., . . . Duprez, D. (2021). Unexpected contribution of fibroblasts to muscle

513 lineage as a mechanism for limb muscle patterning. *Nat Commun*, 12(1), 3851.
 514 <https://doi.org/10.1038/s41467-021-24157-x>

515 Fei, J. F., Schuez, M., Knapp, D., Taniguchi, Y., Drechsel, D. N., & Tanaka, E. M.
 516 (2017). Efficient gene knockin in axolotl and its use to test the role of satellite
 517 cells in limb regeneration. *Proc Natl Acad Sci U S A*, 114(47), 12501-12506.
 518 <https://doi.org/10.1073/pnas.1706855114>

519 Feil, R., Wagner, J., Metzger, D., & Chambon, P. (1997). Regulation of Cre
 520 recombinase activity by mutated estrogen receptor ligand-binding domains.
 521 *Biochem Biophys Res Commun*, 237(3), 752-757.
 522 <https://doi.org/10.1006/bbrc.1997.7124>

523 Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., . . .
 524 Treutlein, B. (2018). Single-cell analysis uncovers convergence of cell identities
 525 during axolotl limb regeneration. *Science*, 362(6413).
 526 <https://doi.org/10.1126/science.aag0681>

527 Hans, S., Kaslin, J., Freudenreich, D., & Brand, M. (2009). Temporally-controlled site-
 528 specific recombination in zebrafish. *PLoS One*, 4(2), e4640.
 529 <https://doi.org/10.1371/journal.pone.0004640>

530 Khattak, S., Schuez, M., Richter, T., Knapp, D., Haigo, S. L., Sandoval-Guzman, T., . . .
 531 Tanaka, E. M. (2013). Germline transgenic methods for tracking cells and
 532 testing gene function during regeneration in the axolotl. *Stem Cell Reports*, 1(1),
 533 90-103. <https://doi.org/10.1016/j.stemcr.2013.03.002>

534 Kroll, K. L., & Amaya, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear
 535 transplantations reveal FGF signaling requirements during gastrulation.

536 *Development*, 122(10), 3173-3183.

537 <https://www.ncbi.nlm.nih.gov/pubmed/8898230>

538 Lin, T. Y., Gerber, T., Taniguchi-Sugiura, Y., Murawala, P., Hermann, S., Grosser,
539 L., . . . Tanaka, E. M. (2021). Fibroblast dedifferentiation as a determinant of
540 successful regeneration. *Dev Cell*, 56(10), 1541-1551 e1546.

541 <https://doi.org/10.1016/j.devcel.2021.04.016>

542 Logan, M., Martin, J. F., Nagy, A., Lobe, C., Olson, E. N., & Tabin, C. J. (2002).
543 Expression of Cre Recombinase in the developing mouse limb bud driven by a
544 Prxl enhancer. *Genesis*, 33(2), 77-80. <https://doi.org/10.1002/gene.10092>

545 Martin, J. F., & Olson, E. N. (2000). Identification of a prx1 limb enhancer. *Genesis*,
546 26(4), 225-229. <https://www.ncbi.nlm.nih.gov/pubmed/10748458>

547 [https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/%28SICI%291526-](https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/%28SICI%291526-968X%28200004%2926%3A4%3C225%3A%3AAID-GENE10%3E3.0.CO%3B2-F?download=true)
548 [968X%28200004%2926%3A4%3C225%3A%3AAID-](https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/%28SICI%291526-968X%28200004%2926%3A4%3C225%3A%3AAID-GENE10%3E3.0.CO%3B2-F?download=true)
549 [GENE10%3E3.0.CO%3B2-F?download=true](https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/%28SICI%291526-968X%28200004%2926%3A4%3C225%3A%3AAID-GENE10%3E3.0.CO%3B2-F?download=true)

550 Metzger, D., & Chambon, P. (2001). Site- and time-specific gene targeting in the
551 mouse. *Methods*, 24(1), 71-80. <https://doi.org/10.1006/meth.2001.1159>

552 Nieuwkoop, P. D., & Faber, J. (1956). *Normal table of Xenopus laevis (Daudin); a*
553 *systematical and chronological survey of the development from the fertilized egg*
554 *till the end of metamorphosis*. North-Holland Pub. Co.

555 Rodrigues, A. M., Christen, B., Marti, M., & Izpisua Belmonte, J. C. (2012). Skeletal
556 muscle regeneration in Xenopus tadpoles and zebrafish larvae. *BMC Dev Biol*,
557 12, 9. <https://doi.org/10.1186/1471-213X-12-9>

558 Sive, H. L., Grainger, R. M., & Harland, R. M. (2000). *Early Development of Xenopus*
 559 *laevis: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

560 Suzuki, M., Satoh, A., Ide, H., & Tamura, K. (2007). Transgenic Xenopus with prx1
 561 limb enhancer reveals crucial contribution of MEK/ERK and PI3K/AKT
 562 pathways in blastema formation during limb regeneration. *Dev Biol*, 304(2),
 563 675-686. <https://doi.org/10.1016/j.ydbio.2007.01.019>

564 Werdien, D., Peiler, G., & Ryffel, G. U. (2001). FLP and Cre recombinase function in
 565 Xenopus embryos. *Nucleic Acids Res*, 29(11), E53-53.
 566 <https://doi.org/10.1093/nar/29.11.e53>

567 Yaseen, W., Kraft-Sheleg, O., Zaffryar-Eilot, S., Melamed, S., Sun, C., Millay, D. P., &
 568 Hasson, P. (2021). Fibroblast fusion to the muscle fiber regulates myotendinous
 569 junction formation. *Nat Commun*, 12(1), 3852. [https://doi.org/10.1038/s41467-](https://doi.org/10.1038/s41467-021-24159-9)
 570 [021-24159-9](https://doi.org/10.1038/s41467-021-24159-9)

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 572

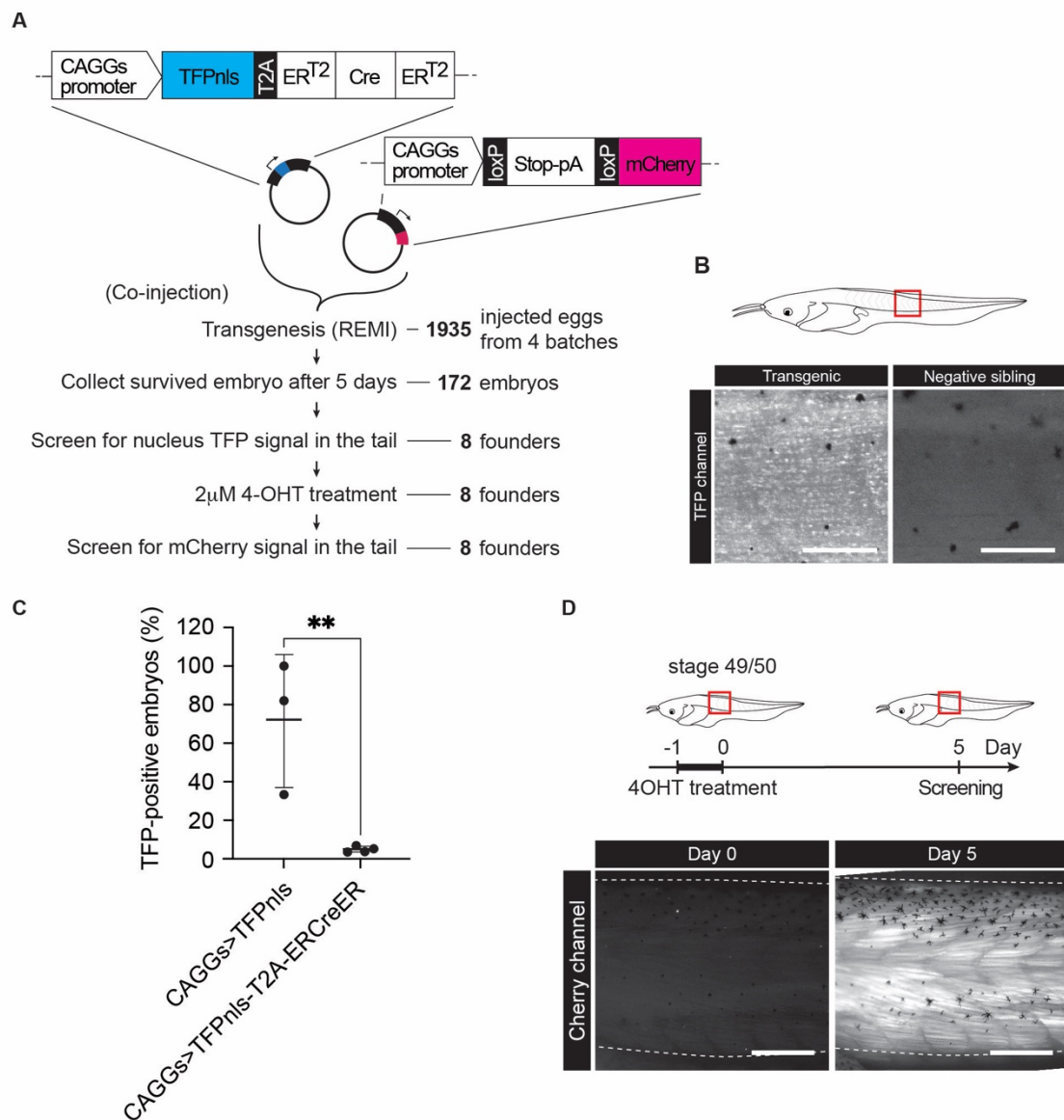


Figure 1. Conditional labeling of tail tissues in F0 *Xenopus laevis* with an ERT^2 -Cre- ERT^2 /loxP system

(A) Overview of the transgenesis workflow. The schematic illustration of the plasmids containing the *CAGGs:ERCreER* and the *CAGGs:lp-Cherry* cassettes are indicated at the top. A summary of the result is indicated at the right side of each step.

(B) Expression of nucleus TFP in the tail region of a transgenic F0 tadpole. Top: schematic illustration of a stage 49 tadpole. The red box indicates the field of view of the bottom row images. Bottom row: whole mount fluorescence images showing TFP signal in a transgenic positive tadpole (left) and a transgenic negative, stage-comparable sibling (right). The white boxes indicate the field of view of the bottom row images. Scale bars represent 200µm.

(C) Scatter plot showing the transgenic efficiencies. Each dot represents an independent REMI experiment, and the lines represent the average efficiency of two different constructs, *CAGGs:TFPnls* (71.8%, n=3) and *CAGGs:ERCreER* (4.9%, n=4). The efficiency was defined by the number of fluorescence-positive tadpoles over the number of total surviving tadpoles. Statistical analysis was determined by one-way ANOVA and the Šidák's multiple comparison test. **: $p < 0.01$. Data are represented as mean $\pm$ SD.

(D) Conditional mCherry expression in the tail region of a transgenic F0 tadpole. Top: a schematic illustration of the 4OHT treatment (black bar) and screening regime. The red boxes indicate the field of view of the middle and the bottom row images. Middle row: whole mount fluorescent images of a TFP-positive tadpole before (left, Day 0) and after (right, Day 5) an overnight 4OHT treatment. mCherry signal in the tail region was detected at Day 5. The white dashed lines outline the tail. Scale bars represent 500µm.

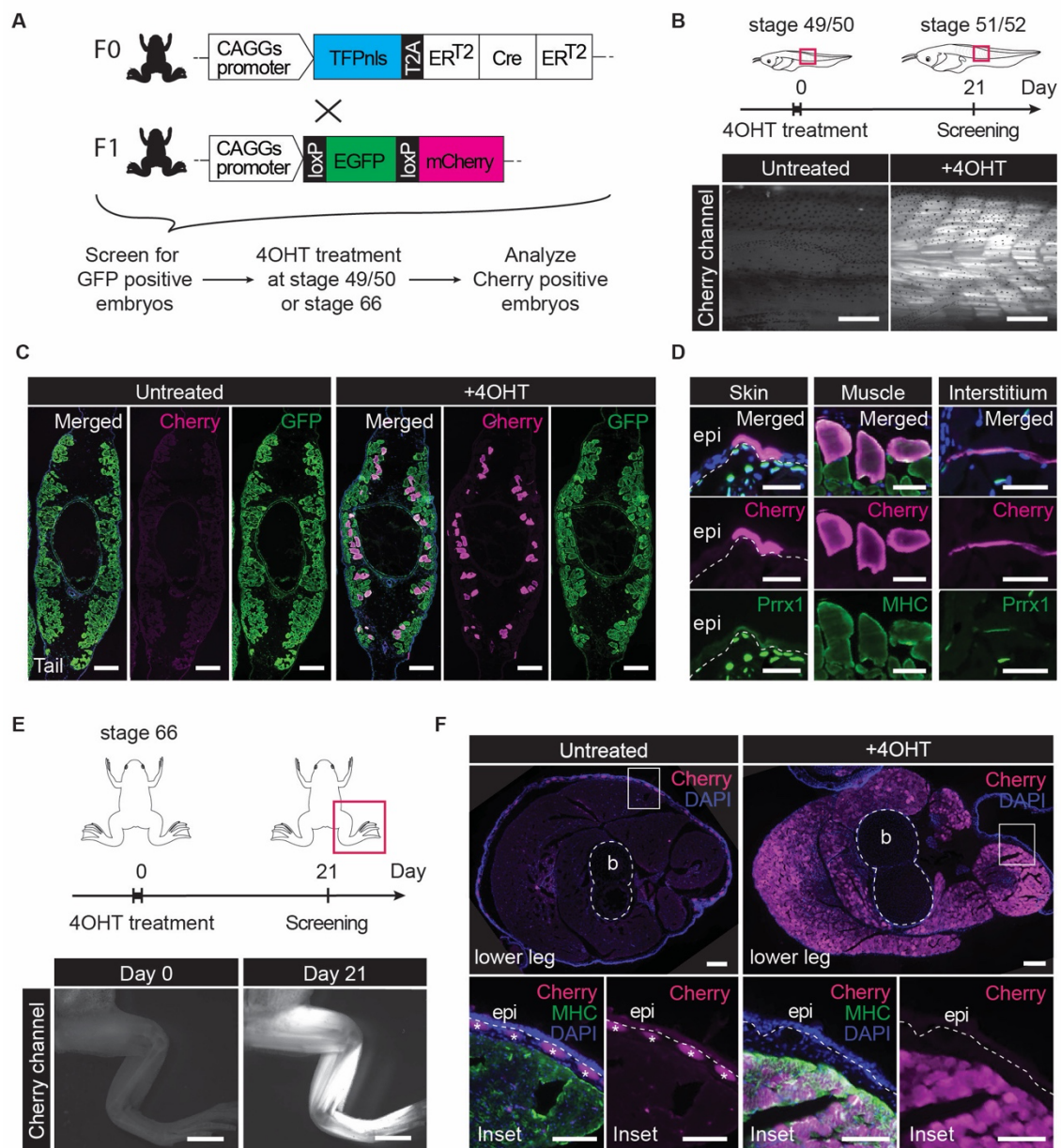


Figure 2. Conditional labeling in stable transgenic *Xenopus laevis* lines

(A) Overview of the screening procedure of the F1 *CAGGS:ERCreER* tadpoles. The schema on the top indicates the parental genotypes.

(B) Conditional mCherry expression in the tail region of a F1 tadpole. Top: schematic illustration of the 4OHT treatment and screening regime. The red

boxes indicate the field of view of the bottom row images. Bottom row: whole mount fluorescent images of a tadpole without (left, Untreated) and with (right, +4OHT) an overnight 4OHT treatment. mCherry signal in the tail region was screened at Day 21. Scale bars represent 500 μ m.

(C) Immunohistochemistry analysis of the conditional mCherry expression in F1 tadpoles. Cross-sections of an untreated (left, Untreated) and a 4OHT-converted (right, +4OHT) F1 tadpole tails were stained with anti-Cherry (Cherry, in magenta), anti-GFP (GFP, in green) antibodies. Nuclei were stained with DAPI (in blue) and showed in the merged image (Merged). Cherry signals were found only after the 4OHT treatment. Scale bars represent 100 μ m.

(D) Immunohistochemistry analysis of the Cherry expression in different tail tissues after 4OHT treatment. Skin tissue (left column, Skin): Cherry (in magenta)-positive and Prrx1 (in green)-negative cells were found in the epidermis. The white dashed line indicates the boundary between the dermis the epidermis (epi). Muscle tissue (middle column, Muscle): Cherry (in magenta)- and MHC (in green)-double positive muscle bundles were found in the muscle region. Interstitial tissue (right column, Interstitium): Cherry (in magenta)- and Prrx1 (in green)-double positive cells were found in the interstitial region. Nuclei were stained with DAPI (in blue) and showed in the merged images (Merged). Scale bars represent 50 μ m.

(E) Conditional mCherry expression in the leg of a stage 66 F1 froglet. Top: schematic illustration of the 4OHT treatment (black bar) and screening regime. The red boxes indicate the field of view of the bottom row images. Bottom row: whole mount fluorescent images of a TFP-positive tadpole before (left, Day 0)

and after (right, Day 21) an overnight 4OHT treatment. Scale bars represent 2mm.

(F) Immunohistochemistry analysis of the conditional mCherry expression in the legs of F1 froglets. Top row: cross-sections of the lower leg region of an untreated (left, Untreated) and a stage-comparable 4OHT-converted (right, +4OHT) F1 froglet were stained with anti-Cherry (Cherry, in magenta) antibody and DAPI (in blue). The white dashed circles indicate the bone region (b). The white boxes indicate the field of view of the bottom row images. Bottom row: the inset images of the top row. Anti-MHC staining (in green) was added to indicate the muscle bundles. Cherry signals were found in the muscle bundles after the 4OHT treatment. The white dashed lines indicate the boundary between dermis and epidermis (epi). White asterisks indicate the autofluorescence signals from gland cells. Scale bars represent 200 μ m (top row) and 100 μ m (bottom row).

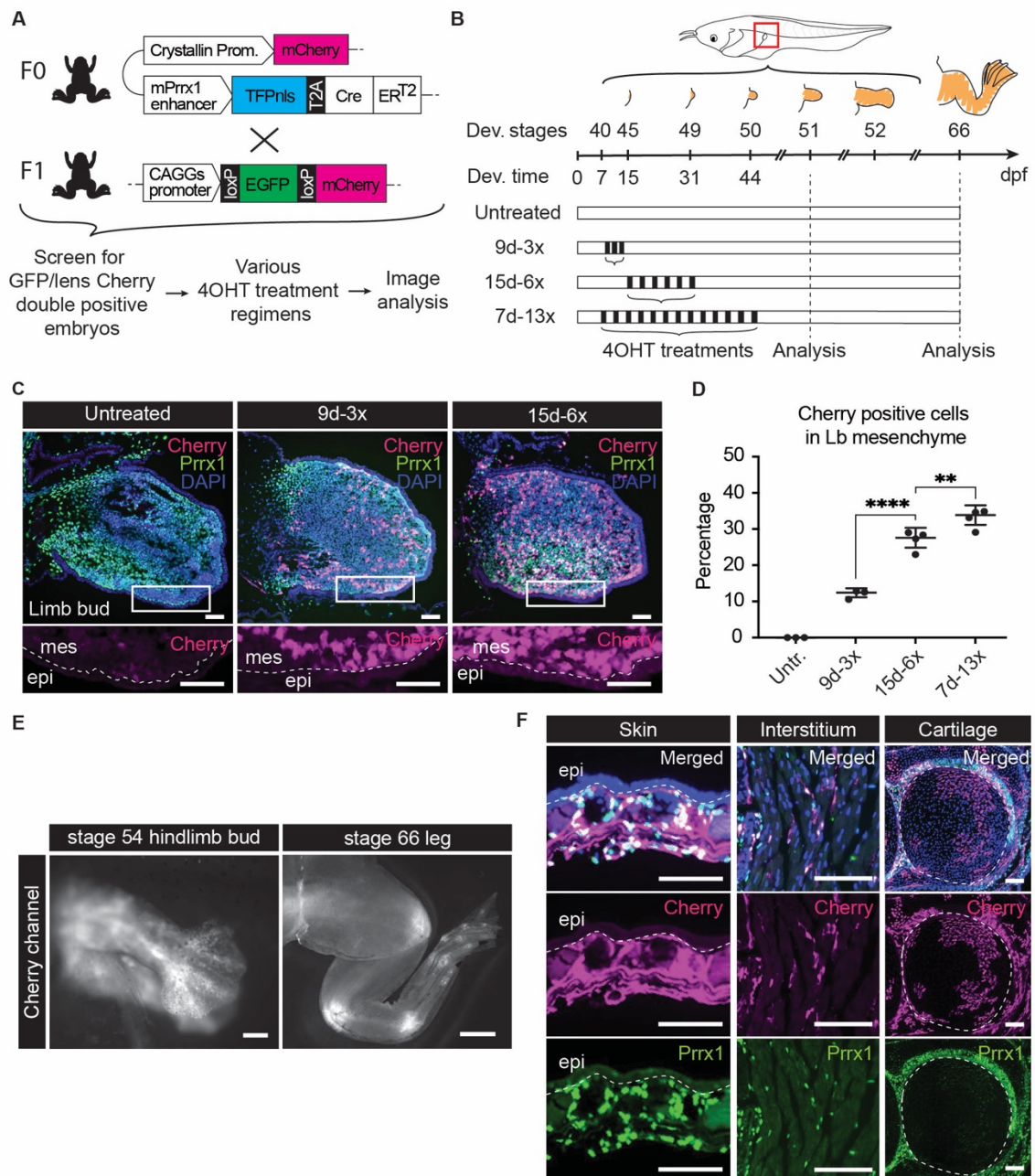


Figure 3. Conditional labeling of limb connective tissue in *Prrx1:CreER* frog lines

(A) Overview of the screening procedure of the F1 *Prrx1:CreER* tadpoles. The schema on the top indicates the parental genotypes.

(B) Overview of four 4OHT treatment regimens. The developmental stages and timing (days post-fertilization, dpf) are indicated with the corresponding limb bud schema on the top. The developing limb is shaded in yellow. The red box indicates the field of view in Figure 3C. Four regimens of 4OHT treatments are indicated as Untreated, 9d-3x (3 times starting from 9dpf), 15d-6x (6 times starting from 15dpf), 7d-13x (13 times starting from 7dpf). The black boxes indicate each overnight 4OHT treatment. Animals were analyzed at stage 51 and stage 66.

(C) Immunohistochemistry analysis of the conditional Cherry expression in limb buds under different 4OHT regimens. Top row: Longitudinal sections of stage 51 limb buds from three 4OHT regimens, Untreated (left column), 9d-3x (middle column), and 15d-6x (right column), were stained with anti-Cherry (in magenta), anti-Prx1 (in green) antibodies and DAPI (in blue). The white boxes indicate the field of view in the bottom row images. Bottom row: the inset images of the top row with the Cherry channel alone. The white dashed line indicates the boundary between mesenchyme (mes) and epidermis (epi). Note that the Cherry signal is only found in the mesenchyme. Scale bars represent 50µm.

(D) Scatter plot showing the conversion efficiencies of different 4OHT regimens. The efficiencies were determined by the percentage of Cherry-positive cells in the limb bud mesenchyme. Each dot represents an independent sample, and the line represents the average efficiency of four different regimens, Untreated (Untr., 0%, n=3), 9d-3x (12.1%, n=3), 15d-6x (26.9%, n=4), and 7d-13x (32.9%, n=4). Statistical analysis was determined by one-way ANOVA and the Šidák's

multiple comparison test. **: $p < 0.01$. ****: $p < 0.0001$. Data are represented as mean $\pm$ SD.

(E) Whole mount fluorescence images of a stage 54 limb bud (left) and a stage 66 leg (right). Scale bars represent 500 μ m (left) and 5mm (right).

(F) Immunohistochemistry analysis of the Cherry expression in different leg tissues after 4OHT treatment. Skin tissue (left column, Skin): Cherry (in magenta)- and Prrx1 (in green)-double positive cells were found in the dermis. The white dashed line indicates the boundary between the dermis the epidermis (epi). Interstitial tissue (middle column, Interstitium): Cherry (in magenta)- and Prrx1 (in green)-double positive cells were found in the interstitial region. Cartilage tissue (right column, Cartilage): patches of Cherry (in magenta)-positive cells were found in the cartilage region, which was identified by its morphology and circled by the white dashed line. Cherry (in magenta)- and Prrx1 (in green)-double positive perichondrium cells were also found at the boundary of cartilage. Nuclei were stained with DAPI (in blue) and showed in the merged images on the top row (Merged). Scale bars represent 100 μ m.

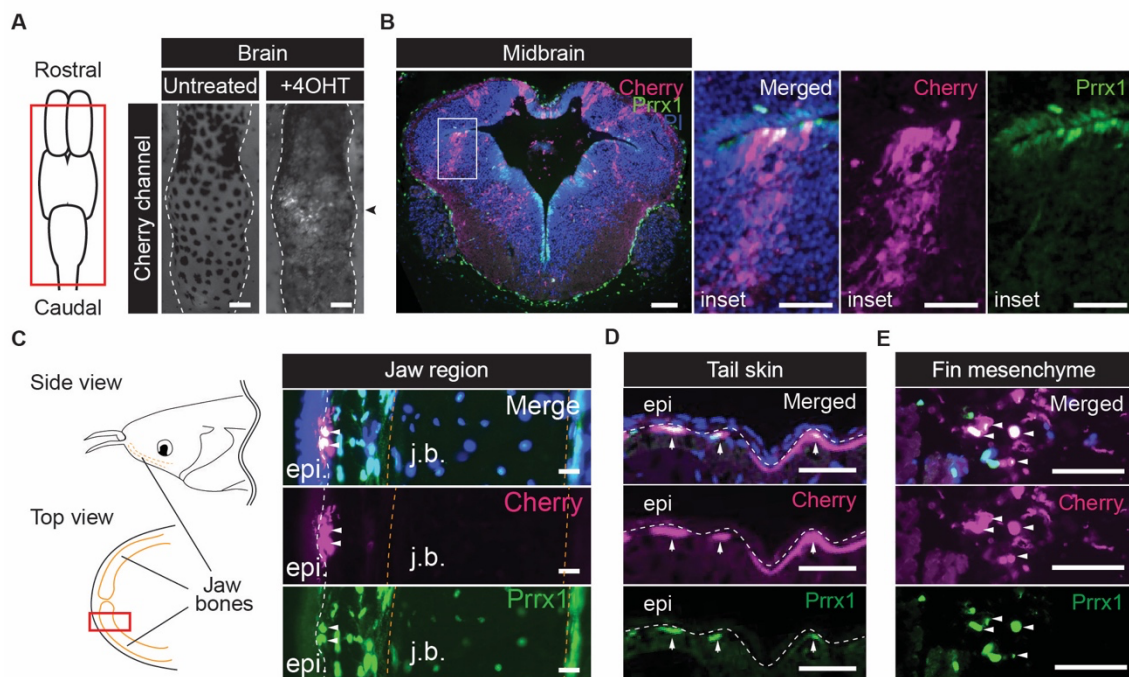


Figure 4. Conditional labeling of cells in other organs in *Prrx1:CreER* frog lines

- (A) Conditional mCherry expression in the brain of a stage 49 tadpole. Left: schematic illustration of the overall brain structure. Top is the rostral side and bottom is the caudal side. The red box indicates the field of view of the right panel. Right: whole mount fluorescence images of the brain region of tadpoles without (Untreated) and with (+4OHT) 4OHT treatment. White dashed lines outline the brain. Black arrowhead indicates the level of section plane in (B). Scale bars represent 200µm.
- (B) Immunohistochemistry analysis of the Cherry expression in a tadpole midbrain region after 4OHT treatment. Left: Cross-section of the midbrain was stained with anti-Cherry (in magenta), anti-Prrx1 (in green) antibodies, and DAPI (in

708 blue). White box indicates the field of view of the inset images on the right.

709 Scale bars represent 100µm (left) and 50µm (inset).

710 (C) Immunohistochemistry analysis of the Cherry expression in the head dermis of a

711 tadpole after 4OHT treatment. Left: schematic illustrations of jaw region

712 viewing from the side (top panel) and top (bottom panel) of a tadpole. Jawbones

713 are outlined by solid or dashed orange lines. The red box indicates the field of

714 view in the bottom images. Bottom: a longitudinal section of the jaw region was

715 stained with anti-Cherry (in magenta), anti-Prrx1 (in green) antibodies, and

716 DAPI (in blue). Cherry-positive cells were found in the dermis, expressing Prrx1

717 (white arrowheads). No Cherry-positive cells were found in the jawbone (j.b.).

718 The white dashed line indicates the boundary between dermis and epidermis

719 (epi). Orange dashed lines outline the jawbone. Scale bars represent 20µm.

720 (D) Immunohistochemistry analysis of the Cherry expression in the tail dermis after

721 4OHT treatment. Cross-sections of the tail were stained with anti-Cherry (in

722 magenta), anti-Prrx1 (in green) antibodies, and DAPI (in blue). White dashed

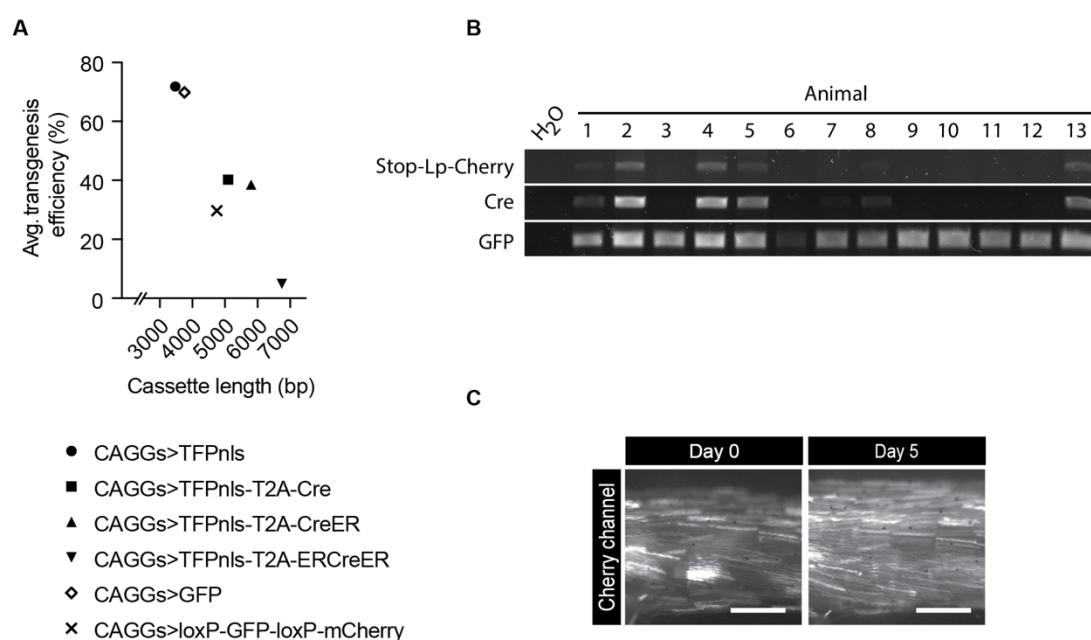
723 line indicates the boundary between dermis and epidermis (epi). White

724 arrowheads indicate the Prrx1/Cherry-double positive tail dermal cells. Scale

725 bars represent 50µm.

726 (E) Same analysis as (F) but in the fin mesenchyme region. Scale bars represent

727 50µm.



Supplementary Figure S1. Transgenic efficiency between different constructs

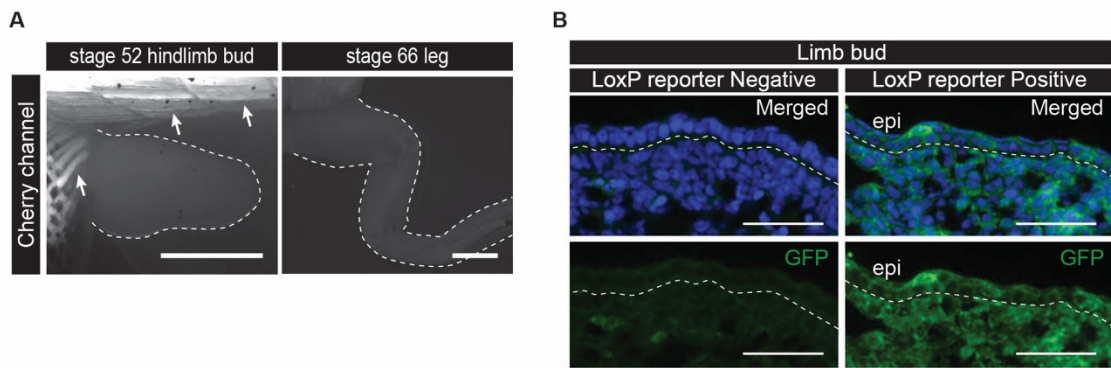
(A) Scatter plot showing the transgenesis efficiencies of different transgenic lines.

Each data point represents the average percentage of fluorescence-positive tadpoles using different DNA constructs indicated on the right. The x-axis represents the length of the DNA cassette of each construct in base pair.

(B) Genotyping results of 13 GFP-positive F1 *CAGGs:ERCreER/CAGGs:lp-Cherry* animals. The names of amplicon regions were on the left (*Stop-lp-Cherry*, *CreER* and *GFP*). Water was used instead of the genomic DNA in the PCR reaction of first lane (H₂O). Note that all the *CreER*-positive animals were also *Stop-lp-Cherry* positive.

(C) Whole mount fluorescence images showing the Cherry expression in the tail region before (left, Day 0) and after (right, Day 5) an overnight 4OHT treatment.

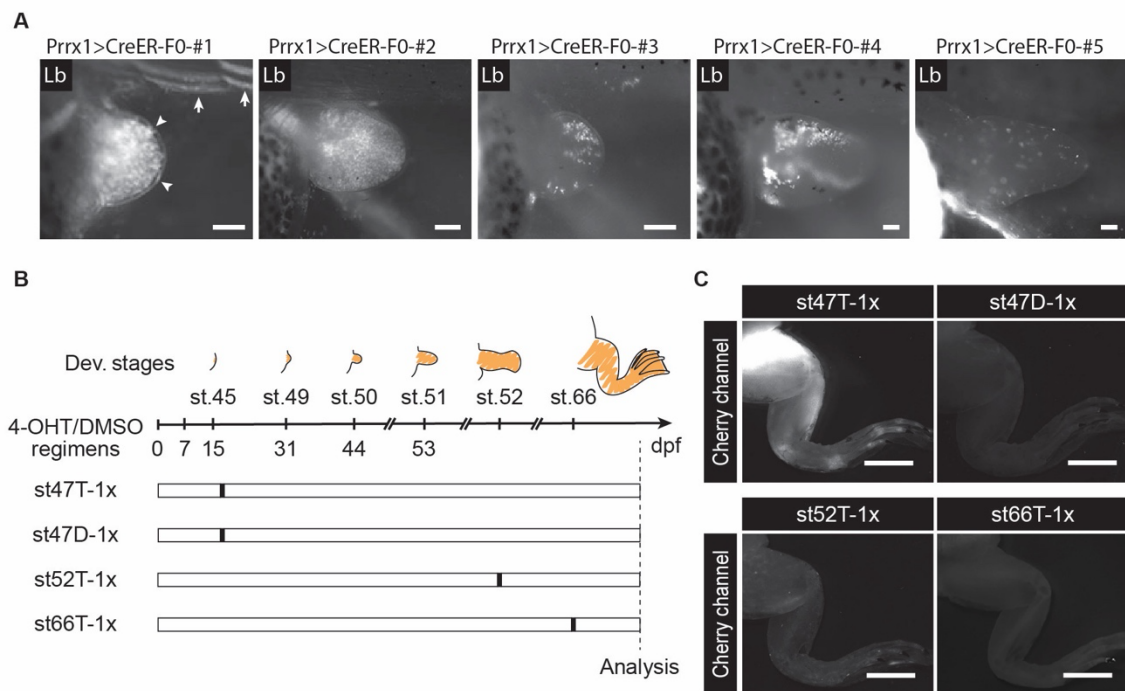
742 Note that Cherry signal was already detectable before 4OHT treatment. Scale
743 bars represent 500 μ m.
744



Supplementary Figure S2. Leg cells were not labeled in *CAGGs:ERCreER*; *CAGGs:lp-Cherry* F1 animals when the 4OHT treatment was at stage 49/50.

(A) Whole mount fluorescence images showing the Cherry signal in the leg region of a stage 52 limb bud (left) and a stage 66 leg (right). Cherry signal was detected in the tail and belly region indicated by white arrows in the left panel, but not in the stage 52 limb bud or the stage 66 leg outlined by white dashed lines in both panels. Scale bars represent 500µm (left) and 2mm (right).

(B) Immunostaining of limb bud longitudinal-sections showing the GFP (in green) expression in a LoxP reporter-negative (left) and positive (right) animals in the stage 51 limb bud region. Cell nuclei were stained with DAPI (in blue) and showed in the top row images (Merged). White dashed lines indicate the border between tail mesenchyme and epidermis (epi). Note that GFP was expressed in both mesenchyme and epidermis. Scale bars represent 50µm.



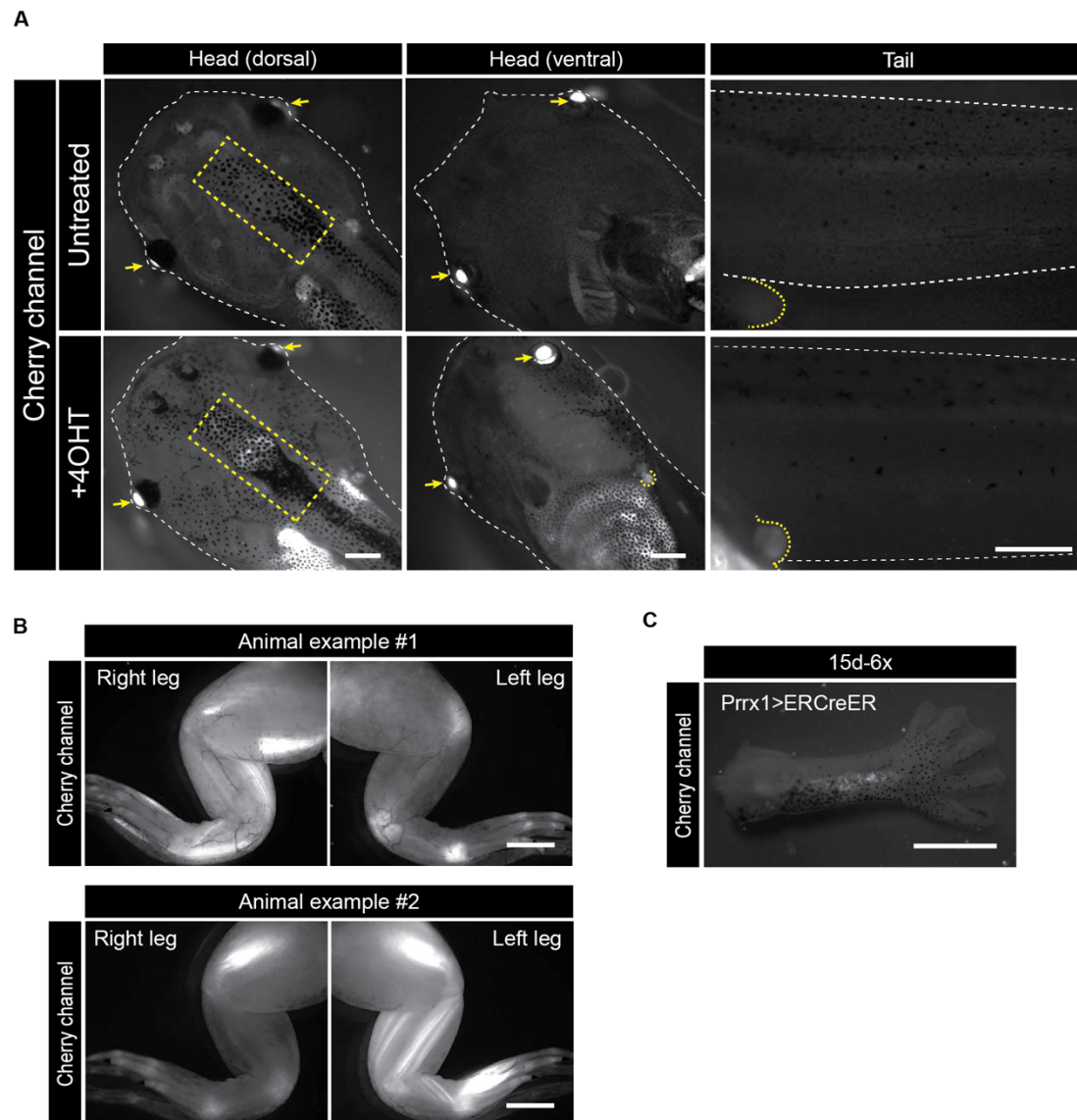
Supplementary Figure S3. The initial optimization of 4OHT treatment strategies and the overall conversion pattern of the *Prrx1:CreER*; *CAGGs:lp-Cherry* F1 tadpoles.

(A) Representative whole mount fluorescence images showing Cherry signal in the limb bud region of the offspring from five different *Prrx1:CreER* founders. Five *Prrx1:CreER* founders were mated with *CAGGs:lp-Cherry* reporter lines. The lens-Cherry/trunk-GFP double positive embryos were treated with 4OHT overnight at stage 49, and the induced Cherry signal in the limb bud region (Lb) was imaged 2-3 weeks later. The first founder (*Prrx1:CreER-F0-#1*) has unspecific expression in the limb epidermis (white arrowheads) and in the trunk muscles (white arrows). The second founder (*Prrx1:CreER-F0-#2*) had robust

and specific Cherry signal in the limb bud mesenchyme. The other three founders labeled fewer limb bud mesenchymal cells. Scale bars represent 50µm.

(B) Overview of four single-4OHT treatment regimens for the offspring of the *Prrx1:CreER-F0-#2* founder. The developmental stages and timing (days post-fertilization, dpf) are indicated with the corresponding limb bud schema on the top. Siblings were treated with 4OHT overnight at stage 47 (st47T-1x), at stage 52 (st52T-1x), or at stage 66 (st66T-1x). A group of siblings were treated with DMSO overnight at stage 47 (st47D-1x) as a control. The black boxes indicate the timing of overnight 4OHT treatment. Animals were analyzed three weeks after the treatment of st66T-1x group.

(C) Whole mount fluorescence images showing the Cherry expression in stage 66 legs of the four different treatment regimens: st47T-1x (top-left), st47D-1x (top-right), st52T-1x (bottom-left), st66T-1x (bottom-right). Scale bars represent 5mm.



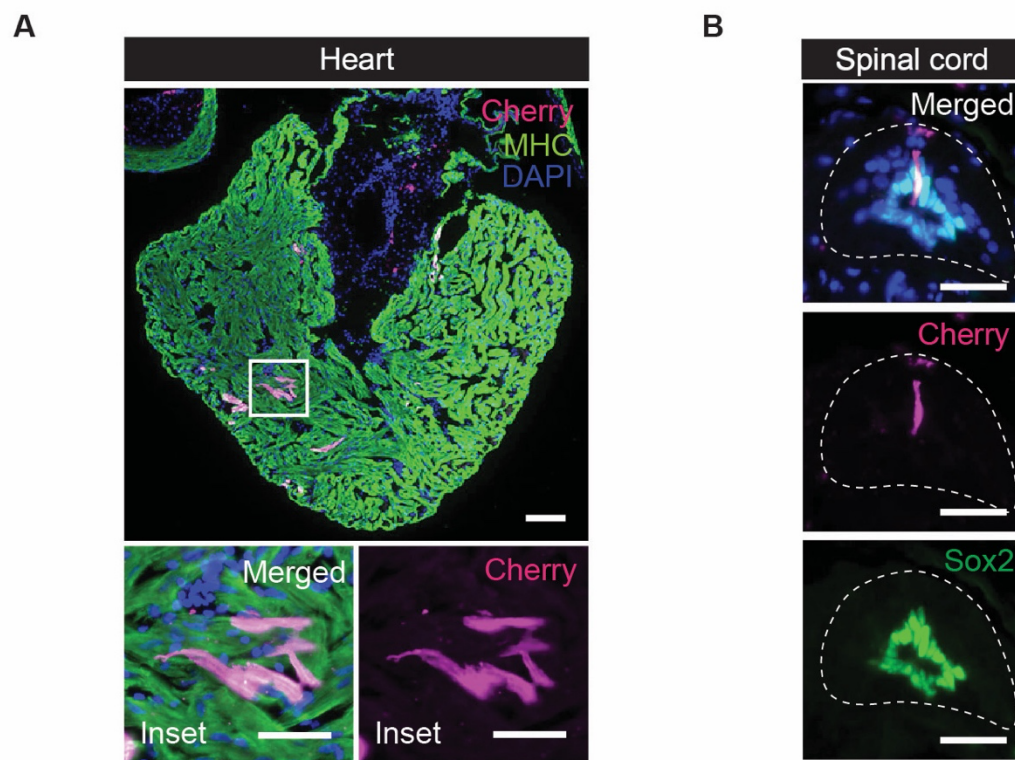
Supplementary Figure S4. Sporadic mCherry expression in leg muscle tissues of *Prrx1:CreER* lines and the conversion pattern of the *Prrx1:ERCreER* lines.

(A) Whole mount fluorescence images showing the Cherry signal of a stage 50 *Prrx1:CreER-F0-#2* tadpole with the 15d-6x 4OHT treatment regime. Top row: an example of the untreated tadpoles (Untreated). Bottom row: an example of

the 4OHT treated tadpole (+4OHT). Left: dorsal view of the head region outlined by white dashed lines. The yellow dashed box shows the field of view in Figure 4A. Middle: ventral view of the head region. The yellow dashed line outlines the forelimb bud. Right: side view of the tail region. The yellow dashed line outlines the hindlimb bud. White dashed lines in all three panels outline the animal. Yellow arrows indicate the Cherry-positive lens. Note that the lens was Cherry-positive regardless of the 4OHT treatment, but the brain and the limb bud expressed Cherry only after 4OHT treatment. Scale bars represent 1mm.

(B) Whole mount fluorescence images showing the differential Cherry expression pattern in the stage 66 legs of the 15d-6x treatment regimen. Leg muscle tissue in some animals was labeled in either right leg (top row) or left leg (bottom row) without a consistent Cherry expression pattern. Scale bars represent 5mm.

(C) A whole mount fluorescence image showing the Cherry signal in a stage 55 *Prrx1:ERCreER; CAGGs:lp-Cherry* animal with the 15d-6x treatment regime. Scale bars represent 1mm.



Supplementary Figure S5. Labeling of heart muscle and spinal cord cells in *Prrx1:CreER* frog lines

- (A) Immunohistochemistry analysis of the Cherry expression in a tadpole heart after 4OHT treatment. A longitudinal section of the heart was stained with anti-Cherry (in magenta), anti-MHC (in green) antibodies, and DAPI (in blue). Cherry- and MHC-double positive cells were found. The white box indicates the field of view of the insets at the bottom row. Scale bars represent 100mm (top) and 50mm (insets).
- (B) Immunohistochemistry analysis of the Cherry expression in a tadpole spinal cord after 4OHT treatment. Cross-sections of spinal cord were stained with anti-Cherry (in magenta), anti-Sox2 (neural progenitor marker, in green) antibodies,

827 and DAPI (in blue). Cherry- and Sox2-double positive cells were found. Scale
828 bars represent 50µm.

II.2 Fibroblast Dedifferentiation as a Determinant of Successful Regeneration

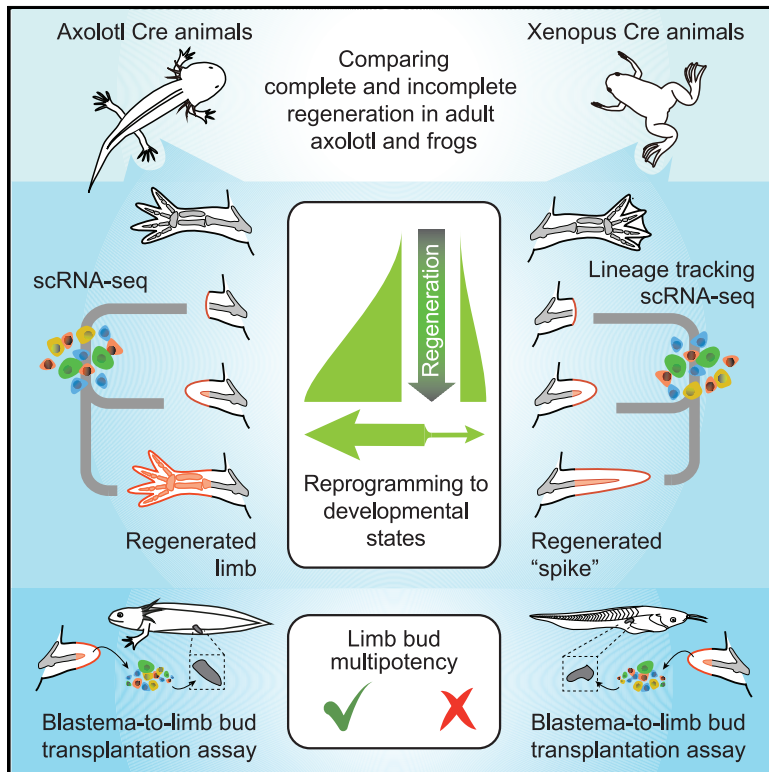
Unlike Axolotls, African clawed frogs (*X. laevis*) do not undergo complete regeneration upon limb amputation, rather they respond by the formation of a “spike” structure which is devoid of digits and proper patterning. In this publication, we investigated the underlying mechanisms that lead to this incomplete limb regeneration with a focus on the limb connective tissue (CT) cells. We found that, although CT-derived cells were the main constituent of a frog limb blastema, their molecular and cellular properties were different from the axolotl counterparts. Using single cell RNA-seq (scRNA-seq) analysis, we showed that the dermal and interstitial CT cell subtypes remained different during the regeneration of frogs, while different axolotl CT cell subtypes converged to a single progenitor population. Quadratic programming analysis on the scRNA-seq datasets revealed that, while axolotl CT cells dedifferentiated to limb bud-like transcriptomic states, frog blastema CT cells only underwent partial dedifferentiation. These results correlated with the fact that frog blastema cells did not acquire the potency to integrate into the cartilages of developing limbs as revealed in transplantation assays. We further showed that the chondrogenesis programs used in the frog limb regeneration and the embryonic development are molecularly distinct. Overall, this study strongly suggests that frog limb CT cells possess cell intrinsic limitations to become fully dedifferentiated and that identifying and resolving these intrinsic barriers should be the direction for future studies.

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Contributions:	<u>TY.L.</u> and S.H. generated transgenic <i>Xenopus laevis</i> lines with assistance from P.M. <u>TY.L.</u> and L.G. performed cell transplantation experiments. <u>TY.L.</u> , S.H. and Y.TS. performed immunohistochemistry. <u>TY.L.</u> and T.G. performed single-cell experiments. T.G. analyzed scRNA-seq data. <u>TY.L.</u> , T.G., Y.TS., B.T., and E.M.T. designed the study, analyzed data and wrote the manuscript.

Developmental Cell

Fibroblast dedifferentiation as a determinant of successful regeneration

Graphical abstract



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In brief

Lin et al. systematically compared the response to limb amputation in axolotls and African clawed frogs. Unlike in axolotls, frog limb fibroblasts are incapable of fully re-activating developmental programs required for complete limb regeneration. This work defines intrinsic restrictions in fibroblasts as a limitation to adult regeneration.

Highlights

- Fibroblast-derived $Prrx1^+$ cells are the main constituent of a frog limb blastema
- Frog fibroblasts only undergo partial dedifferentiation due to intrinsic limitations
- Adult chondrogenesis is distinct from the embryonic program



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Article

Fibroblast dedifferentiation as a determinant of successful regeneration

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SUMMARY

Limb regeneration, while observed lifelong in salamanders, is restricted in post-metamorphic *Xenopus laevis* frogs. Whether this loss is due to systemic factors or an intrinsic incapability of cells to form competent stem cells has been unclear. Here, we use genetic fate mapping to establish that connective tissue (CT) cells form the post-metamorphic frog blastema, as in the case of axolotls. Using heterochronic transplantation into the limb bud and single-cell transcriptomic profiling, we show that axolotl CT cells dedifferentiate and integrate to form lineages, including cartilage. In contrast, frog blastema CT cells do not fully re-express the limb bud progenitor program, even when transplanted into the limb bud. Correspondingly, transplanted cells contribute to extraskeletal CT, but not to the developing cartilage. Furthermore, using single-cell RNA-seq analysis we find that embryonic and adult frog cartilage differentiation programs are molecularly distinct. This work defines intrinsic restrictions in CT dedifferentiation as a limitation in adult regeneration.

INTRODUCTION

Limb regeneration capability varies among tetrapods, and the underlying causes for this diversity are unclear. Among amphibians, salamanders such as the axolotl (*Ambystoma mexicanum*) regenerate limbs as sexually mature adults, whereas frogs such as *Xenopus laevis*, studied here, lose regeneration capability during metamorphosis (Simon and Tanaka, 2013). In axolotls, limb amputation triggers the formation of a blastema, an accumulation of mesenchymal progenitors covered by an epithelium that rebuilds the missing limb, including fully patterned skeletal elements. Interestingly, the amputation of a post-metamorphic frog (stage 66) limb yields a proliferative response and the formation of a blastema that abortively forms a spike of unsegmented cartilage (Simon and Tanaka, 2013) (Figure 1A). Correspondingly, at the blastema stage, collagen and limb development factors are present in frog but show some divergent patterns to the axolotl blastema (Ohgo et al., 2010). Here, an important question is whether this abortive phenotype is mainly due to a deficient adult environment or an intrinsic incapability of blastema cells themselves (Sessions and Bryant, 1988). Multiple studies have attempted to enhance frog regeneration by supplementing the adult environment with additional cell types or extracellular factors (Herrera-Rincon et al., 2018; Lin et al., 2013; Mitogawa et al.,

2018; Satoh et al., 2005; Yakushiji et al., 2009; Zhang et al., 2018). Other studies have stressed the potential negative impact of developing cartilage (Aztekin et al., 2020) or an adult immune system (Harty et al., 2003; King et al., 2012; Mescher et al., 2013). Correlative evidence shows a higher methylation state of a *shh* gene enhancer in adult frog cells compared with embryonic cells (Yakushiji et al., 2007). However, it has been challenging to disentangle the complex cohort of cells, tissues, and systemic environment to ascertain the defined limitations to regeneration.

One hindrance has been a lack of clarity of whether the same cell types build the axolotl and frog blastema and execute regeneration. During axolotl limb regeneration, descendants of the lateral plate mesoderm, collectively called as the connective tissue (CT), are major contributors to the early- and mid-bud limb blastema (Gerber et al., 2018; Kragl et al., 2009; Muneoka et al., 1986). The mature limb CT consists of multiple subtypes including dermal fibroblasts, interstitial fibroblasts, cartilage, bone, periosteum/perichondrium, ligaments, and tendons (Gerber et al., 2018; McCusker et al., 2015). Recently, genetic fate mapping combined with single-cell RNA sequencing (scRNA-seq) of CT cells in axolotls showed that dermal and interstitial CT fibroblasts are the major contributors to the blastema (Gerber et al., 2018). These fibroblasts dedifferentiate toward common, multipotent CT progenitors that rebuild the patterned



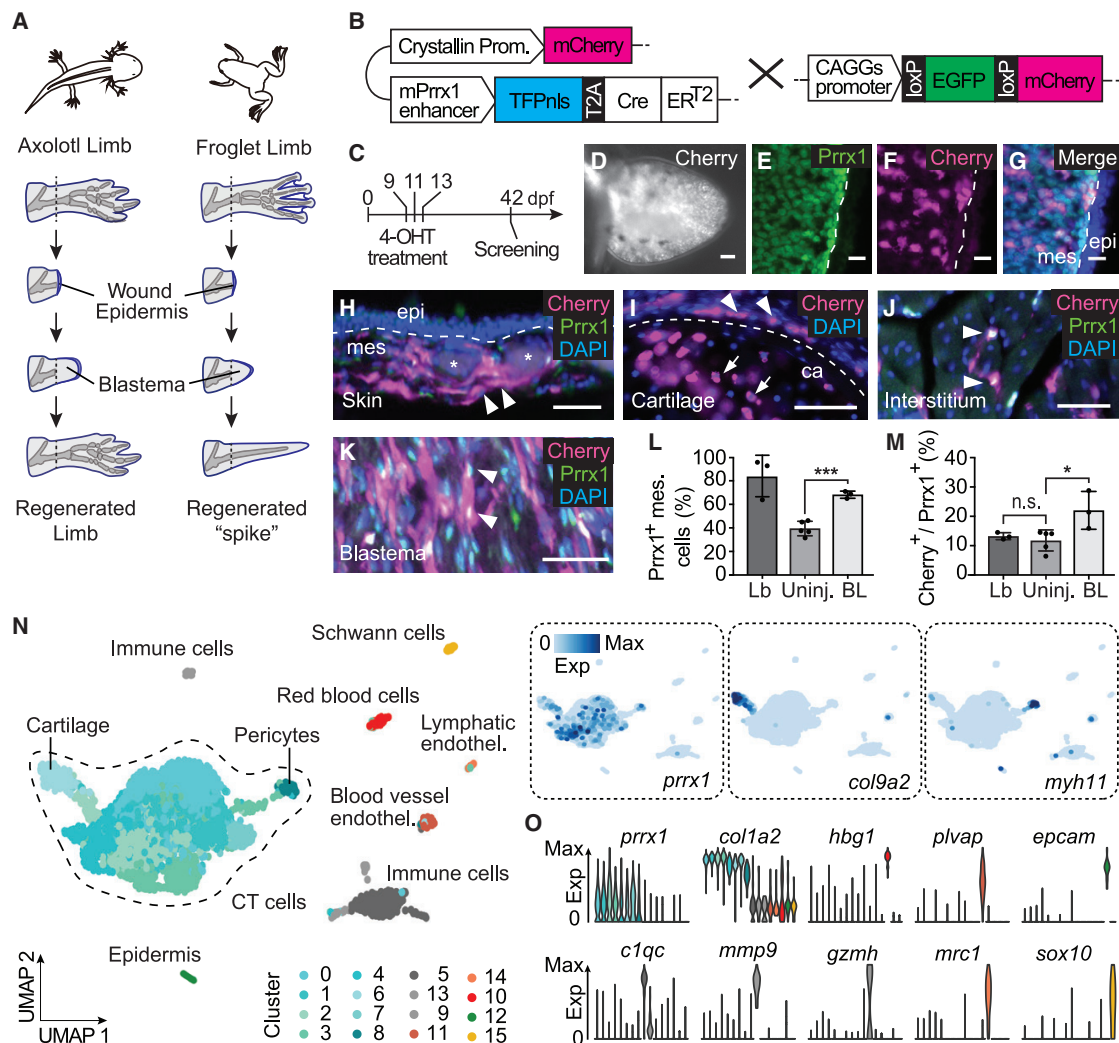


Figure 1. Connective tissue cells are source cells of the adult frog blastema

(A) Schematic comparison of limb regeneration after amputation in axolotls versus post-metamorphic frogs. Axolotls regenerate a complete limb while post-metamorphic frogs form a blastema but generate an abortive cartilage spike.

(B) Schematic illustration of the *Prrx1:CreER* line (left) and the *CAGGs:lp-Cherry* reporter line (right). The *CreER* cassette was codon optimized and each genotype was generated individually with the REMI protocol (see STAR methods for details). Germline transmitted *Prrx1:CreER* founders were mated with a *CAGGs:lp-Cherry* reporter line. The F1 carrying both *CreER* and reporter cassettes were screened and used for the experiments in this study.

(C) 4-hydroxytamoxifen (4-OHT) treatment paradigm used to convert the reporter cassette in F1.

(D) Whole-mount image of mCherry fluorescence of a hindlimb bud from a converted *Prrx1:CreER;CAGGs:lp-Cherry* transgenic stage 51 tadpole. Proximal: left; Distal: right. Scale bar represents 20 μ m.

(E-G) Magnified fluorescence images of a longitudinal section of (D) immunostained for *Prrx1* (mesenchymal CT cells, green) (E), *Cherry* (converted cells, magenta) (F), and the merged image with DAPI (nuclei, blue) (G). White dashed line denotes the border between *Prrx1*-positive mesenchyme (mes) and *Prrx1*-negative epidermis (epi). mCherry-expressing cells are confined to mesenchyme. Scale bars represent 20 μ m.

(H) Transverse section of uninjured upper hindlimb skin from a converted *Prrx1:CreER;CAGGs:lp-Cherry* transgenic froglet, immunostained for *Cherry* (converted cells, magenta), *Prrx1* (dermal CT cells, green), and DAPI (nuclei, blue). White arrowheads indicate examples of converted dermal fibroblasts identified as *Cherry*- and *Prrx1*-double positive cells at the mesenchyme (mes)-epidermis (epi) border (white dashed line). Asterisks indicate non-specific tissue autofluorescence. Scale bars represent 50 μ m.

(I) Transverse section of cartilage region from a converted *Prrx1:CreER;CAGGs:lp-Cherry* transgenic froglet, immunostained for *Cherry* (converted cells, magenta) and DAPI (nuclei, blue). White dashed line denotes the border of cartilage (ca). White arrows indicate examples of converted *Cherry*-positive chondrocytes and white arrowheads the converted perichondria. Scale bars represent 50 μ m.

(J) Transverse section of muscle bundle region from a converted *Prrx1:CreER;CAGGs:lp-Cherry* transgenic froglet, immunostained for *Cherry* (converted cells, magenta), *Prrx1* (interstitial CT cells, green), and DAPI (nuclei, blue). White arrowheads indicate examples of converted interstitial fibroblasts identified as *Cherry*- and *Prrx1*-double positive cells. Scale bars represent 50 μ m.

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skeleton and CT. Such dedifferentiated blastema cells regain an embryonic gene expression program comparable to limb bud cells, including the patterning gene network. Here, we address the question whether frog CT fibroblasts make comparable contributions to the frog blastema and spike, and whether they dedifferentiate to fully potent limb bud progenitors. We reveal that the incapability of frog blastema cells to dedifferentiate back to limb-bud-like states is a key difference compared with axolotl blastema cells.

RESULTS

CT cells are source cells of the adult frog blastema

To determine if frog blastema cells have a comparable origin to axolotl blastema cells, we generated germline transgenic frog lines that express the tamoxifen-inducible Cre-ERT2 cassette under the control of the mouse *Prrx1* enhancer element (*Cry stallin:mCherry;mPrrx1: xen(TFPnls-T2A-Cre-ERT2);CAGGs:lp-GFP-3pA-lp-mCherry-pA*, hereafter referred to as *Prrx1:Cre ER;CAGGs:lp-Cherry* animals) (Figures 1B and 1C) and performed genetic fate mapping. *Prrx1* has been established as a marker of CT precursors in the limb bud, and our previous work showed that CT-specific labeling could be achieved in axolotls using a mouse *Prrx1*-enhancer-driven Cre-ERT2 system with the administration of tamoxifen at limb bud stages (Gerber et al., 2018). Immunostaining with anti-PRRX1 antibody showed that *Prrx1* is expressed in the majority of mesenchymal cells in developing stage 51 frog limb buds, as in the case of axolotls (Figures 1D, 1E, and 1L).

To track the contribution of CT cells to the blastema, we first induced recombination by tamoxifen administration at early embryonic stages (Figure 1C). 13.2% $\pm$ 1.2% of the limb bud *Prrx1*-positive cells underwent genomic recombination to express mCherry (Figures 1D–1G and 1M). The converted cells remained labeled after metamorphosis and were found in dermal, interstitial, perichondrial, and cartilage regions (Figures 1H–1J). Analysis of limb sections immunostained to detect *Prrx1* protein showed that 11.7% $\pm$ 3.6% of *Prrx1* protein-expressing cells expressed the Cherry transgene (Figure 1M), consistent with the embryonic conversion efficiency. We followed these converted cells during regeneration, where, after post-metamorphic limb amputation, mCherry-positive cells constituted 22.0% $\pm$ 6.5% of the *Prrx1*-expressing blastema cells that made up to 68.2% $\pm$ 3% of the blastema (Figures 1K–1M). This 2-fold enrichment of Cherry-positive cells indicated that the labeled *Prrx1*-descendant cells are potent contributors to the blastema. To

further characterize the composition of the frog blastema, we performed scRNA-seq of cells dissociated from a frog blastema 14 days post-amputation (dpa) and visualized the data in two dimensions using uniform manifold approximation and projection (UMAP) embedding (Figures 1N and 1O). Clustering and marker gene expression analysis revealed that 85.3% of the dissociated cells showed CT signatures with 84.6% of CT cells expressing *prrx1* (Figures 1N and 1O). These blastema CT cells could be subcategorized into eight discernable subpopulations, including cartilage spike cells expressing *col9a2*, pericytes expressing *myh11*, and other blastema subpopulations. The remaining non-CT cell clusters included immune, endothelial, epidermal, and Schwann cells. Given that tissue dissociation can lead to distortions of cell type proportions, these observations concur satisfactorily with the 68.2% $\pm$ 3% of frog blastema mesenchyme cells observed to express *Prrx1* protein (Figure 1L).

In axolotls, the *Prrx1*-descendants from the interstitium and dermis enter the blastema and converge to a common multipotent progenitor type that can regenerate all CT subtypes including skeleton, tendons, ligaments, interstitium, and dermis (Gerber et al., 2018). To ask whether frog CT subpopulations follow a similar program, we performed scRNA-seq analysis on blastema CT cells in a time course of limb regeneration spanning 0, 3, 7, 10, 14, 20, and 52 dpa (Figures 2A, 2B, S1A, and S1B), encompassing key time points of blastema establishment. Since our tracing results showed that *Prrx1*-converted Cherry-positive cells contribute potently to the blastema, and due to a lack of obvious blastema structure at 0 and 3 dpa, we FAC-sorted *Prrx1*-converted mCherry-positive cells from these time points from our *Prrx1:CreER;CAGGs:lp-Cherry* transgenic frogs for scRNA-seq. Importantly, the sorted cells clustered together with CT cells from an unsorted sample and we observed all representative CT subtypes in the sorted fraction (Figure S1C). The 0 and 3 dpa datasets were then combined with the extracted CT cell clusters of 7, 10, 14, 20, and 52 dpa datasets collected from whole blastemas (Figure S1A) (see STAR methods for details). We used Harmony (Korsunsky et al., 2019), a tool designed to integrate single-cell transcriptomic data across experiments and batches, to integrate the data across the different time points and UMAP to visualize the data in two dimensions. Clustering and marker gene expression analysis revealed four major mature CT subpopulations (excluding pericytes) in the intact tissue: interstitial cells (86.4%), dermal cells (4.9%), tenocytes (4.1%), and skeletal cells (3.6%) (Figure 2C; Table S1). The regeneration time course revealed a gradient of regenerating cell states across all blastema time points emerging from the

(K) Magnified fluorescence image of a longitudinal section of a 28 dpa blastema from a converted *Prrx1:CreER;CAGGs:lp-Cherry* transgenic froglet, immunostained for Cherry (converted cells, magenta), *Prrx1* (blastema CT cells, green), and DAPI (nuclei, blue). Arrowheads show examples of the *Prrx1*-converted Cherry-positive cells contributing to the blastema. Scale bars represent 50 μ m.

(L) Percentage of *Prrx1*-expressing mesenchymal cells in limb buds (Lb, 84.4% $\pm$ 17.7%, $n = 3$), uninjured leg (Uninj, 39.9% $\pm$ 5.6%, $n = 5$), and blastema (BL, 68.2% $\pm$ 3.0%, $n = 3$), determined by immunohistochemistry. Statistical significance is calculated using unpaired Student's *t* test. *** $p < 0.001$. Data are represented as mean $\pm$ SD.

(M) Percentage of converted Cherry+ CT precursors (Cherry+/Prrx1+) in limb buds (Lb, 13.2% $\pm$ 1.2%, $n = 3$), uninjured leg (Uninj, 11.7% $\pm$ 3.6%, $n = 5$), and blastema (BL, 22.0% $\pm$ 6.5%, $n = 3$), determined by immunohistochemistry. Statistical significance is calculated using unpaired Student's *t* test. n.s.: $p > 0.05$; * $p < 0.01$. Data are represented as mean $\pm$ SD.

(N) Uniform manifold approximation and projection (UMAP) embedding of scRNA-seq data of 3,817 cells from a 14-dpa frog blastema. Cells are colored by cluster and cell type annotations are noted. Insets show the expression of blastema cells with a CT signature (*prrx1*), pericyte (*myh11*), and cartilage (*col9a2*) markers projected onto the UMAP embedding.

(O) Violin plots show the expression of canonical marker genes defining cluster cell type annotations in the 14 dpa blastema as shown in (N).

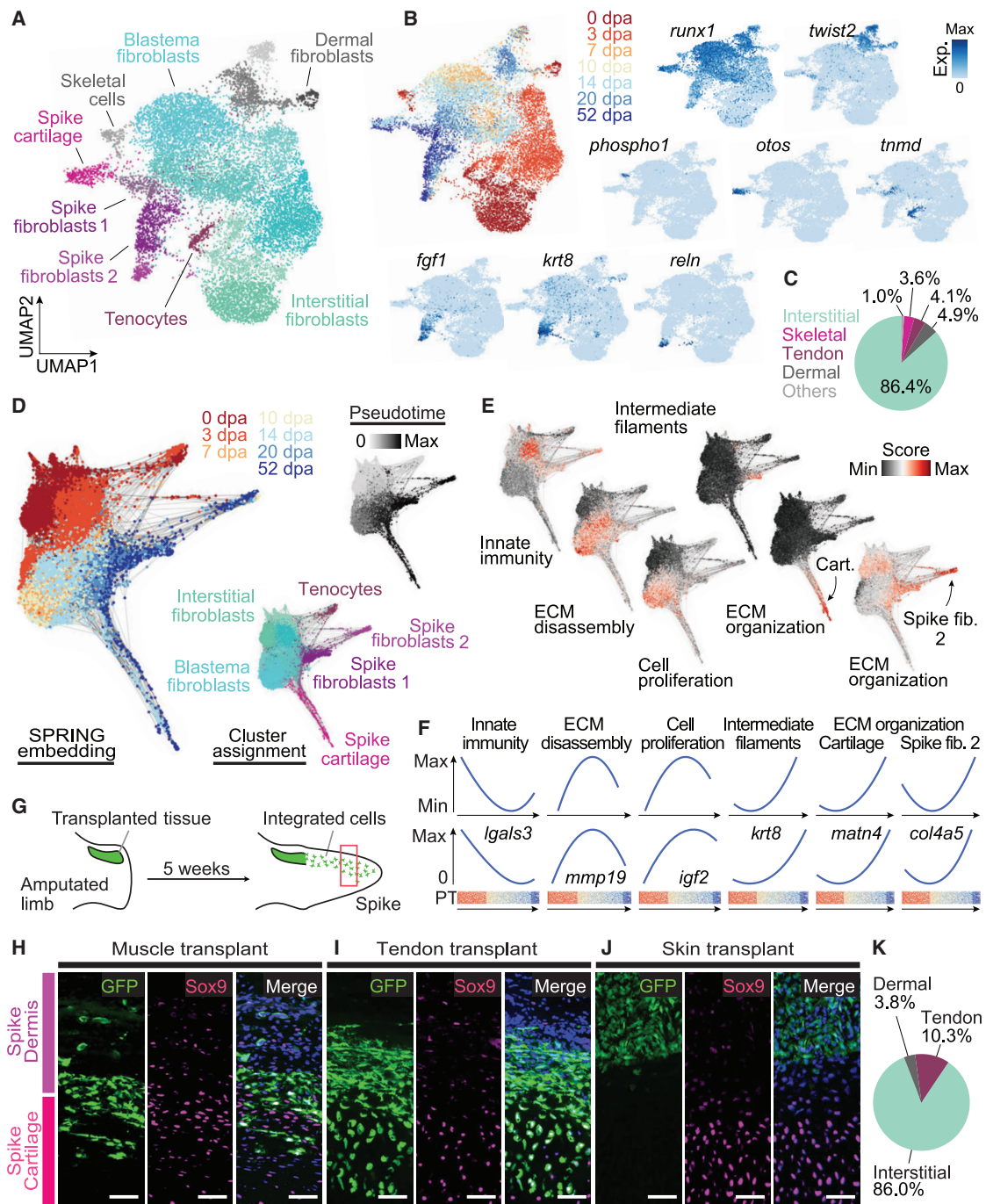


Figure 2. Dermis- and interstitium-derived blastema cells contribute differentially to spike cartilage

(A) UMAP embedding of scRNA-seq data of 15,014 cells from a frog blastema time course after data integration using Harmony (Korsunsky et al., 2019). Cells are colored by cluster and cell type annotations are noted. CT cells shown here were extracted from the blastema cell atlases shown in Figures S1A and 1N, or were experimentally enriched by FACS (0 and 3-dpa cells).

(B) Left: UMAP embedding as shown in (A) with cells being colored by sampling time point. Right and Bottom: feature plots visualizing the expression of cluster marker genes.

(C) Pie chart showing fractions of cell types composing the CT in the mature uninjured state (0 dpa).

(D) Spring plot (Weinreb et al., 2018) of Harmony-integrated scRNA-seq data of 13,124 CT cells excluding skeletal and dermal cells. Left: cells colored by time point. Center: cells are colored by cluster with cell types being noted. Right: cells colored by ranked pseudotime estimates obtained from a diffusion map analysis (Angerer et al., 2016).

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mature interstitial CT and tendon clusters toward spike cartilage and mesenchyme clusters. Interestingly, the dermal cluster was distinct and included cells from all blastema time points, suggesting that, unlike in the case of axolotls, dermal cells do not funnel into the main blastema cluster and therefore do not contribute to spike cartilage and mesenchyme. We next performed a pseudotime analysis to align all CT cells, excluding dermal and skeletal cells, along a regeneration trajectory and visualized the cells in a force-directed layout of a k-nearest neighbor graph (Weinreb et al., 2018) (Figure 2D). We found that interstitial CT and tendon-associated cell types converged to a common phenotype in the early blastema (3 dpa), which then progressed through various blastema stages prior to a bifurcation into spike mesenchyme and spike cartilage around 10 and 14 dpa (Figure 2D). Gene ontology (GO) enrichment analysis for gene sets marking different phases along the pseudotime course revealed the presence of an initial innate immune response followed by signatures of ECM disassembly, cell proliferation, intermediate filaments, and finally, ECM organization (Figures 2E and 2F). Overall, this progression resembles the progression found in axolotls during regeneration (Gerber et al., 2018).

Dermis- and interstitium-derived blastema cells contribute differentially to spike cartilage

To functionally validate the cell trajectory inferences from the single-cell sequencing data, we performed tissue scale transplantations to determine the contributions of bone (periosteum), interstitial-cell-, dermis-, and tendon-derived CT cells to the regenerated spike cartilage (Figures 2G–2J and S2A–S2H). Interestingly, similar to the axolotl, bone-derived CT cells contributed to callus formation at the amputation plane but not significantly to spike outgrowth (Figures S2A and S2B). Tendon or interstitial fibroblast transplants (through muscle transplantation) readily contributed to Sox9-expressing spike cartilage (Figures 2H, 2I, and S2C–S2F). Dermal transplants showed extensive migration in a distinct dermis-like layer at the tip of the blastema with minimal contribution to cartilage (Figures 2J, S2G, and S2H). We carefully examined two samples for each type of transplantations and observed a similar trend in the contribution of donor cells to chondrogenic lineage between samples of the same type (Figure S2I). Quantitation of each tissue's contribution to cartilage (taking into account the proportional representation of each tissue at the amputation plane) revealed that interstitial fibroblasts contributed 85.6% of cells to spike cartilage, whereas tendon- and dermis-derived cells contributed only 10.6% and 3.8%, respectively (Figure 2K; Table S1). These results show that while interstitial, tendon, and dermal CT can contribute to the

frog blastema, dermis remains largely as a separate tissue layer with very little, if any, contribution to regenerated cartilage, which contrasts with the successful regeneration in axolotls.

Frog blastema cells show intrinsic limitations in dedifferentiation

These results raised the question of whether frog CT cells are fully reprogrammed to limb bud progenitor potential, as seen in axolotls. To compare axolotl and frog cells, we generated scRNA-seq data for similarly staged axolotl and frog limb buds and blastemas (Figures 3A and S3A–S3D) and used quadratic programming analysis to diagnose to what extent frog blastema cells as compared with axolotl blastema cells acquire a limb bud progenitor identity *in situ*. For each species, we used a set of adult-CT- versus embryonic-limb-bud-specific genes to decompose each cell's identity into a fractional adult and fractional embryonic identity. This analysis showed that between 7 and 14 dpa, frog blastema cells express a fraction of the embryonic limb bud program. However, in contrast to axolotls in which a significant proportion of cells between 11 and 25 dpa approached almost 100% limb bud character, none of the frog cells reached 100%, with a very small fraction reaching a maximum of 60% limb bud identity (Figure 3A). In addition, when integrating limb bud and mature CT cells for each species using Harmony (Korsunsky et al., 2019), the same integration parameters led to a complete mixing of blastema and limb bud cells for axolotls but not for frog CT cells (Figures S3E and S3F). These findings indicated that the adult frog blastema does not fully dedifferentiate to an embryonic-like phenotype *in situ*.

We wanted to further ask whether this incomplete reprogramming was due to an intrinsic restriction within the fibroblasts' potential, or due to limited or even inhibitory environmental cues. We therefore developed a functional assay to test axolotl and frog CT potentials (Figure 3B). Transplantation of frog blastema to axolotl blastema, which would have been ideal, showed rapid tissue rejection (Figures S3G and S3H); therefore, we adopted transplantation into the embryonic limb bud as the test bed to assay regenerative capacities. Upper leg axolotl blastema cells that regain a full embryonic limb bud state should contribute to patterned skeleton, including the foot when transplanted into an axolotl limb bud. If frog blastema cells that show incomplete regeneration are limited by signals in their adult environment but are actually intrinsically capable of regenerating a limb, they should show the same characteristics when transplanted into frog limb buds. On the other hand, if frog CT cells undergo incomplete reprogramming and intrinsically lack the capability of regenerating a limb, their contribution to patterned skeleton in this assay would be limited. We first validated the

(E) GO enrichment scores identified based on genes differentially expressed along the pseudotime course. Scores are visualized as feature on the SPRING embedding. Innate immune response (GO:0045087), extracellular matrix (ECM) disassembly (GO:0022617), Positive regulation of cell proliferation (GO:0008284), Intermediate filament (GO:0005882), ECM organization (GO:0030198).

(F) Top: GO enrichment scores are visualized as a function of pseudotime by a loess fitted curve through the data points. Bottom: expression intensities of example genes for each GO term are shown.

(G) Schematic illustration of tissue transplantation assays. See Figure S2 for details. Red box indicates the regions shown in (H–J).

(H–J) Magnified insets from representative longitudinal sections of muscle (H) (n = 2), tendon (I) (n = 2), and skin (J) (n = 2) transplantation samples. Sections were immunostained for GFP (donor tissue, green), Sox9 (chondrocytes, magenta), and the merged image with DAPI (nuclei, blue). The field of view was further divided into spike dermis (upper region, purple) and spike cartilage (lower region, magenta) according to the expression of Sox9. Scale bars represent 20 μ m.

(K) Pie chart showing percentage contributions of interstitial cells, tendon cells and dermal cells to Sox9-expressing cartilage in the spike.

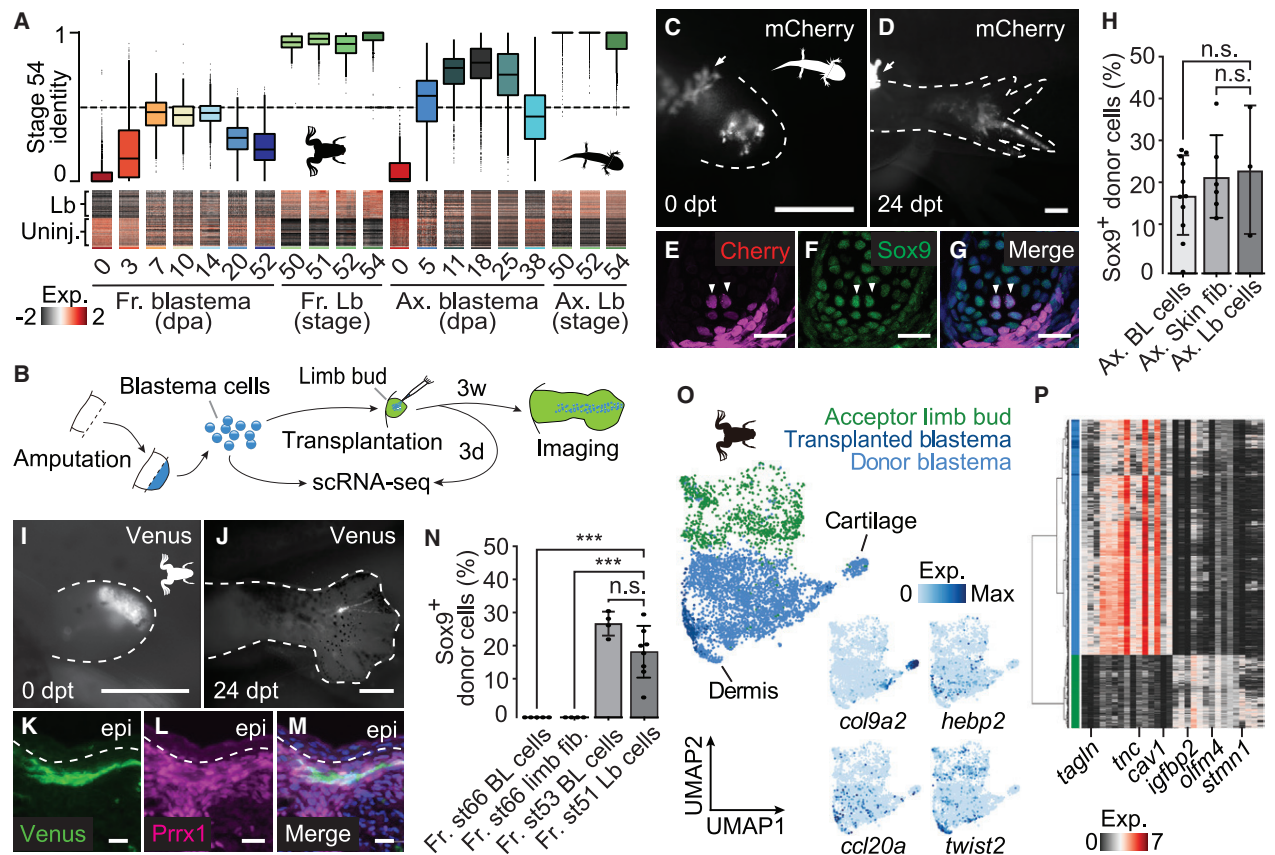


Figure 3. Froglet blastema cells show incomplete reprogramming to a limb-bud-like progenitor state

(A) Quadratic programming shows limited conversion of frog blastema cells to embryonic phenotype. Top: violin plots show fractional embryonic (stage 54) identity in comparison to mature identity for frog (left) and axolotl (right) CT blastema and limb bud cells. See Figures S3E and S3F for cellular heterogeneity. 0 = 100% mature identity; 1 = 100% limb bud identity. Bottom: heatmap representation of scaled gene expression across single cells (columns) for genes (rows) used as input for the quadratic programming analysis in frog and axolotl, respectively. Differences in cell numbers per time point were neglected and genes were sorted by timepoint specificity.

(B) Schematic of the transplantation assay. Unsorted blastema cells of axolotls or froglets were dissociated and injected into developing limb buds of the respective species. For a frog blastema to limb bud transplantation scRNA-seq data was acquired for the donor blastema population and the limb bud cells 3 days after transplantation in addition to imaging data.

(C and D) Fluorescent images of a *d/d* axolotl limb bud 0 day post-transplantation (dpt) (C) and 24 dpt (D) after transplantation of unsorted mCherry-expressing axolotl 9 dpa blastema cells. White dashed lines outline the limb buds. Arrows show non-specific tissue autofluorescence. Scale bars represent 500 μ m.

(E–G) Transverse section of (D) at the digit level, immunostained for Cherry (donor cells, magenta) (E), Sox9 (chondrocytes, green) (F), and the merged image with DAPI (nuclei, blue) (G). Arrowheads indicate examples of Cherry⁺/Sox9⁺ cells, which are transplanted cells that have integrated into cartilage. Scale bars represent 20 μ m.

(H) Bar plot showing the ratio of Sox9-expressing Cherry-positive cells from unsorted axolotl blastema-to-limb bud (Ax. BL cells, $n = 11$), FAC-sorted axolotl skin fibroblasts-to-limb bud (Ax. Skin fib. $n = 6$), and unsorted axolotl limb bud-to-limb bud (Ax. Lb cells, $n = 3$) transplantation experiments. Statistical significance is calculated using unpaired Student's *t* test. n.s.: $p > 0.05$. Data are represented as mean $\pm$ SD.

(I and J) Fluorescent images of a wild-type frog tadpole limb bud 0 dpt (I) and 24 dpt (J) after transplantation of unsorted Venus-expressing frog 14 dpa blastema cells. White dashed lines outline the embryonic limb buds. Scale bars represent 500 μ m.

(K–M) Epidermal region of a section of a transplanted frog sample from (J) immunostained for Venus (donor cells, green) (K) and Prrx1 (CT cells, magenta) (L) and the merged image with DAPI (nuclei, blue) (M). Scale bars represent 20 μ m.

(N) Bar plot showing the ratio of Sox9-expressing Cherry⁺ cells from unsorted stage 66 froglet blastema-to-limb bud (Fr. st66 BL cells, $n = 5$), FAC-sorted stage 66 froglet limb fibroblasts-to-limb bud (Fr. st66 limb fib., $n = 4$), unsorted stage 53 regenerative limb-bud-blastema-to-limb-bud (Fr. st53 BL cells, $n = 4$), and unsorted stage 51 frog limb-bud-to-limb-bud (Fr. st51 Lb cells, $n = 8$) transplantation experiments. Statistical significance is calculated using unpaired Student's *t* test. *** $p < 0.001$. Data are represented as mean $\pm$ SD.

(O) UMAP embedding of scRNA-seq data from the host limb bud acceptor cells (green) and transplanted blastema cells (dark blue) 3 days after transplantation, and blastema donor cells before transplantation (light blue) after data integration using Harmony (Korsunsky et al., 2019). Cells are colored by sample type. Feature plots (right) visualize expression of a cartilage (*col9a2*) and a dermis marker gene (*twist2*) and two transplant-specific genes on the UMAP embedding. (P) Heatmap shows the expression of main limb bud and donor blastema cluster markers (columns) for cells (rows) shown in (O). Cells were hierarchically clustered using ward.D2. Example genes are highlighted underneath the heatmap.

heterochronic transplantation approach in axolotls by transplanting unsorted, fluorescently labeled 9 dpa upper leg axolotl blastema cells into stage 51-*d/d* axolotl hindlimb buds (Figures 3C and 3D). Transplanted blastema cells contributed to patterned skeleton along the entire proximal-distal axis comparable with transplanted limb bud cell controls (Figures 3E–3H and S4A–S4C). These results reflect acquisition of a multipotent state with a robust contribution to limb development. We then tested the potency of frog blastema cells by transplanting unsorted, fluorescently labeled frog blastema cells into stage 50 wild-type tadpole limb buds (Figures 3I and 3J). Interestingly, while control unsorted frog limb bud cell transplants showed a similar integration profile as in axolotl, indicating comparability of the assays (Figures S4G–S4I), the frog blastema cells did not contribute to the developing skeleton but rather associated with dermis-like elements in the foot (Figures 3K–3N and S4D–S4F). Frog fibroblasts directly dissociated and FAC sorted from the mature limb also showed a similar lack of contribution to developing skeleton (Figures 3N and S4J). The starting number of injected donor cells was comparable across transplantation experiments (Figure S4K). To confirm the competence of the isolated frog blastema cells to integrate with host tissue, we transplanted the unsorted frog blastema cells back into the blastema of thy-mectomized post-metamorphic hosts where they robustly contributed to the spike (Figures S4L and S4M). To further rule out the possibility that the wound-healing response in frog prohibited the chondrogenic contribution of donor cells in general, we transplanted unsorted blastema cells from regenerative, stage 53 frog limb bud blastemas to stage 50 limb buds (Figures 3N and S4N–S4Q). The blastema cells of the regenerative stage contributed abundantly into host cartilage in a similar fashion compared with the control limb bud transplants (Figures 3N and S4N–S4Q).

These functional experiments point to an incomplete reprogramming of frog CT cells after limb amputation. We next used scRNA-seq on frog donor blastema, host limb bud and transplanted blastema cells to directly measure at the molecular level the extent of reprogramming. In a UMAP embedding of Harmony-integrated data as well as in a hierarchical clustering analysis (Figures 3O and 3P), the transplanted frog blastema cells clustered together with the donor blastema cells rather than the host limb bud cells indicating that they do not acquire the phenotype of surrounding limb bud cells but closely resemble the original blastema donor cells 3 days after transplantation. Only few transplant-specific marker genes could be identified and comprised immune-related genes such as *ccl20a* or necrotic factors such as *hebp2* (Figure 3O). Notably, these genes were also detected in a fraction of blastema cells but not in limb bud cells. In summary, our combined functional and transcriptomic analysis shows that frog blastema cells are incompletely reprogrammed and intrinsically incapable of undertaking a full limb development program.

Frog blastema cells harness a distinct cartilage differentiation program compared with embryonic tadpole limb bud cells

An interesting aspect of our experiments is the capability of adult frog blastema cells to differentiate into a cartilage rod, but their incompetence to contribute to patterned cartilage when trans-

planted to the limb bud. We therefore compared the cartilage differentiation programs in the frog limb bud (Figures 4A and S4R) and the frog blastema (Figure 4B) at the single-cell transcriptomic level. In both scenarios, differentiation into cartilage cells was defined by expression of cartilage-specific genes including *otos*, *col9a2*, and *matn4*. However, we found a discrepancy in the expression of patterning-related genes such as *meis1*, *hoxa11*, and *hoxa13*. While patterning along the proximal-distal axis in the frog limb bud cartilage was defined by an anticorrelation in expression of the proximal marker *meis1* and the distal markers *hoxa13* and *hoxd13*, no clear correlation or anticorrelation in expression of these genes was detected in cartilage cells of the frog blastema (Figure 4C). In addition, the overall expression levels of these patterning genes were greatly reduced in differentiating cartilage cells in the frog blastema compared with the limb bud (Figures 4D, S4S, and S4T). This shows that the cartilage differentiation program in the frog blastema lacks proper proximal-distal patterning, consistent with previous reports examining selected transcripts (Ohgo et al., 2010). In contrast, we found a clear signature of proximal-distal patterning for differentiating cartilage cells in the axolotl blastema and limb buds (Figures 4E, 4F, and S4U). The deranged *hox* gene expression in the frog blastema is likely functionally linked to the inability of these cells to participate in limb developmental chondrogenesis. Previous work in chicken and mice showed that *hoxa* and *hoxd* genes confer segment-specific chondrocyte condensation and skeletal morphogenesis properties to limb progenitors during development (Boulet and Capecchi, 2004; Fromental-Ramain et al., 1996; Yokouchi et al., 1995).

To further compare cartilage differentiation in different scenarios, we first ordered cells based on a cartilage score, which was calculated using shared cartilage development markers (e.g., *otos*, *col9a2*, see STAR methods for full lists) (Figure 4G), and then performed a differential expression analysis for cells of matching cartilage scores between frog blastema and limb buds. As a comparison, we also performed this analysis on differentiating cartilage cells in axolotl limb bud versus blastema (Figure 4H). We identified various genes that were specifically expressed during cartilage development in the frog limb bud but not in the frog blastema (Figure 4I), including the known cartilage development marker *nog*, the bone mineralization associated gene *irx3*, and the Wnt-pathway-related gene *dact1*. In contrast, *matrilines* (*matn1* and *matn2*) as well as genes associated with cell adhesion (GO:0007155), such as *hapln3* and *edil1*, were only found to be expressed in the frog blastema and not the frog limb bud, highlighting further molecular distinctions in the cartilage differentiation program in the frog blastema compared with the frog limb bud. Remarkably, when we examined the orthologs of these genes in the axolotl, we found that the axolotl limb bud and blastema cartilage cells showed similar expression profiles validating the conclusion that axolotl CT cells attain a bona fide limb-bud-like state in the blastema (Figure 4I). A potential gene network guiding the correct patterning of cartilage in limb buds and the axolotl blastema might be the coordinated expression of *sox8* and *dlx5*, two transcription factors potentially interacting with each other (Wissmüller et al., 2006), that are absent or only weakly expressed in the frog blastema. We found that only a few genes (*rarres1*, *myc*, *pim1*) differentially expressed between axolotl limb bud and blastema cells

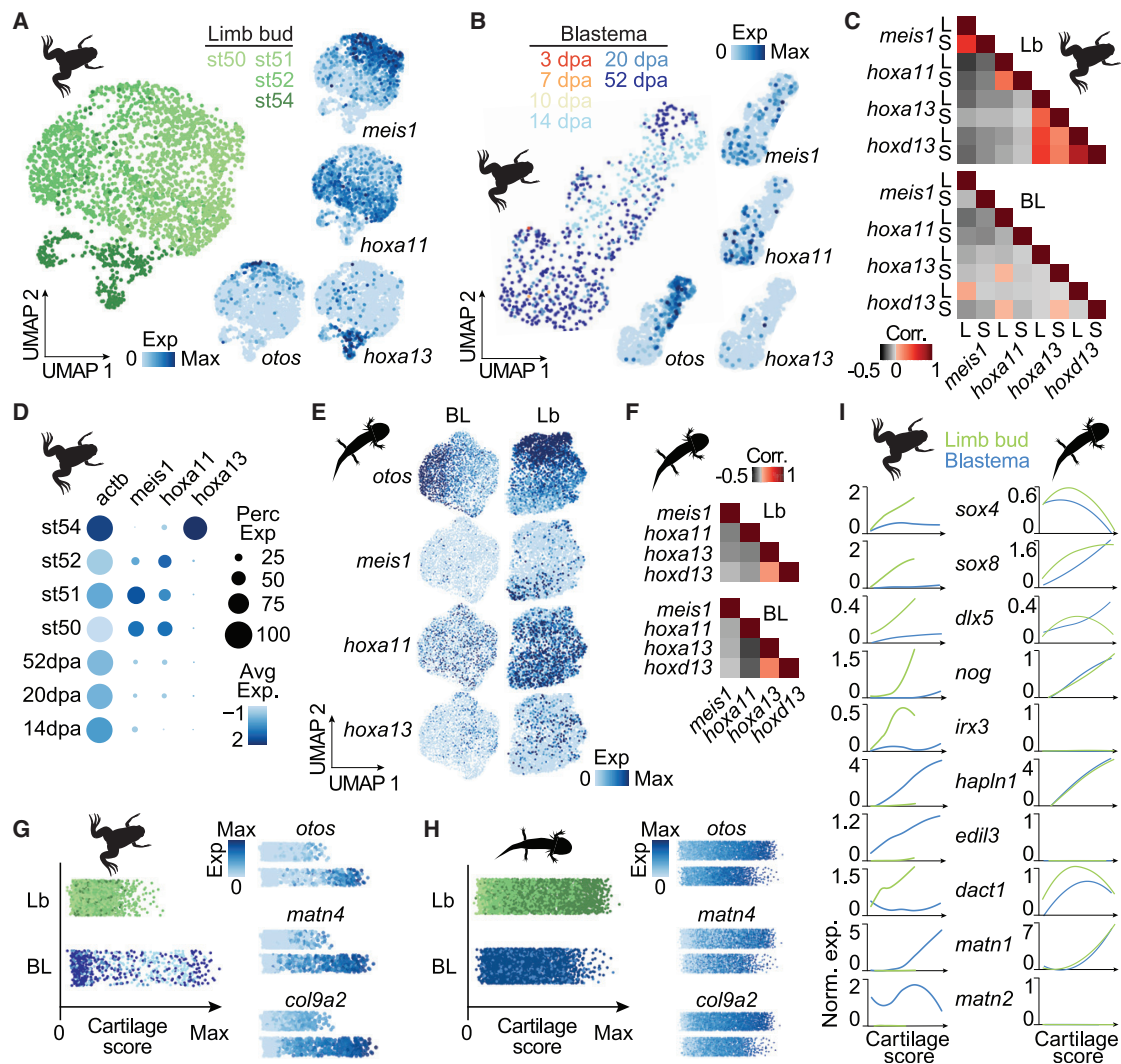


Figure 4. Proximal-distal stratification of patterning factors during cartilage formation in axolotl blastema and tadpole limb buds, but not in froglet blastema

(A) UMAP embedding of scRNA-seq data from limb bud cartilage cells ranging from stage 50 to stage 54 limb buds. Cells are colored by limb bud stages. Feature plots (right) show the expression of maturing cartilage marker (*otos*) and proximal-distal patterning factors (*meis1*, *hoxa11*, *hoxa13*). Cartilage cells were isolated based on the clustering of cells in Figure S4R.

(B) UMAP embedding of scRNA-seq data from frog blastema cartilage cells with cartilage cells being isolated based on the clustering of cells in Figure 2A. Cells are colored by blastema time points. Expression of *otos*, *meis1*, *hoxa11*, and *hoxa13* are visualized as feature plots.

(C) Correlation matrix for proximal-distal patterning genes in the developing cartilage of tadpole limb bud (Lb, top) and frog blastema (BL, bottom).

(D) Dot plot showing gene expression intensity and frequency differences between developing cartilage cells in the tadpole limb bud and the frog blastema. Avg Exp: average expression values, Perc Exp: percent of cells expressing each gene. See Figures S4S and S4T for more information.

(E) Feature plots showing expression level of *otos*, *meis1*, *hoxa11* and *hoxa13* on UMAP embeddings of scRNA-seq data from the cartilage cells of an 18-dpa axolotl blastema (BL, left) and limb buds (Lb, right). Cartilage cells were isolated based on the clustering of cells in Figure S4U.

(F) Correlation matrix for proximal-distal patterning genes in the developing cartilage cells of an 18-dpa axolotl blastema (BL, bottom) and axolotl limb buds (Lb, top).

(G) Cartilage scores (x axis) of frog limb bud (Lb, green) and blastema (BL, blue) cells, respectively, based on common markers of cartilage development (left, see STAR methods). Cells are colored by sample with colors matching the ones in (A) and (B) and are jittered along the y axis. Right: Exemplary feature plots of common cartilage development markers.

(H) Cartilage scores (x axis) of axolotl limb bud (Lb, green) and blastema (BL, blue) cells, respectively, based on common markers of cartilage development (left, see STAR methods). Cells are colored by sample with colors matching the ones in Figure S4U and are jittered along the y axis. Right: exemplary feature plots of common cartilage development markers.

(I) Gene expression as a function of cartilage score for selected genes identified to be differentially expressed between frog limb bud and blastema cartilage differentiation is visualized for frog (left) and axolotl (right) limb bud and blastema cartilage cells. A loess fitted curve through the data points is shown. Green: limb bud. Blue: blastema.

(Figure S4V) and these genes did not show similar expression curves in the corresponding frog tissues. Only one gene, *adrenomedullin 2* (*adm2*), showed a consistently high expression in the blastema compared with limb bud for frog and axolotl, suggesting a blastema specific role in cartilage development not needed in embryonic limb buds.

DISCUSSION

To understand how frogs fail to regenerate a fully patterned limb, it is essential (1) to study the cellular and molecular mechanisms underlying the abortive regeneration process, (2) to compare these mechanisms to a successful model of limb regeneration, and (3) to test which of the differences that we find between the two models that could account for the regeneration failure. Here, by establishing previously unavailable methods, such as lineage tracing with a stable transgenic CreERT/LoxP line, single-cell RNA-seq, and a transplantation-based functional assay, we systematically compared the differences between the limb regeneration processes of frogs and axolotls.

One previously unresolved question is whether the frog blastema is composed of similar cell types and derives from a similar origin as blastema cells in the axolotl. A rigorous treatment of this question requires genetic fate mapping of candidate source tissues. To date, only a doxycycline-inducible labeling system targeting tadpole chondrogenic cells was reported (Mochii et al., 2007), and an attempt to label tadpole trunk and leg muscles using the CreERT/LoxP system was unsuccessful (Rodrigues et al., 2012). Here, we successfully established *Prrx1:CreER;CAGGS:lp-Cherry* stable transgenic frog lines that allow conditional labeling and tracing of limb CT cells. Combining lineage tracing with both single-cell RNA-seq analysis and immunostaining, we confirmed that, similar to an axolotl blastema, the frog blastema is composed of a comparable proportion of soft connective tissue-derived fibroblasts (Figure 1). Our scRNA-seq result also suggests that there is no pre-existing stem cell pool in frog limbs.

In contrast, frog blastema cells behave differently compared with axolotl blastema cells at the molecular level. Unlike axolotl dermal fibroblasts, which undergo dedifferentiation and contribute to multiple CT types, frog dermal fibroblasts maintained their dermal identity throughout the regeneration process. In addition, even though frog interstitium-derived blastema fibroblasts progressed through similar phases as axolotl blastema cells based on a GO enrichment analysis of the regeneration time course (Figures 2E and 2F; Gerber et al., 2018), they did not fully dedifferentiate to limb-bud-like progenitors before they differentiated into chondrocytes (Figure 3A). According to morphological data and bulk transcriptomic analysis, axolotl regeneration proceeds through 3 distinct stages: wound healing, blastema formation (dedifferentiation), and re-development (patterning) (Bryant et al., 2002; Knapp et al., 2013). Due to its unbranched and unsegmented appearance, the frog spike phenotype has long been regarded as a patterning defect (Suzuki et al., 2006). Our observation, however, suggests that the frog spike phenotype is not merely a patterning problem but rather an outcome of incomplete dedifferentiation prior to patterning. It is important to identify missing factors and/or pathways during frog limb regeneration, and to develop tools to determine

whether complete dedifferentiation could lead to complete patterning in future studies. Our single-cell transcriptomic comparison of cartilage formation in the limb bud versus the limb blastema also points to molecular distinctions between the two processes above and beyond the very noticeable suppression and derangement of the patterning program.

To date, rescuing the spike phenotype by ectopically applying or expressing extracellular factors, such as *fgf* (fibroblast growth factor), *bmp* (bone morphogenic protein), *shh* (sonic hedgehog), and progesterone, has been a main focus of the field (Herrera-Rincon et al., 2018; Lin et al., 2013; Mitogawa et al., 2018; Satoh et al., 2005; Yakushiji et al., 2009). The assumption behind this strategy is that frog blastema cells have capacities to correctly respond to extrinsic signals, and thus, the environmental cues are the determinants for successful limb regeneration. While our result does not exclude the importance of extracellular factors, it strongly suggests that cell intrinsic, most likely epigenetic, limitations are the key hinderance to complete regeneration. Characterizing and overcoming those epigenetic limitations will be crucial to improve regeneration in future studies.

In summary, our findings have important implications for regenerative medicine as they show that generic chondrogenic or osteogenic potential (i.e., *in vitro* differentiation) is an insufficient criterium for regenerative potential *in vivo* when aiming to rebuild functional skeleton. Strategies to restore embryonic, position-appropriate qualities to CT descendants should become a major focus of future regenerative medicine efforts.

Limitations of the study

Although our heterochronic transplantation experiments distinguished between the cell intrinsic potency of frog and axolotl limb blastema cells, we cannot completely rule out the possibility that the dedifferentiation observed in axolotl blastema is directed by “regeneration inducers” in the axolotl limb environment that are not present in the frog. Such hypothetical inducers, either secreted, circulating, and/or ECM molecules, could potentiate axolotl limb fibroblasts and push the cells through the dedifferentiation process. Ideally, transplanting frog fibroblasts into the axolotl limb blastema would be an elegant way to test this hypothesis. Unfortunately, we were not able to establish such assays in this study due to the rapid rejection of frog donor cells (Figure S3G). Similar results were obtained when using thymectomized axolotls as hosts (data not shown), suggesting that mechanisms other than immune rejection, such as the incompatibility of growth factors, might play roles in the failure of integration. Overcoming the compatibility problem would be a valuable direction in future studies.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.devcel.2021.04.016>.

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AUTHOR CONTRIBUTIONS

T.-Y.L. and S.H. generated transgenic *Xenopus laevis* lines with assistance from P.M. T.-Y.L. and L.G. performed cell transplantation experiments. Y.T.-S. performed tissue scale transplantation experiments. E.S. performed cross-species blastema transplantation experiments. T.-Y.L., S.H., and Y.T.-S. performed immunohistochemistry. T.-Y.L. and T.G. performed single-cell experiments. T.G. analyzed scRNA-seq data. T.-Y.L., T.G., Y.T.-S., B.T., and E.M.T. designed the study, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

E.M.T. is a member of the advisory board for developmental cell but did not participate in the editorial process of this manuscript.

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REFERENCES

Angerer, P., Haghighi, L., Büttner, M., Theis, F.J., Marr, C., and Büttner, F. (2016). destiny: diffusion maps for large-scale single-cell data in R. *Bioinformatics* 32, 1241–1243.

Aztekin, C., Hiscock, T.W., Gurdon, J.B., Jullien, J., Marioni, J.C., and Simons, B.D. (2020). Secreted inhibitors drive the loss of regeneration competence in *Xenopus* limbs. *bioRxiv*. <https://doi.org/10.1101/2020.06.01.127654>.

Bankhead, P., Loughrey, M.B., Fernández, J.A., Dombrowski, Y., McArt, D.G., Dunne, P.D., McQuaid, S., Gray, R.T., Murray, L.J., Coleman, H.G., et al. (2017). QuPath: open source software for digital pathology image analysis. *Sci. Rep.* 7, 16878.

Boulet, A.M., and Capecchi, M.R. (2004). Multiple roles of Hoxa11 and Hoxd11 in the formation of the mammalian forelimb zeugopod. *Development* 131, 299–309.

Bryant, S.V., Endo, T., and Gardiner, D.M. (2002). Vertebrate limb regeneration and the origin of limb stem cells. *Int. J. Dev. Biol.* 46, 887–896.

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21.

Fromental-Ramain, C., Warot, X., Lakkaraju, S., Favier, B., Haack, H., Birling, C., Dierich, A., Dollé, P., and Chambon, P. (1996). Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. *Development* 122, 461–472.

Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., Gac-Santel, M., Nowoshilow, S., Kageyama, J., Khattak, S., et al. (2018). Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science* 362, eaaq0681.

Haghighi, L., Büttner, M., Wolf, F.A., Büttner, F., and Theis, F.J. (2016). Diffusion pseudotime robustly reconstructs lineage branching. *Nat. Methods* 13, 845–848.

Harty, M., Neff, A.W., King, M.W., and Mescher, A.L. (2003). Regeneration or scarring: an immunologic perspective. *Dev. Dyn.* 226, 268–279.

Herrera-Rincon, C., Golding, A.S., Moran, K.M., Harrison, C., Martyniuk, C.J., Guay, J.A., Zaltsman, J., Carabello, H., Kaplan, D.L., and Levin, M. (2018). Brief local application of progesterone via a wearable bioreactor induces long-term regenerative response in adult *Xenopus* Hindlimb. *Cell Rep* 25, 1593–1609.e7.

Horton, J.D., Horton, T.L., Dzialo, R., Gravenor, I., Minter, R., Ritchie, P., Gartland, L., Watson, M.D., and Cooper, M.D. (1998). T-cell and natural killer cell development in thymectomized *Xenopus*. *Immunol. Rev.* 166, 245–258.

Huang, da W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57.

Karimi, K., Fortriede, J.D., Lotay, V.S., Burns, K.A., Wang, D.Z., Fisher, M.E., Pells, T.J., James-Zorn, C., Wang, Y., Ponferrada, V.G., et al. (2018). Xenbase: a genomic, epigenomic and transcriptomic model organism database. *Nucleic Acids Res* 46, D861–D868.

Khattak, S., Murawala, P., Andreas, H., Kappert, V., Schuez, M., Sandoval-Guzmán, T., Crawford, K., and Tanaka, E.M. (2014). Optimized axolotl (*Ambystoma mexicanum*) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nat. Protoc.* 9, 529–540.

Khattak, S., Schuez, M., Richter, T., Knapp, D., Haigo, S.L., Sandoval-Guzmán, T., Hradlikova, K., Duemmler, A., Kerney, R., and Tanaka, E.M. (2013). Germline transgenic methods for tracking cells and testing gene function during regeneration in the axolotl. *Stem Cell Rep* 1, 90–103.

King, M.W., Neff, A.W., and Mescher, A.L. (2012). The developing *Xenopus* limb as a model for studies on the balance between inflammation and regeneration. *Anat. Rec. (Hoboken)* 295, 1552–1561.

Knapp, D., Schulz, H., Rascon, C.A., Volkmer, M., Scholz, J., Nacu, E., Le, M., Novozhilov, S., Tazaki, A., Protze, S., et al. (2013). Comparative transcriptional profiling of the axolotl limb identifies a tripartite regeneration-specific gene program. *PLoS One* 8, e61352.

Korsunsky, I., Millard, N., Fan, J., Slowikowski, K., Zhang, F., Wei, K., Baglaenko, Y., Brenner, M., Loh, P.R., and Raychaudhuri, S. (2019). Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat. Methods* 16, 1289–1296.

- 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.
- Kroll, K.L., and Amaya, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122, 3173–3183.
- Lin, G., Chen, Y., and Slack, J.M. (2013). Imparting regenerative capacity to limbs by progenitor cell transplantation. *Dev. Cell* 24, 41–51.
- McCusker, C., Bryant, S.V., and Gardiner, D.M. (2015). The axolotl limb blastema: cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods. *Regeneration (Oxf)* 2, 54–71.
- Mescher, A.L., Neff, A.W., and King, M.W. (2013). Changes in the inflammatory response to injury and its resolution during the loss of regenerative capacity in developing *Xenopus* limbs. *PLoS One* 8, e80477.
- Mitogawa, K., Makanae, A., and Satoh, A. (2018). Hyperinnervation improves *Xenopus laevis* limb regeneration. *Dev. Biol.* 433, 276–286.
- Mochii, M., Taniguchi, Y., and Shikata, I. (2007). Tail regeneration in the *Xenopus* tadpole. *Dev. Growth Differ.* 49, 155–161.
- Muneoka, K., Fox, W.F., and Bryant, S.V. (1986). Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. *Dev. Biol.* 116, 256–260.
- Nieuwkoop, P.D., and Faber, J. (1956). *Normal Table of Xenopus Laevis (Daudin); a Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis* (North-Holland Publishing).
- Nowoshilow, S., and Tanaka, E.M. (2020). Introducing www.axolotl-omics.org - an integrated -omics data portal for the axolotl research community. *Exp. Cell Res.* 394, 112143.
- Ohgo, S., Itoh, A., Suzuki, M., Satoh, A., Yokoyama, H., and Tamura, K. (2010). Analysis of *hoxa11* and *hoxa13* expression during patternless limb regeneration in *Xenopus*. *Dev. Biol.* 338, 148–157.
- R Core Team (2019). R: A language and environment for statistical computing (Vienna, Austria: R Foundation for Statistical Computing). <https://www.R-project.org/>.
- Rodrigues, A.M., Christen, B., Martí, M., and Izpisua Belmonte, J.C. (2012). Skeletal muscle regeneration in *Xenopus* tadpoles and zebrafish larvae. *BMC Dev. Biol.* 12, 9.
- Satoh, A., Suzuki, M., Amano, T., Tamura, K., and Ide, H. (2005). Joint development in *Xenopus laevis* and induction of segmentations in regenerating froglet limb (spike). *Dev. Dyn.* 233, 1444–1453.
- Schloissnig, S., Kawaguchi, A., Nowoshilow, S., Falcon, F., Otsuki, L., Tardivo, P., Timoshevskaya, N., Keinath, M.C., Smith, J.J., Voss, S.R., and Tanaka, E.M. (2021). The giant axolotl genome uncovers the evolution, scaling, and transcriptional control of complex gene loci. *Proc. Natl. Acad. Sci. USA* 118. e2017176118. <https://doi.org/10.1073/pnas.2017176118>.
- Sessions, S.K., and Bryant, S.V. (1988). Evidence that regenerative ability is an intrinsic property of limb cells in *Xenopus*. *J. Exp. Zool.* 247, 39–44.
- Simon, A., and Tanaka, E.M. (2013). Limb regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 291–300.
- Sive, H.L., Grainger, R.M., and Harland, R.M. (2000). *Early Development of Xenopus laevis: A Laboratory Manual* (Cold Spring Harbor Laboratory Press).
- Sobkow, L., Epperlein, H.H., Herklotz, S., Straube, W.L., and Tanaka, E.M. (2006). A germline GFP transgenic axolotl and its use to track cell fate: dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration. *Dev Biol* 290, 386–397.
- Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H., and Tamura, K. (2006). Limb regeneration in *Xenopus laevis* froglet. *ScientificWorldJournal* 6 (suppl 1), 26–37.
- Weinreb, C., Wolock, S., and Klein, A.M. (2018). SPRING: a kinetic interface for visualizing high dimensional single-cell expression data. *Bioinformatics* 34, 1246–1248.
- Wissmüller, S., Kosian, T., Wolf, M., Finzsch, M., and Wegner, M. (2006). The high-mobility-group domain of Sox proteins interacts with DNA-binding domains of many transcription factors. *Nucleic Acids Res* 34, 1735–1744.
- Yakushiji, N., Suzuki, M., Satoh, A., Ide, H., and Tamura, K. (2009). Effects of activation of hedgehog signaling on patterning, growth, and differentiation in *Xenopus* froglet limb regeneration. *Dev. Dyn.* 238, 1887–1896.
- Yakushiji, N., Suzuki, M., Satoh, A., Sagai, T., Shiroishi, T., Kobayashi, H., Sasaki, H., Ide, H., and Tamura, K. (2007). Correlation between *Shh* expression and DNA methylation status of the limb-specific *Shh* enhancer region during limb regeneration in amphibians. *Dev. Biol.* 312, 171–182.
- Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H., and Kuroiwa, A. (1995). Misexpression of *Hoxa-13* induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev* 9, 2509–2522.
- Zhang, M., Chen, Y., Xu, H., Yang, L., Yuan, F., Li, L., Xu, Y., Chen, Y., Zhang, C., and Lin, G. (2018). Melanocortin receptor 4 signaling regulates vertebrate limb regeneration. *Dev. Cell* 46, 397–409.e5.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-GFP	Rockland	Cat# 600-401-215; RRID: AB_828167
mouse anti-GFP	MPI-CBG protein facility, Dresden	Cat# 106-A20-Mix
goat anti-mCherry	Acris Antibodies	Cat# AB0040-200; RRID: AB_2333092
Rabbit anti-RFP	Rockland	Cat# 600-401-379; RRID: AB_2209751
rabbit anti-Sox9	Chemicon	Cat# Ab5535; RRID: AB_2239761
goat anti-Sox9	R&D Systems	Cat# AF3075; RRID: AB_2194160
rabbit anti-Prrx1	Gerber et al., 2018	N/A
mouse anti-Pax7	MPI-CBG protein facility, Dresden	AB-022
mouse Anti-Col1A2	Developmental Studies Hybridoma bank	Cat# SP1.D8; RRID: AB_528438
Donkey anti-mouse Alexa 488	Invitrogen	Cat# A-21202; RRID: AB_141607
Donkey anti-mouse Alexa 568	Invitrogen	Cat# A-10037; RRID: AB_2534013
Donkey anti-rabbit Alexa 568	Invitrogen	Cat# A-10042; RRID: AB_2534017
Donkey anti-rabbit Alexa 647	Invitrogen	Cat# A-31573; RRID: AB_2536183
Donkey anti-goat Alexa 568	Invitrogen	Cat# A-11057; RRID: AB_2534104
Donkey anti-goat Alexa 647	Invitrogen	Cat# A-21447; RRID: AB_2535864
Chemicals, peptides, and recombinant proteins		
DAPI	Sigma-Aldrich	Cat# D9542
Mowiol 4-88	Sigma-Aldrich	Cat# 9002-89-5
4-hydroxytamoxifen	Sigma-Aldrich	Cat# 7940
DMSO	Sigma-Aldrich	Cat# D2650
tricaine methanesulfonate (MS222)	Sigma-Aldrich	Cat# A5040
Benzocaine	Sigma-Aldrich	Cat# E1501
pregnant mare serum gonadotropin	ProSpec	Cat# HOR-272
human chorionic gonadotropin	Sigma-Aldrich	Cat# CG10
Liberase TM	Roche	Cat# 5401127001
DNase I	Roche	Cat# 4716728001
OCT compound	Science Service	Cat# 62550-12
Critical commercial assays		
QIAGEN Maxi kit	Qiagen	Cat# 12163
Chromium Single Cell 3' GEM, Library & Gel Bead Kit v3, 16 rxns	10X Genomics	Cat# PN-1000075
Chromium Chip B Single Cell Kit, 48 rxns	10X Genomics	Cat# PN-1000073
Chromium i7 Multiplex Kit, 96 rxns	10X Genomics	Cat# PN-120262
SPRIselect	Beckman Coulter	Cat# B23318
Bioanalyzer High Sensitivity DNA Kit	Agilent	Cat# 5067-4626
Deposited data		
Raw and analyzed data	This paper	GEO: GSE165901
Xenopus reference genome NCBI build v9.2, Xenbase v9.2 gene models	Xenbase	http://www.xenbase.org/other/static-xenbase/ftpDatafiles.jsp
Axolotl reference genome	Nowoshilow and Tanaka, 2020	www.axolotl-omics.org
Experimental models: organisms/strains		
<i>Xenopus laevis</i> : wildtype	NASCO	Cat# LM00531 (Female); Cat# LM00715 (Male); RRID: XEP_Xla100
<i>Xenopus laevis</i> : wildtype	EXRC	N/A

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Xenopus laevis</i> : CAGGs:Venus	EXRC	RRID: EXRC_0048
<i>Xenopus laevis</i> : CMV:tdTomato	EXRC	RRID: EXRC_0033
<i>Xenopus laevis</i> : CAGGs:nucTFP	This paper	N/A
<i>Xenopus laevis</i> : CAGGs:mCherry	This paper	N/A
<i>Xenopus laevis</i> : CAGGs:lp-Cherry	This paper	N/A
<i>Xenopus laevis</i> : Prrx1:CreER	This paper	N/A
<i>Ambystoma mexicanum</i> : d/d	IMP animal facility	N/A
<i>Ambystoma mexicanum</i> : CAGGs:mCherry	This paper	N/A
<i>Ambystoma mexicanum</i> : CAGGs:GFP	Sobkow et al., 2006	N/A

Oligonucleotides

Primer Crystallin 5' forward:ACGCGTGG ATCACCTGAAAGCAGGTTG	This paper	N/A
Primer Crystallin 3' reverse:CAGCTCC TCGCCCTTGCTCACCATGGTGG CGCCTAGGTGGCGACCGGTGGATCC	This paper	N/A
Primer mCherry 5' forward: GTGAGCAAGGGCGAGGAG	This paper	N/A
Primer mCherry 3' reverse: CTTGTACAGCTCGTCCATGCC	This paper	N/A
Primer SV40pA 5' forward: GGCAT GGACGAGCTGTACAAGTAACACGT GCTGATCATAATCAGCCATAC	This paper	N/A
Primer SV40pA 3' reverse: GGGCCCGGAATTGCGTTAAGATAC	This paper	N/A

Recombinant DNA

pMAT/X(TFPnls-T2A-Cre-hERT2)	GeneArt	N/A
CAGGs:nucTFP	This paper	N/A
CAGGs:mCherry	This paper	N/A
CAGGs:loxP-GFP-loxP-mCherry	Khattak et al., 2013	N/A
Crystallin:mCherry; mPrrx1:xen(TFPnls-T2A-Cre-ERT2)	This paper	N/A
Plasmid: CL EGFP DI attB	Daniel L. Weeks Lab, Department of Biochemistry, University of Iowa	N/A

Software and algorithms

Fiji version 2.1.0/1.53c	Fiji	RRID: SCR_002285
QuPath	Bankhead et al. 2017	RRID: SCR_018257
Zen 3.2 (blue edition)	Zeiss	RRID: SCR_013672
Prism version 9.0.2	GraphPad Software	RRID: SCR_002798
Cellranger 3.1	10xGenomics	RRID: SCR_017344
STARsolo v2.7.5a	Dobin et al., 2013	N/A
R v3.6	R Core Team, 2019	N/A

Other

Filcon, sterile, (size: 10 µm)	BD bioscience	Cat# 340732
MACS SmartStrainers: 70 µm	Miltenyi Biotec	Cat# 130-098-45
MACS SmartStrainers: 30 µm	Miltenyi Biotec	Cat# 130-098-462
Chromium Controller	10X Genomics	Cat# 1000204; RRID: SCR_019326

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elly M. Tanaka (elly.tanaka@imp.ac.at).

Materials availability

All the plasmids and transgenic frog lines generated in this study are available upon request.

Data and code availability

The accession number for the mapped bam files and gene/cell matrix files reported in this paper is GEO: GSE165901. R scripts used for the scRNA-seq analysis are available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Xenopus strains and maintenance

Wildtype frogs were obtained from the European Xenopus Resource Center (EXRC), UK and NASCO, USA. Transgenic lines (CAGGs:*Venus* (EXRC line #33) and *CMV:tdTomato* (EXRC line #48) were obtained from EXRC. Frog transgenic lines (CAGGs:*nucTFP*, CAGGs:*mCherry*, and *Prrx1:CreER*;CAGGs:*lp-Cherry*) were generated in CRTD animal facilities. All lines were bred and group housed in CRTD and IMP facilities. All animal handling and surgical procedures were carried out in accordance with local ethics committee guidelines. Animal experiments were performed as approved by the State Authorities Saxony and the Magistrate of Vienna. Animals were anesthetized either in 0.003% benzocaine (Sigma-Aldrich, E1501), or 0.03% tricaine methanesulfonate (MS222, Sigma-Aldrich, A5040) before amputation and surgery. *Xenopus laevis* husbandry followed the guidelines as described (Sive et al., 2000). In brief, the female and the male were injected with 500 IU and 50 UI of human chorionic gonadotropin (hCG, Sigma-Aldrich, CG10), respectively, before natural mating. The pair were put together in 7L 20mM NaCl in room temperature overnight. The embryos were collected for the next two days before the mating pair was separated. To obtain frog eggs for transgenesis, wildtype females were primed with 50 IU of pregnant mare serum gonadotropin (PMSG, ProSpec, HOR-272) one week before the injection of 500 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich, CG10) in the evening before egg collection. For surgical experiments in this study, frogs were used at the juvenile stage (see Method details for more information) and were not biased toward specific sex.

Axolotl strains and maintenance

White (*d/d*) axolotls were used as the host of transplantation experiments. The CAGGs:*GFP* axolotl line was previously described (Sobkow et al., 2006), and the CAGGs:*Cherry* axolotl line was established previously in the lab. All lines were bred and maintained in CRTD and IMP facilities and each animal is kept individually. All the handling and surgical procedures were carried out in accordance with local ethics committee guidelines. Animal experiments were performed as approved by the State Authorities Saxony and the Magistrate of Vienna. Animals were anesthetized in 0.003% benzocaine (Sigma-Aldrich, E1501) before amputation and surgery. Axolotl husbandry was described in detail in a previous paper (Khattak et al., 2014). For surgical experiments in this study, axolotls were used at the juvenile stage (see Method details for more information) and were not biased toward specific sex.

METHOD DETAILS

Codon optimization and molecular cloning

TFPnls-T2A-Cre-ERT2 cassette was codon-optimized based on the codon usage of *Xenopus laevis* (GeneArt). To replace the original TFPnls-T2A-Cre-ERT2 cassette, the codon-optimized cassette (xen(TFPnls-T2A-Cre-ERT2)) was subcloned into the *Prrx1:CreER* construct (Gerber et al., 2018) with restriction enzymes *NheI* and *EcoRI*. Fragments of the Crystallin:*mCherry* cassette (Crystallin promoter, *mCherry*, SV40 poly-A sequences) was assembled using over-extension PCR before subcloned into the *Prrx1:CreER* construct using restriction enzymes *Apal* and *MluI*. The reporter CAGGs:*lp-GFP-3pA-lp-mCherry-pA* construct was described previously (Khattak et al., 2013). All plasmids used in transgenesis were prepared with QIAGEN Maxi kit (Qiagen, 12163).

Transgenesis and 4-hydroxytamoxifen treatment

CAGGs:*nucTFP* and CAGGs:*mCherry* transgenic *Xenopus laevis* lines were established independently using REMI protocol (Kroll and Amaya, 1996) using existing plasmids in the lab. *Crystallin:mCherry*; *Prrx1:xen(TFPnls-T2A-Cre-ERT2)-pA* (*Prrx1:CreER* in short) and CAGGs:*loxP-GFP-3pA-loxP-mCherry-pA* (CAGGs:*lp-Cherry* in short) transgenic *Xenopus laevis* lines were generated independently using REMI protocol (Kroll and Amaya, 1996). *Prrx1:CreER* and CAGGs:*lp-Cherry* F0 transgenic tadpoles were screened by virtue of red lens and green trunk muscle, respectively. *Prrx1:CreER*;CAGGs:*lp-Cherry* double positive F1 embryos were treated with 1 μ M 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, H7940) overnight at 9-, 11- and 13-days post-fertilization (dpf), and were screened for *mCherry* signals in the limb bud around stage 50 (42 dpf) to assess conversion efficiency and specificity.

Thymectomy

To suppress the potential immune response of frogs in allograft transplantations, we developed a thymectomy method similar to a published method (Horton et al., 1998; Lin et al., 2013). In brief, tadpoles younger than stage 47 were anesthetized and their thymus glands were removed using surgical scissors and forceps (Fine science tools, 15000-08 and 11251-35). The animals were raised and used as hosts for cell transplantation and bulk tissue grafting mentioned below.



Bulk tissue grafting

For bulk tissue grafting, both Venus⁺ transgenic donor and thymectomised wild-type host froglets were anesthetized in 0.03% MS-222. For bone transplantations, tibio-fibula were isolated from Venus⁺ transgenic froglets. To remove the surrounding tissue, isolated bone was treated with Liberase solution for 20 minutes at 37 degrees (15μl of Liberase TM (Roche, 5401127001), 26 Wüsch units/ml, 30μl of DNaseI (Roche, 4716728001), up to 3ml with diluted DMEM (300ml of DMEM, 4ml of Penstrep, up to 400ml with water)). After adding the same amount of diluted DMEM with 10% FBS, the remaining surrounding tissue was removed mechanically using fine forceps. Clean bone was washed with 0.8x PBS and transplanted into a wild type host from which the tibio-fibula was removed. For muscle transplantations, a small incision was made on the host calf skin using surgical scissors and a small piece of Venus⁺ muscle was transplanted into the host calf region. For tendon transplantations, the Achilles tendon was isolated from a Venus⁺ froglet and transplanted into the host calf region. For skin transplantation, half of the host calf/shank skin was replaced with Venus⁺ donor skin. Bone was not injured by this procedure. The incision and replaced skin were closed using surgical sutures (B.Braun, G111434). During operation, donor and host froglets were covered with clean paper towels wetted with MS-222 solution. The host animals were placed into sterile water with Pen/Strep after operation. 9-days after operation, the tissue grafted froglet was anesthetized in 0.03% MS-222 and their lower hind limb was amputated through the grafted site. 4-weeks after limb amputation, animals were anesthetized in 0.03% MS-222 and the regenerated spike was fixed in modified Zamboni fixative (2%PFA, 0.1% Saturated picric acid, 40mM NaH₂PO₄, 120mM Na₂HPO₄) overnight at 4°C, washed in PBS, treated with 8% EDTA/1xPBS pH7.4, incubated in 15% and 30% sucrose/1x PBS overnight at 4°C, and frozen in OCT compound (Science Service, 62550-12) and stored in -80°C ready for cryosection and further analysis.

Blastema collection and cell isolation

All tools were sterilized beforehand by autoclave. For cell transplantation experiments, 2.0 - 2.2 cm snout-to-cloaca stage 66 froglets and 8 - 9 cm snout-to-tail axolotls were used as the blastema donors. Regenerative blastema were prepared using stage 53 developing tadpoles. Green- (*CAGGs:Venus* for frogs; *CAGGs:GFP* for axolotls) or red- (*CMV:tdTomato* or *CAGGs:mCherry* for frogs; *CAGGs:mCherry* for axolotls) fluorescent animals of the correct stage and size were collected under a microscope. Legs of the selected frogs or axolotls were amputated with disposable scalpels at the upper leg level close to the knee. After the extruded bone was trimmed using spring scissors (Fine Scientific Tools, 15000-4), animals were allowed to recover in clean water briefly until bleeding stopped. They were then transferred to clean water with 0.5x Pen-Strep. Daily feeding and cleaning paradigms were applied until the blastema samples were ready to be collected and the animals were sacrificed after blastema collection.

On the day of blastema collection, blastema samples (blastema plus stump) were collected under a stereoscope (Olympus, SZX16) in a hood and washed twice in 0.7x PBS at room temperature. They were then dipped in 70% ethanol for 10 seconds and immediately washed twice in 0.7x PBS at room temperature. To expose the blastema mesenchyme, the muscle bundles surrounding the blastema were carefully removed by spring scissors under green or red fluorescence channel depending on the genotype of the animals. Blastema mesenchyme can be distinguished at the tip of the sample and was harvested using a disposable scalpel before being transferred to fresh 0.7x PBS. The blastema mesenchyme was chopped into <0.5mm cubes and immediately transferred to a tube of digesting solution (0.26WU/ml Liberase TM in 0.7x PBS). The enzymatic digestion was placed on a rotating wheel at room temperature for 45 minutes and then the cells were mechanically dissociated by pipetting 10-15 times with a P1000 pipet tip. Enzymatic reactions were stopped by adding 1x volume high-serum culture medium (HS-AMEM, 125ml MEM, 20ml heat inactivated FBS (Sigma-Aldrich, F7524), 2ml insulin (1mg/mL, Sigma-Aldrich, I5000), 2ml glutamine (200mM, Gibco, 25030-024), 2ml Pen/Strep (10000U/mL, Sigma-Aldrich, P0781), 50ml ddH₂O), and samples were filtered sequentially through 70 μm and 30 μm MACS Smart-Strainers (Miltenyi Biotec, 130-098-458 and 130-098-462). Dissociated cells were then pelleted using 300x rcf centrifugation for 5 minutes at room temperature, then washed twice with 0.7x PBS before resuspension in serum-free medium (114.3ml F12:DM EM⁺ Glutmax (Gibco, 10565018), 1.5ml Sodium pyruvate (Gibco, 11360070), 1.5ml B27 supplement, 1.5ml MEM NEAA (Gibco, 11140050), 1.5ml insulin (1mg/mL, Sigma-Aldrich, I5000), 1.5ml Pen/Strep (10000U/mL, Sigma-Aldrich, P0781), 28.2ml ddH₂O) on ice. The concentration of cells was determined using a hemocytometer. For limb bud donor cells, stage 51 limb bud cells of fluorescent tadpoles were collected and prepared with the same procedure. For scRNA-seq library prep, dissociated frog cells were further filtered through a 10μm cell strainer (BD Biosciences, 340732).

Cell transplantation

The number of cells required for transplantation was calculated, and then pelleted in a sealed yellow Pipetteman tip by centrifugation. The yellow tip was then cut open near the level of cell pellet and placed on a transparent tape in a 3-cm petri-dish ready for loading. To load the injecting glass pipet with a high concentration of cells, the remaining medium in the yellow tip was first removed as much as possible without resuspending the pellet. The cells were then sucked directly into a glass pipet connected to a nanoject II system (Drummond SCI, 3-000-204). Meanwhile, stage 50 thymectomised wildtype tadpoles (Nieuwkoop and Faber, 1956) were anesthetized and laid belly-up on a piece of wet tissue paper in a 10-cm petri-dish. The limb bud was oriented under a stereoscope, the head and the tail regions were covered with additional pieces of wet tissue paper to prevent the animal from drying. The glass pipet pierced the epidermis at the proximal region of the limb bud and was moved distally until the tip of the pipet reached the distal mesenchyme without piercing the epidermis. Cells were then carefully injected into the distal region of the limb bud. Cells were injected into either one or both limb buds of the same tadpole. The transplantation procedure was repeated until the donor cells in the glass pipet were depleted. More than 10000 cells were estimated to be injected per limb bud (Figure S4K). The transplanted limb buds were

immediately imaged after transplantation (0-day post-transplantation, 0 dpt), and were traced two times per week until collection at around 4–5 weeks. Mature legs of frogs and axolotl were collected and fixed in 4% MEMFA (1x MEM (41.86g MOPS, 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.52g EGTA, dissolve in H_2O and fill up to 200mL, pH 7.4), 3.7% formaldehyde (Sigma-Aldrich, 1040031000)) on a rotor in a cold room overnight. Samples were washed three times in PBS before decalcification in 8% EDTA/PBS overnight in the cold room. The next day, the samples were washed three times in PBS and dehydrated with 30% sucrose overnight in the cold room. They were then embedded in OCT and stored in -20°C ready for cryosection and further analysis.

Cryosection, immunostaining and microscopy

Samples were sectioned into 8 μm thickness on loading slides with a cryostat (NX70, Thermo Scientific). Cryosections were dried and stored in -20°C . Before immunostaining, slides were thawed and were stained with primary antibodies against GFP (Rockland, 600-401-215, 1:1000, and MPI-CBG, Dresden, 1:1000), mCherry (Acris Antibodies, AB0040-200, 1:1000), Sox9 (Chemicon, Ab5535, 1:200, and R&D, AF3075, 1:200), Prrx1 (Gerber et al., 2018), Pax7 (MPI-CBG, Dresden, 1:200) and Col1A2 (Developmental Studies Hybridoma Bank, SP1.D8, 1:50). Alexa 488, Alexa 568, Alexa 647 (Invitrogen, 1:500, see key resources table for catalog details) were used as secondary antibodies. All sections were stained with DAPI (Sigma-Aldrich, D9542, 1:1000) and mounted in Mowiol 4-88/DABCO mounting medium (Sigma-Aldrich, 9002-89-5).

For samples of the bulk tissue transplantation, images were taken using an Olympus VS110 microscope and analyzed using FIJI and QuPath software (Bankhead et al., 2017). The number of Venus-labeled cells in the stump (500 micrometers from amputation plane) and Venus-labeled cells in the spike were counted. The number of Venus-labeled cells was calculated as a ratio of the percentages of Venus labeled cartilage cells in the spike over the total number of Venus labeled cells in the spike (Table S1). Two sections each from two different samples were used for counting. For the cell transplantation samples, images were taken with either a Zeiss Axio Imager Z2 (upright) with sCMOS camera, or a Zeiss LSM780 Axio Observer (inverted). At least 3 sections of each sample were counted using the ZEN Blue software.

Single-cell RNA sequencing

For frog blastema and limb bud samples of different stages, cells were isolated as mentioned above and cDNA generated following the Chromium Single Cell 3' Kit protocol (10X Genomics, 3' library kit, v3 chemistry). For one of the mature uninjured and the 3 dpa samples, converted *Prrx1:CreER;CAGGs:lp-Cherry* cells from the upper legs were dissociated and isolated by FACS (FACSaria III, BD Biosciences) with a 100 μm nozzle prior to cDNA generation. Cell concentration was estimated manually using a hemocytometer and cells were diluted to obtain approximately 5,000–7,000 cells for non-pooled experiments and to obtain 12000–15000 cells in pooled experiments per lane of a 10x microfluidic chip device. Pooled experiments were performed for the blastema time course where cells of 3 different time points with each animal batch expressing a different fluorescent color signal were pooled and for the stage 54 limb bud cells that were pooled with a non-sorted mature uninjured tissue (0 dpa). Cells of blastema time points 7, 10 and 14 dpa (early blastema) were pooled and cells of blastema time points 14, 20 and 52 dpa (late blastema) were pooled, respectively. Single-cell cDNA and sequencing libraries were generated following the manufacturer recommendations (10X Genomics, 3' library kit, v3 chemistry). The quality of the cDNA and resulting sequencing libraries were checked by either bioanalyzer (High Sensitivity DNA Kit, Agilent, 5067-4626). The libraries were sequenced using Illumina NovaSeq platforms PE100 or PE150.

For axolotl limb bud samples, the stages of axolotl embryos were confirmed under a stereomicroscope before stage 50, 52, and 54 limb buds were collected. Cells of each stage, including epidermis, were dissociated and prepared using the same Liberase protocol described above. scRNA-seq libraries were prepared following the Chromium Single Cell 3' Kit protocol (10X Genomics, 3' library kit, v3 chemistry), and were sequenced using Illumina NovaSeq platforms PE100. For axolotl blastema samples, cells of two blastema time points (5 and 11 dpa) were isolated from *Prrx1:CreER* axolotl strains as described in (Gerber et al., 2018) without FACS enrichment. scRNAseq experiments were performed following the Chromium Single Cell 3' Kit protocol (10X Genomics, 3' library kit, v2 chemistry) as described in Gerber et al. (2018). Three 10x lanes were used for 5 dpa blastema cells and two 10x lanes for 11 dpa cells. The quality of the cDNA and resulting sequencing libraries were checked by a bioanalyzer. The libraries were sequenced on an Illumina HiSeq2500.

Computational analysis

Cell Ranger (10x Genomics) was used to align sequencing reads using default parameters to the *Xenopus laevis* v9.2 reference genome with fluorescent marker genes being manually added to the reference. The genome was downloaded from Xenbase (<http://www.xenbase.org/>, RRID:SCR_003280) (Karimi et al., 2018). Reads originating from axolotl libraries were mapped against the latest axolotl genome release (Nowoshilow and Tanaka, 2020; Schloissnig et al., 2021) using STARsolo (STAR 2.7.5a) (Dobin et al., 2013) with parameters mimicking Cell Ranger's (10x Genomics) transcript counting strategy. Fluorescent marker genes were manually added to the references, respectively. Different versions of Seurat (Butler et al., 2018) were used throughout the analysis. Seurat v2 was used for raw data characterization and cell type assignment. Seurat v3.1 was used to generate all panels shown in the manuscript. Ribosomal protein genes and pseudogenes were excluded from the analysis besides the transplantation experiment analysis. Generally, cells with more than 60,000 or less than 1,000 detected transcripts, as well as those with a mitochondrial transcript proportion higher than 20% were excluded with some exceptions (see full information in Tables S2 and S3). When clusters were identified to be still driven only by low RNA counts in a first screening, cells belonging to these clusters were removed. For CT-enriched sorted cells some contaminating cell types such as epidermal cells were identified and removed after an initial screening using Seurat. *Xenopus laevis* carries a tetraploid genome



and encodes for almost all genes a L and S version of the same gene. Whenever L or S is not designated in the manuscript and both genes were expressed, the version with the higher expression intensity (most often L) is represented.

For visualizations in the main figures and the supplementary cell atlases, Seurat's built-in scale function was used to regress out differences in nUMI, nGene, percent.mt and cell cycle effects. Cell cycle effects were not considered in the first round of data screening. Scaling and PCA were always performed on all genes. Uniform Manifold Approximation and Projection (UMAP) was applied to the top principal components (PCs) for visualization. Data integration was performed for the blastema time course, the transplantation experiment, and combined limb bud and blastema data (frog and axolotl) using Seurat's built in Harmony (Korsunsky et al., 2019) function to reduce the confounding effect of different batches and samples. Running Harmony was always performed with default parameters and 50 input PCs. For the blastema time course the top 50 harmony components obtained were converted to positive values by adding the absolute minimum for each harmony component across the cells for that component. The corrected matrix was used as input into SPRING (Weinreb et al., 2018) using the SPRING webpage (<https://kleintools.hms.harvard.edu/tools/spring.html>). SPRING was run with 50 input PCs, 4 nearest neighbors and a gene variability percentile of 20% and no other filters were applied. The dynamic mode was used to visualize the SPRING embedding. The pseudotime estimate was calculated by a diffusion map analysis (Haghverdi et al., 2016) using the R package *destiny* (Angerer et al., 2016) followed by ranking cells based on their raw diffusion pseudotime. Gene ontology (GO) enrichment analyses were performed by using DAVID Bioinformatics Resources 6.7 (Huang et al., 2009) using time point specific cluster markers as input genes. Quadratic programming was performed using the R package *quadprog*. Top and bottom fold-change genes between mature and stage 54 limb bud samples based on time-point-averaged gene expression values were estimated by subtracting values of respective time points. Top and bottom 100 hits, respectively, were used as input gene set for quadratic programming. Fluorescent markers were removed when found on the list. Cartilage scores were obtained by summing expression values of shared cartilage marker genes for frog and axolotl blastema and limb bud developing cartilage cells. Common cartilage markers for frog developing cartilage were *col2a1.L*, *col2a1.S*, *col9a1.L*, *col11a2p1.S*, *col9a2.S*, *col9a3.L*, *col9a2.L*, *LOC108702653*, *matn4.L*, *matn4.S*, *col9a1-b*, *otos.S*, *otos.L*, *LOC101730276.L*, *fgfr3.L*, *spock3.S*, *rspo3.L*, *fgfr1.S*, *plod2.L*, *ftth1.L*, *LOC108717350*. Common cartilage markers for axolotl developing cartilage were *matn1*, *acan*, *crip1*, *hapln1*, *otos*, *cnmd*, *fgfbp2*, *epyc*, *c17orf67*, *loc108704014*, *col11a2*, *col9a3*, *col2a1*, *papss2*, *col9a1*, *ecrg4*, *loc115080956*, *col9a2*, *pdcd6*, *col11a1*, *rbp4*, *ucma*, *clec3a*, *matn4*, *fbn1*, *papss1*, *cthr1*, *nog*, *lox13*, *plod2*, *hapln3*, *pcolce*, *xylt1*, *fgfr3*, *sox9*, *tgm2*, *loc115477099*, *sox6*, *pdgfrl*, *cox4i2*, *scrg1*, *gyg1*, *sox8*, *smoc1*, *aldb*, *cpa6*, *lmcd1*, *chst3*, *sfrpx*, *loc104277804*, *lox14*, *ldhb*, *slc35d1*, *mob2*, *srxp*. Differentially expressed genes between frog or axolotl limb bud and blastema cartilage development were identified by using Seurat's default DE analysis function, respectively, by comparing cells having a high cartilage score in either frog limb buds or blastema against cells that have a lower score in the same sample type as well as all cells of the other tissue type and vice versa.

In addition, a recently published scRNA-seq data set (GEO accession number: GSM3206455) on axolotl blastema formation was re-analyzed (Samples: GSM3206452, GSM3206453, GSM3206454, GSM3206455, GSM3206456, GSM3206457, GSM3206458, GSM3206459) (Gerber et al., 2018).

Identifying samples in pooled scRNA-seq

For pooled *Xenopus* scRNA-seq experiments samples were identified by expression of fluorescent markers in an initial screening using Seurat and doublet discrimination was performed based on fluorescent label expression. However, some fluorescent markers were weakly expressed and showed therefore a poor detection rate, hampering sample and doublet assignment. For the blastema time course, cells of the 10 dpa sample (early blastema pool) and the 20 dpa sample (late blastema pool) had almost no fluorescent signal detected. As the separation of early and late blastema was possible through the different batches we assigned all cells without a detected fluorescent label to 10 dpa for the early blastema and to 20 dpa for the late blastema. Potential doublets and misassigned blastema time points that were potentially not identified due to missing fluorescent signal do not critically interfere with the conclusions we state in the manuscript. Similarly, the fluorescent label for the mature cells in the stage 54 limb bud-mature pooled experiment was affected. In contrast to the blastema pool, cells could be easily differentiated by their expression profile and mature CT cells could be isolated from stage 54 limb bud cells by selecting cells from sample specific clusters.

After isolating individual samples from pooled experiments, cells that passed quality control were clustered as mentioned above. The CT cell clusters of each sample (pooled and non-pooled experiments) were identified by CT-specific markers (e.g. *prrx1*) and then isolated using the subset function in Seurat. The extracted CT cells of respective time points were computationally pooled prior to proceeding to time course analyses shown in Figures 2 and 3A.

QUANTIFICATION AND STATISTICAL ANALYSIS

At least three independent experiments were performed for each quantification unless specified. All quantifications were performed with at least three animals, with a minimal of three representative and distinct sections being imaged and counted per animal. Two-tailed unpaired Student's t test was performed using GraphPad Prism version 8 and 9 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com). Data were presented as mean $\pm$ standard deviation (SD). Asterisks indicate statistically significant difference between groups. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. The statistical details can also be found in the corresponding figure legends.

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Supplemental information

**Fibroblast dedifferentiation as a determinant
of successful regeneration**

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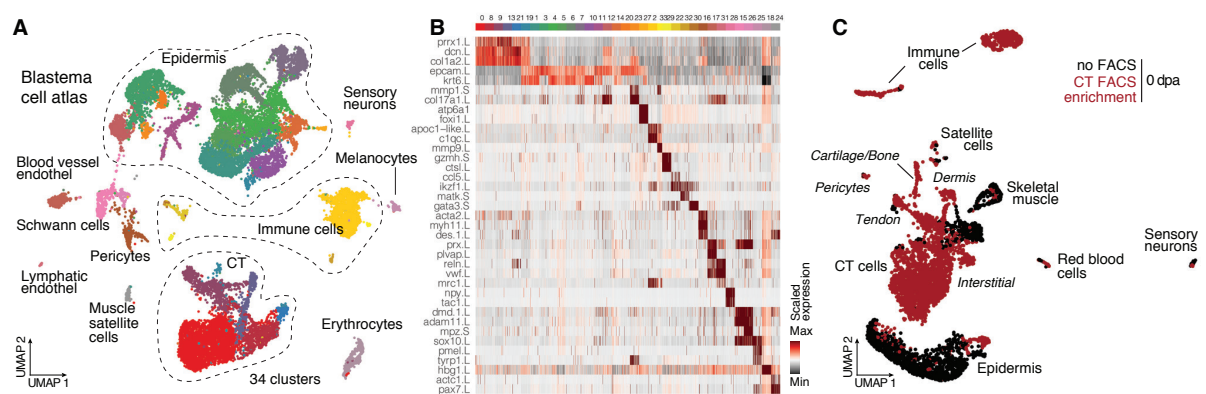


Figure S1. Frog Limb Blastema Cell Atlases. Related to Figure 2.

(A) UMAP embedding of scRNA-seq data of 20,139 froglet blastema cells over a time course of 7, 10, 14, 20 and 52 dpa. Cells are colored by cluster. Cell type annotations based on canonical markers are shown next to corresponding cells.

(B) Heatmap visualization of canonical marker gene expression used to assign cell types in (A) for cells. Colors in top bar correspond to cluster colors in (A). Only 50 cells are visualized per cluster.

(C) UMAP embedding of scRNA-seq data of uninjured mature frog CT cells with one set of cells being enriched through FACS (dark red) and one without FACS (black). Data integration using Harmony (Korsunsky et al., 2019) was performed.

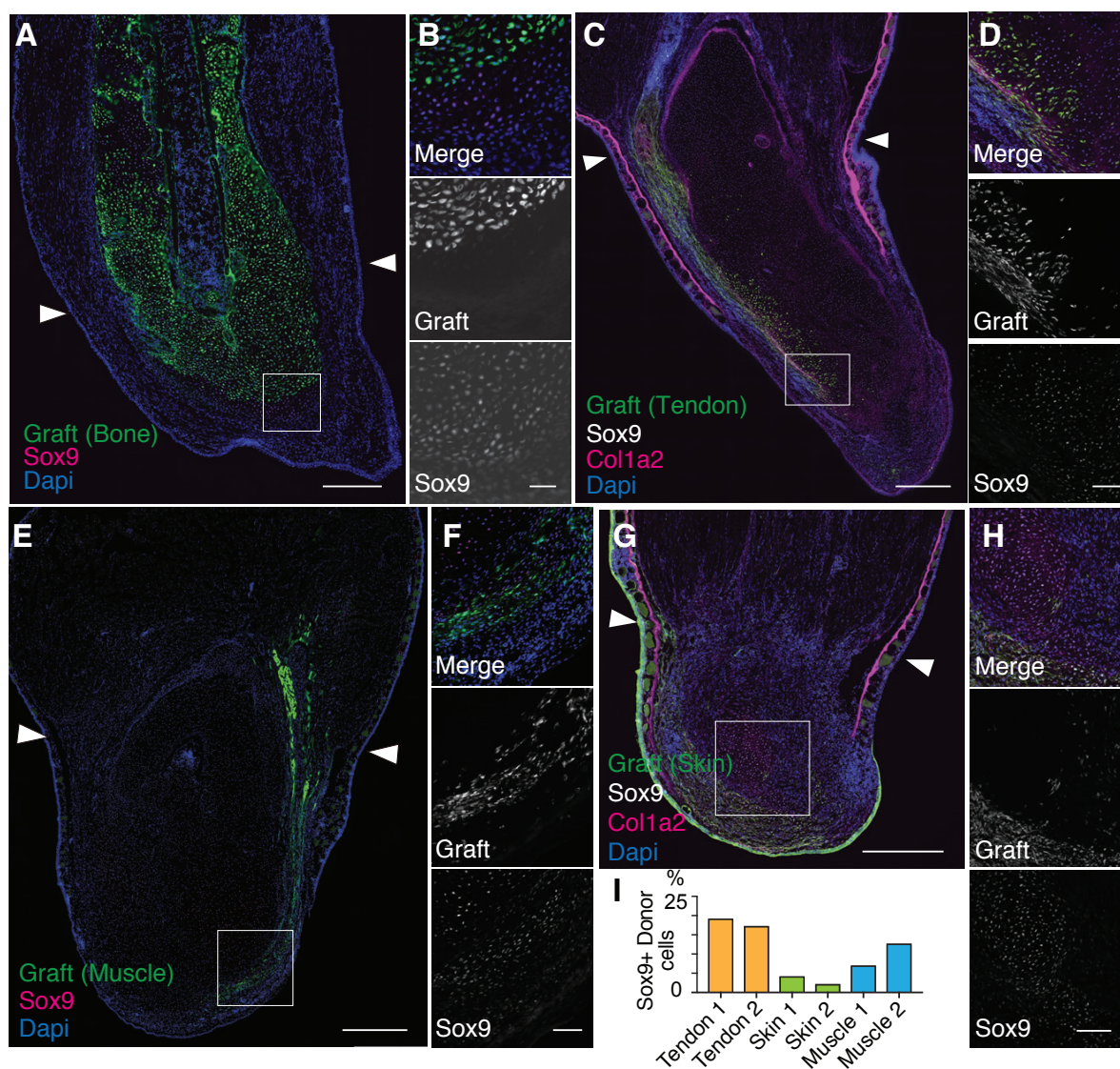


Figure S2. Tissue Scale Transplantation Experiments in *Xenopus laevis* Froglets. Related to Figure 2.

(A, C, E and G) Example image of longitudinal sections from froglets transplanted with GFP+ bone (A), tendon (C), muscle tissue (E), and skin (G). Blue: DAPI-stained nuclei. Magenta/white: Sox9 immunostaining (chondrocytes). Green: GFP immunostaining (donor cells). Magenta: Col1a2 immunostaining. White boxes indicate the respective field of view in (B), (D), (F) and (H). Arrowheads indicate amputation plane. Scale bars represent 500 μ m.

(B, D, F and H) Insets from (A), (C), (E) and (G), respectively, showing overlay of immunofluorescence signals (top), only GFP (donor cells from the grafted tissue, center) or Sox9 (chondrocytes, bottom). Scale bars represent 100 μ m.

(I) A bar plot showing the percentage of Sox9-expressing GFP+ donor cells in tendon (orange), skin (green), and muscle (blue) transplants. Two samples of each transplantation type were analyzed in this study.

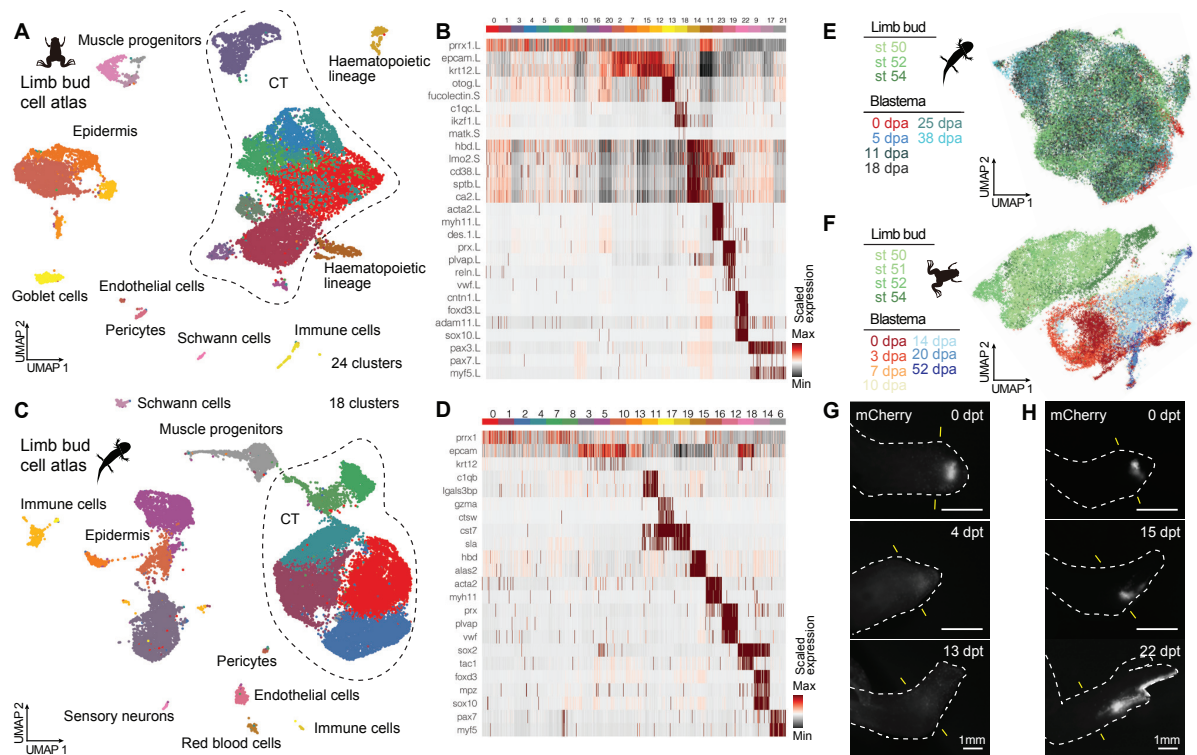


Figure S3. Frog and Axolotl Limb Bud Cell Atlases. Related to Figure 2 and Figure 3.

(A) UMAP embedding of scRNA-seq data of 16,120 tadpole cells originating from embryonic stage 50, 51 and 52. Cells are colored by cluster. Cell type annotations based on canonical markers are shown next to corresponding cells.

(B) Heatmap visualization of canonical marker gene expression used to assign cell types in (A). Only 50 cells are visualized per cluster.

(C) UMAP embedding of scRNA-seq data of 29,768 larval axolotl limb bud cells originating from embryonic stage 50, 52 and 54. Cells are colored by cluster. Cell type annotations based on canonical markers are shown next to corresponding cells.

(D) Heatmap visualization of canonical marker gene expression used to assign cell types in (C). Only 50 cells are visualized per cluster.

(E) UMAP embedding of scRNA-seq data of 43,915 axolotl CT cells originating from different limb bud stages, blastema states and mature uninjured CT cells. Cellular transcriptomes were integrated using Harmony (Korsunsky et al., 2019).

(F) UMAP embedding of scRNA-seq data of 26,152 frog CT cells originating from different limb bud stages, blastema states and mature uninjured CT cells. Cellular transcriptomes were integrated using Harmony (Korsunsky et al., 2019) with exactly the same parameters as used for axolotl data integration shown in (E).

(G) Fluorescence images of cross-species transplantation. Unsorted frog mCherry⁺ blastema cells were transplanted to non-fluorescent axolotl blastemas and the mCherry signal were traced for 13 days. Fluorescence signal rapidly disappeared at day 4 (n=4). White dashed lines outline the samples. Yellow lines indicate the amputation planes. Scale bars represent 1 mm.

(H) Fluorescence images of control transplantation experiments of (G). Unsorted axolotl mCherry⁺ blastema cells were transplanted to non-fluorescent axolotl blastemas and the mCherry signal were traced for 22 days. Donor mCherry⁺ cells were consistently observed in the patterning limb (n=4). White dashed lines outline the samples. Yellow lines indicate the amputation planes. Scale bars represent 1 mm.

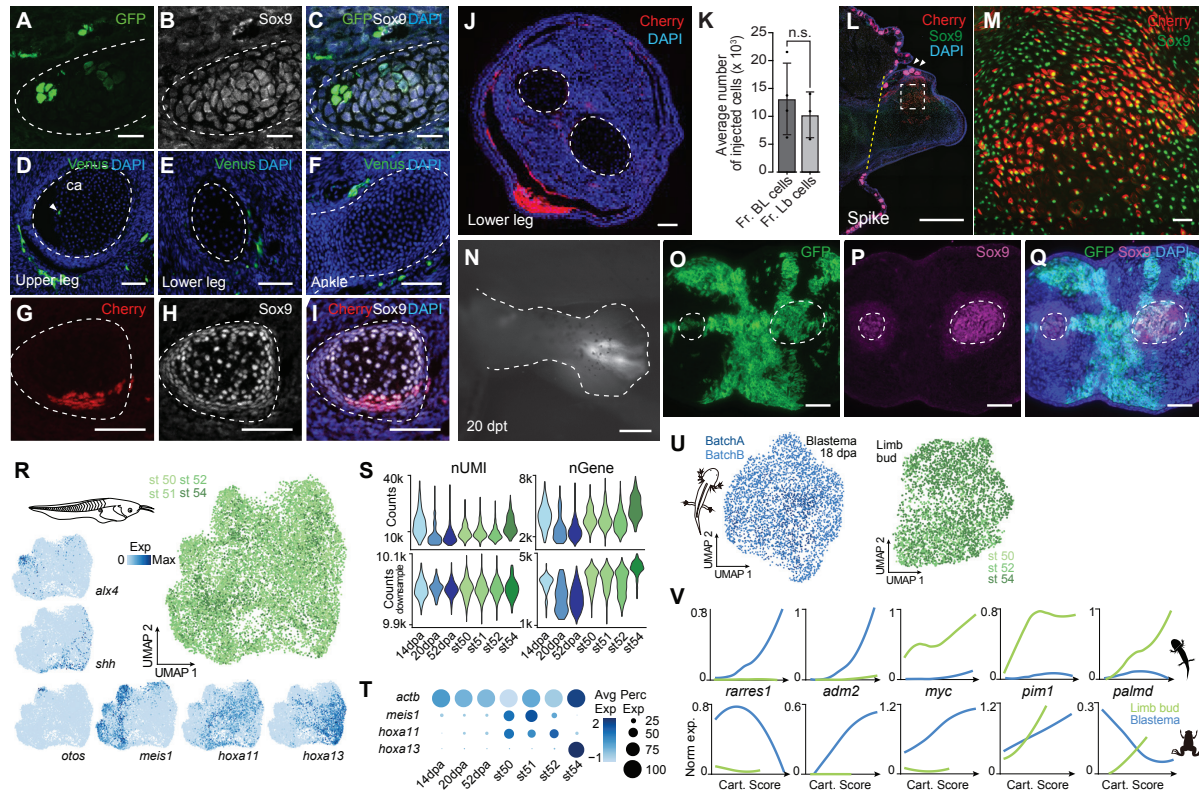


Figure S4. Heterochronic Transplantation Assay and Limb Bud Single-Cell RNA-seq Analysis of Frog and Axolotls. Related to Figure 3 and Figure 4.

(A–C) A transverse section at the foot level from a developing axolotl limb transplanted with unsorted axolotl early embryonic limb bud cells (GFP⁺) 22 days earlier. Sections were immunostained for GFP (donor cells, green) (A), Sox9 (Chondrocytes, white) (B) and the combined overlay with DAPI (nuclei, blue) (C). Dashed circles indicate the cartilage region. Scale bars represent 50 μ m.

(D–F) Transverse sections at the levels of upper leg (D), lower leg (E), and foot (F) regions from a developing tadpole limb transplanted with unsorted frog 14 dpa blastema cells (Venus⁺) 21 days earlier. Sections were immunostained for GFP (donor cells, green) and DAPI (nuclei, blue). Dashed circles indicate the cartilage regions. The white arrowhead indicates autofluorescence. Scale bars represent 50 μ m.

(G–I) A transverse section at the foot level from a developing tadpole limb transplanted with unsorted stage 50 tadpole limb bud cells (mCherry⁺) 21 days earlier. Sections were immunostained for Cherry (donor cells, red) (G), Sox9 (Chondrocytes, white) (H) and the combined overlay with DAPI (nuclei, blue) (I). Dashed circle outlines the cartilage regions. Scale bars represent 50 μ m.

(J) A transverse section at the lower leg level of a developing tadpole limb transplanted with FAC-sorted Prrx1-converted cells (mCherry⁺) from uninjured frog upper legs 21 days earlier. Sections were immunostained for Cherry (donor cells, red) and DAPI (nuclei, blue). Dashed circles indicate the cartilage regions. Scale bar represents 100 μ m.

(K) Bar plot showing the average number of injected frog donor cells per transplant sample. The number of cells transplanted per sample is comparable for both frog blastema-to-limb bud (Fr. BL cells, N=4 experiments) and frog limb bud-to-limb bud (Fr. Lb cells, N=3 experiments). Statistical significance is calculated using unpaired Student's t test. n.s.: $p > 0.05$.

(L) A transverse section from a froglet spike transplanted with unsorted frog 14 dpa blastema cells (mCherry⁺) 30 days earlier. The section was immunostained for Cherry (donor cells, red), Sox9 (Chondrocytes, green), and DAPI (nuclei, blue). White arrowheads indicate autofluorescence. Yellow dashed line indicates the amputation plane. White dashed box indicated the field of view in (M). Scale bar represents 1mm.

(M) Inset of (L). Only the overlay of Cherry (red) and Sox9 (green) signals is shown. Scale bar represents 50 μ m.

(N) Fluorescent images of a developing tadpole limb 20 days post-transplantation of unsorted Venus-expressing 10 dpa blastema cells of stage 53 frog limb buds (regenerative stage). White dashed lines outline the host limb buds. Scale bar represents 500 μ m.

(O–Q) Transverse sections at the digit level of (N) immunostained for GFP (donor cells, green) (O), Sox9 (chondrocytes, magenta) (P), and the combined overlay with DAPI (nuclei, blue) (Q). Dashed circles indicate the cartilage regions. Scale bars represent 50 μ m.

(R) UMAP embedding of scRNA-seq data of 13,028 CT cells from a tadpole limb bud time course after data integration using Harmony (Korsunsky et al., 2019). Cells are colored by embryonic stage. Feature plots show expression of proximal-distal (*meis1*, *hoxa11*, *hoxa13*) and anterior-posterior (*alx4*, *shh*) patterning genes as well as *otoc* as mature cartilage marker.

(S) Violin plots show the detected number of transcripts (UMI) and genes per sample across 3 frog blastema time points and 4 frog limb bud stages. Top: Raw transcript counts for the gene-cell-matrix that was used to generate the dotplot in Figure 4D. Bottom: Transcript counts and corresponding gene counts for the same but downsampled gene-cell-matrix that was used to generate the dotplot in (T).

(T) Dotplot showing gene expression intensity and frequency differences between developing frog tadpole limb bud cartilage and frog blastema cartilage cells after downsampling transcripts to similar values (see Figure S4S). Avg Exp: Average expression values, Perc. Exp: Percent of cells expressing each gene.

(U) UMAP embeddings of scRNA-seq data of 3,720 cartilage cells from an 18 dpa axolotl blastema (left) and 3643 cartilage cells from 3 different axolotl limb bud stages (right). Cells are colored by batch or stage, respectively.

(V) Gene expression visualization for differentially expressed genes between axolotl limb bud and blastema cartilage differentiation across the cartilage scores in frog (bottom) and axolotl (top) limb bud and blastema samples. A loess fitted curve through the data points is shown. Green: limb bud. Blue: blastema.

Tissue type	Fractions of <i>Prrx1</i> ⁺ cells in the uninjured upper leg ¹	Expand ratio ²	Contribution to blastema ³	Fraction of Sox9 ⁺ cells in the expanded population ⁴	Contribution to Sox9 ⁺ cartilage in blastema ⁵
Interstitial CT	86.4%	3.15	82.9%	10%	85.6%
Tendon	4.1%	4.62	5.8%	19%	10.6%
Dermis	4.9%	7.59	11.3%	3%	3.8%
Bone	3.6%	ND	ND	ND	ND
Others	1%	ND	ND	ND	ND

Table S1. Contribution of CT Cell Populations to Frog Blastema and Developing Cartilage. Related to Figure 2.

¹based on scRNA-seq analysis of uninjured upper leg (0 dpa);

²calculated by dividing the GFP⁺ cells in the blastema region over GFP⁺ cells within the 500 µm zone proximal to the amputation plane;

³calculated by multiplying (1) and (2) and then divided by the three populations (interstitial CT, tendon, and dermis) combined;

⁴calculated by dividing the GFP⁺/Sox9⁺ cells over total GFP⁺ cells in the blastema region;

⁵calculated by multiplying (1), (2), and (3) and then divided by the three populations (interstitial CT, tendon, and dermis) combined; ND: not determined.

Feature	0 dpa	3 dpa	Early BL	Late BL	14 dpa	Trans-plant	Stage 50 Lb	Stage 51 Lb	Stage 52 Lb	Stage 54 Lb/ 0 dpa
nUMI high	30,000	60,000	40,000	40,000	40,000	60,000	40,000	40,000	40,000	40,000
nUMI low	2,000	2,000	3,000	2,000	3,000	2,000	2,000	2,000	2,000	3,000
mt	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.1	0.1
Sample mix	Single	Single	Pool	Pool	Single	Single	Single	Single	Single	Pool
Genotype	Prrx1	Prrx1	Venus (7dpa); Cherry (10dpa); TFP (14dpa)	TFP (14dpa); Cherry (20dpa); Venus (52dpa)	Venus	Cherry (Host); Venus (Donor)	GFP	GFP	GFP	TFP (st54); tdTom (0dpa)
FACS	Yes	Yes	No	No	No	No	No	No	No	No

Table S2. Metadata for *Xenopus* scRNAseq Analysis. Related to STAR methods.

Number of transcripts (nUMI) and Fraction of mitochondrial genes (mt) were used to remove low quality cells as well as cell multiplets from the data sets. Some experiments were run as cell pools with cells expressing different fluorescent labels (see STAR Methods for details). CT enrichment by FACS was performed for some experiments. Prrx1: *Prrx1:CreER;CAGGs:lp-Cherry*. Venus: *CAGGs:Venus*. Cherry: *CAGGs:mCherry*. TFP: *CAGGs:nucTFP*, tdTom: *CMV:tdTomato*.

Feature	0 dpa	5 dpa	11 dpa	18 dpa	25 dpa	38 dpa	Stage 50	Stage 52	Stage 54
nUMI high	15,000	30,000	30,000	10,000	20,000	15,000	25,000	25,000	25,000
nUMI low	1,000	500	500	1,000	1,000	1,000	1,000	1,000	1,000
mt	0.1	0.1	0.1	0.05	0.05	0.05	0.2	0.2	0.2
FACS	Yes	No	No	Yes	Yes	Yes	No	No	No

Table S3. Metadata for Axolotl scRNAseq Analysis. Related to STAR methods.

Number of transcripts (nUMI) and Fraction of mitochondrial genes (mt) were used to remove low quality cells as well as cell multiplets from the data sets. CT enrichment by FACS was performed for some experiments. Samples 0, 18, 25 and 38 dpa are published data sets and were reanalyzed from Gerber et al., 2018. (see STAR Methods for details).

III. Discussion & Outlook

The purpose of my study was to uncover the mechanisms of limb regeneration by systematically comparing the molecular and cellular behaviors of limb CT cells during the limb regeneration of two amphibian species: axolotls and the African clawed frogs. I first established transgenic frog lines that allow the labeling of limb CT cells in a temporal manner. The combination of this genetic tool and the single cell RNA-seq (scRNA-seq) analysis revealed that in contrast to axolotl, frog CT cells only undergo a partial dedifferentiation. Notably, this correlates with their inability to form a patterned limb. Using a universal cell transplantation assay, I confirmed the existence of cell intrinsic limitations of frog blastema cells. The specifics of these limitations are yet to be determined, but it is likely to be acting at the epigenetic level. The work was published as two scientific research articles, where the conclusions for each specific topic were discussed. In this section, I will discuss the challenges and outlook in a broader dimension.

Limb regeneration involves multiple cell types and to gain a comprehensive understanding of this process, investigating the behavior and function of individual cell types is inevitable and important. This need becomes evident when considering an emerging methodology to improve frog limb regeneration by ectopically applying drugs or molecules. For example, host frogs transplanted with growth factor-soaked beads (FGF10, SHH, Thymolysin b) and embryonic limb cells at the amputation site regenerated multiple bone segments (Lin 2013). More recently, a “biodome” device was developed to locally apply progesterone or a combination of five different molecules (1,4-DPCA, growth hormone, bone-derived neurotropic factor, resolvin D5 and retinoic acid) at the amputation site of frogs and an increased bone length as well as the branching of spike was observed (Herrera-Rincon 2018 and Murugan 2022). While these proof-of-concept studies provide valuable insights and demonstrate the potential of medical applications, more questions were raised than solved. What are the target cell types of each molecule? Which signaling pathways are activated/deactivated by those molecules? How do the affected cell types impact the overall regeneration process? Such questions will have to be addressed to move forward.

Although the merit of having *Prrx1:CreER* frog lines was not fully utilized in my published work, the establishment of such lines is a key, long-term contribution of my PhD project to the research field of limb regeneration. This is because the ability to specifically label limb CT cells opens the doors to many possibilities. For example, in addition to lineage tracing, those labeled CT cells could be physically isolated, profiled and transplanted. One could perform *in vitro* assays on the cultured CT cells, such as drug screening to identify components of certain signaling pathways involving in regeneration. The temporal control of the *Cre-ERT²* system allows us to manipulate cells *in vivo* with minimal disruption of embryonic development. For example, one could replace the *mCherry* sequence at the second position of the *loxP* cassette with a gene of interest (GOI) to study the cell autonomous effect of GOI overexpression in the CT cells. One could also ablate CT cells using a nitroreductase-mediated cell ablating system (Sanchez-Iranzo 2018) or similar methods to study the effect of CT cell

depletion on limb regeneration. Perhaps the most exciting aspect of the *Prrx1:CreER* frog lines is in the perspective of cross-species comparison. Because *Prrx1:CreER* axolotl and mouse lines both exist (Gerber 2018 and Kawanami 2009), one could simultaneously conduct the same analysis on the limb CT cells of the three species and draw conclusions from their direct comparison. The possibilities are immense.

Dedifferentiation was defined as a qualitative term describing a reversion of cell state from a differentiated state to a progenitor state. However, in the era of single cell transcriptomic analysis, a new definition of dedifferentiation is needed to describe the process based on the transcriptome. In the Dev Cell paper, we used the similarity score calculated using quadratic programming analysis to quantitatively depict the extent of dedifferentiation. Comparing the scores across frog samples over the regeneration time points, we concluded that frog limb CT cells only partially dedifferentiated to the limb bud state, in contrast to the full dedifferentiation observed in axolotl CT cells. One dilemma is that the extent of CT cell dedifferentiation, as defined by the similarity scores, and the regenerative potency of the CT cells, as defined by the transplantation assay, did not seem to correlate with each other, even though they are both influenced by the gene expression profiles. For example, neither the frog CT cells from the uninjured tissue nor the CT cells from a 14dpa blastema were able to contribute to the host cartilage at all, despite the former having 0% similarity score and the later around 50% on average. The mismatch between the dedifferentiation status and cell potency calls for a closer examination.

A simple explanation could be that 50% dedifferentiation is not enough to reach the state required to have the potency of developmental cartilage progenitor, therefore the difference between 0% and 50% could not be distinguished by the transplantation assay. If this is the case, boosting the dedifferentiation status of frog blastema cells should help them acquire the potency to integrate into the developing cartilage, and hence searching for the methods to boost dedifferentiation should be the focus of future studies. It also would be informative to determine at which level of dedifferentiation do the frog blastema cells obtain such potency. Another approach would be to decrease the dedifferentiation level of axolotl blastema cells to determine the minimum level of dedifferentiation required for the chondrogenic potency.

Another consideration is needed to understand the full picture because the abovementioned hypothesis could not explain the fact that axolotl CT cells from the uninjured limbs were able to integrate into the host limb bud cartilage, despite having near 0% scores. After transplantation, the donor axolotl CT cells from the uninjured limb started to dedifferentiate under the influence of the permissive limb bud environment, and eventually led to their contribution to the host cartilages. In the frog transplantation experiments, although the transplanted frog CT cells started the dedifferentiation process, they could only partially dedifferentiate and thus did not acquire the potency to contribute to the developing cartilages of the limb buds. To test the hypothesis, one could isolate and profile the donor CT cells at various time points following transplantation and determine whether the donor CT cells increase their similarity scores. In the Dev Cell paper, we showed that the transcriptome

of the donor frog CT cells remained similar to the uninjured state 3 days after transplantation, but whether those cells have higher similarity scores was not examined. A more thorough design, including samples from multiple time points after transplantation and from the necessary controls, is required in the future studies.

While these hypotheses can explain the outcomes of the transplantation assays, they also imply that the similarity score of individual time points do not fully reflect the potency of cells defined by the current assay. This problem shows that we do not fully understand the molecular representation of dedifferentiation and that we haven't been able to extract relevant information from the transcriptome of a single time point to predict cell potencies. Improving our understanding of the transcriptomic representation of dedifferentiation that is relevant to regenerative potencies would be a key aspect in future studies.

We also need new measurements to assess cell potencies. One problem is that the readout of the current potency assay is binary while the nature of similarity scores is graded. For example, the current assay does not distinguish the potency of cells with 0% dedifferentiation and those with 50%. New measurements of cell potency that represent different dedifferentiation status are needed to better define dedifferentiation. In addition, having measurable milestones with smaller intervals would be beneficial if the goal is to improve regeneration by boosting dedifferentiation of CT cells. For example, it would be arguably easier to improve dedifferentiation of a cell from 0% to 25% than from 0% directly to 100%.

What is clear is that frog CT cells possess intrinsic limitations that prevent them from acquiring the full regenerative potency. Such limitations could exist at the multiple levels and require a further breakdown into testable hypotheses. One possibility is that frog CT cells perceive the environmental cues differently than the limb bud cells, i.e., certain receptors are either mis-expressed or expressed in a different ratio so that they invoke different downstream signaling cascades. To test this hypothesis, one could investigate the differentially expressed receptors using my scRNA-seq datasets and determine if certain signaling pathway is differentially activated in frog limb CT cells, and if manipulating the activity of such pathway would lead to an increase in the regenerative potency. Another approach is to perform a screen on the cultured frog CT cells and frog limb bud cells with a panel of common ligands to investigate the immediate early response by RNA-seq analyses. A parallel experiment using axolotl CT cells and axolotl limb bud cells should be included as another control dataset to confirm that the ligand-receptor interactome is the same between axolotl CT cells and limb bud cells.

The second possibility is that the genetic network required for a full dedifferentiation is not present in the frog limb CT cells. Specifically, one could identify differentially expressed genes between frog blastema and frog limb bud CT cells using the scRNA-seq datasets and look for genes that do not turn on/off during regeneration, with a specific focus on transcription factors and chromatin modulators in the candidate list. Once the candidates are identified, downstream analysis, such as overexpression or downregulation of the candidate genes, could be performed on the frog limb CT cells to determine whether the manipulation

of such genes could increase the potency of cells. The gene manipulation method could be either overexpression by electroporation, or gene knock-out by the CRISPR/Cas9 system. The readout for this strategy would be the transplantation assay, in which the cells are transplanted into a limb bud following the manipulation of gene expression.

The third possibility is that the epigenomic landscape of the frog limb CT cells does not change in favor of the acquisition of the full regenerative potency. It is likely that certain genomic regions that are important for cell potency remain inaccessible or inactive in the blastema. One example is the hypermethylation status of the sonic hedgehog enhancer sequence (Yakushiji 2007). To understand how the epigenomic landscape changes, one could perform ATAC-seq to map the open chromatin regions in the frog CT cells during regeneration or ChIP-seq to map the active and inactive chromatin regions by histone marks, with a focus on the non-coding chromosome regions. Two challenges remain to be addressed. First, there is no *de facto* controls with such analyses because the non-coding sequences are largely different between axolotls and frogs. The comparison therefore has to be confined to the conserved non-coding elements (CNEs). Identifying CNEs in both species would be the first task. Second, once we have the map of CNEs of both axolotls and frogs, and perhaps discovered the differentially activated CNEs between both species, new technologies are needed to manipulate the epigenetic status of CNEs to establish the casual effect of the CNE activity and the regenerative potency.

These three possibilities are not independent. Once having a substantial understanding on each of them, one inevitably has to look at them as one system to gain the full picture.

Rather than examining lack of regeneration at a vague, tissue-scale level, we have defined CT cells dedifferentiation as one key limitation in the partial regeneration of frogs. This allows for the analysis of regeneration restriction on a more precise, molecular level. Of course, there could be other cellular restrictions, such as differences in immune cells, or epidermal cell potency, and these would have to be further explored by others. What started as a seemingly simple question: how frogs fail to regenerate a patterned limb, led to the discovery of fundamental difference of the limb CT cells between frogs and axolotls. More questions emerged than answered. We are not interested in studying how frogs grow a spike *per se* but using their partial limb regeneration as a model to understand the principles of limb regeneration and what mechanisms may be limiting this process that arose during evolution. These principles can only be fully understood through the lens of the evolution, and it will be fascinating to determine if those mechanisms limiting regeneration in an amphibian are conserved in amniotes, or whether restrictions to regeneration arose independently along metazoan evolution.

IV. References

- Agarwal, S. K., & Niazi, I. A. (1980). Hind limb regeneration capacity in tadpoles of the Indian bull frog *Rana tigerina* Daud. *Indian J Exp Biol*, 18(2), 140-145. <https://www.ncbi.nlm.nih.gov/pubmed/7399594>
- Aztekin, C., Hiscock, T. W., Gurdon, J., Jullien, J., Marioni, J., & Simons, B. D. (2021). Secreted inhibitors drive the loss of regeneration competence in *Xenopus* limbs. *Development*, 148(11). <https://doi.org/10.1242/dev.199158>
- Brockes, J. P., & Kumar, A. (2005). Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science*, 310(5756), 1919-1923. <https://doi.org/10.1126/science.1115200>
- Butler, E. G. (1933). The effects of X-radiation on the regeneration of the fore limb of *Amblystoma* larvae. *Journal of Experimental Zoology*, 65(3), 271-315. <https://doi.org/DOI> 10.1002/jez.1400650302
- Butler, E. G., & Schotte, O. E. (1941). Histological alterations in denervated non-regenerating limbs of urodele larvae. *Journal of Experimental Zoology*, 88(1-2), 307-341. <https://doi.org/DOI> 10.1002/jez.1400880208
- Carlson, B. M. (1975). The effects of rotation and positional change of stump tissues upon morphogenesis of the regenerating axolotl limb. *Developmental Biology*, 47(2), 269-291. [https://doi.org/10.1016/0012-1606\(75\)90282-1](https://doi.org/10.1016/0012-1606(75)90282-1)
- Carlson, B. M. (1982). The Regeneration of Axolotl Limbs Covered by Frog-Skin. *Developmental Biology*, 90(2), 435-440. <https://doi.org/DOI> 10.1016/0012-1606(82)90393-1
- Chalkley, D. T. (1954). A Quantitative Histological Analysis of Forelimb Regeneration in *Triturus Viridescens*. *Journal of Morphology*, 94(1), 21-70. <https://doi.org/DOI> 10.1002/jmor.1050940103
- Christensen, R. N., Weinstein, M., & Tassava, R. A. (2002). Expression of fibroblast growth factors 4, 8, and 10 in limbs, flanks, and blastemas of *Ambystoma*. *Dev Dyn*, 223(2), 193-203. <https://doi.org/10.1002/dvdy.10049>
- Currie, J. D., Kawaguchi, A., Traspas, R. M., Schuez, M., Chara, O., & Tanaka, E. M. (2016). Live Imaging of Axolotl Digit Regeneration Reveals Spatiotemporal Choreography of Diverse Connective Tissue Progenitor Pools. *Dev Cell*, 39(4), 411-423. <https://doi.org/10.1016/j.devcel.2016.10.013>
- David, H., Winkelmann, E., & Marx, I. (1963). [Electron Microscope Studies on Degenerative and Regenerative Processes in Severed Spinal Cord of *Ambystoma Mexicanum*]. *J Hirnforsch*, 6, 235-255. <https://www.ncbi.nlm.nih.gov/pubmed/14126249> (Elektronenmikroskopische untersuchungen degenerativer und regenerativer vorgaenge am durchtrennten ruckenmark von ambystoma mexicanum.)
- Dent, J. N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *Journal of Morphology*, 110, 61-77. <https://doi.org/10.1002/jmor.1051100105>
- Dunis, D. A., & Namenwirth, M. (1977). The role of grafted skin in the regeneration of x-irradiated axolotl limbs. *Developmental Biology*, 56(1), 97-109. [https://doi.org/10.1016/0012-1606\(77\)90157-9](https://doi.org/10.1016/0012-1606(77)90157-9)

- Elewa, A., Wang, H., Talavera-Lopez, C., Joven, A., Brito, G., Kumar, A., . . . Simon, A. (2017). Reading and editing the *Pleurodeles waltl* genome reveals novel features of tetrapod regeneration. *Nat Commun*, 8(1), 2286. <https://doi.org/10.1038/s41467-017-01964-9>
- Endo, T., Bryant, S. V., & Gardiner, D. M. (2004). A stepwise model system for limb regeneration. *Developmental Biology*, 270(1), 135-145. <https://doi.org/10.1016/j.ydbio.2004.02.016>
- Endo, T., Tamura, K., & Ide, H. (2000). Analysis of gene expressions during *Xenopus* forelimb regeneration. *Developmental Biology*, 220(2), 296-306. <https://doi.org/10.1006/dbio.2000.9641>
- Endo, T., Yokoyama, H., Tamura, K., & Ide, H. (1997). Shh expression in developing and regenerating limb buds of *Xenopus laevis*. *Dev Dyn*, 209(2), 227-232. [https://doi.org/10.1002/\(SICI\)1097-0177\(199706\)209:2<227::AID-AJA8>3.0.CO;2-K](https://doi.org/10.1002/(SICI)1097-0177(199706)209:2<227::AID-AJA8>3.0.CO;2-K)
- Fei, J. F., Schuez, M., Knapp, D., Taniguchi, Y., Drechsel, D. N., & Tanaka, E. M. (2017). Efficient gene knockin in axolotl and its use to test the role of satellite cells in limb regeneration. *Proc Natl Acad Sci U S A*, 114(47), 12501-12506. <https://doi.org/10.1073/pnas.1706855114>
- Flink, I. L. (2002). Cell cycle reentry of ventricular and atrial cardiomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, *Amblystoma mexicanum*: confocal microscopic immunofluorescent image analysis of bromodeoxyuridine-labeled nuclei. *Anatomy and Embryology*, 205(3), 235-244. <https://doi.org/10.1007/s00429-002-0249-6>
- Fry, A. E. (1966). Hind Limb Regeneration in *Rana Pipiens* Larvae. *Copeia*(3), 530-&. <https://doi.org/Doi 10.2307/1441078>
- Gargioli, C., & Slack, J. M. (2004). Cell lineage tracing during *Xenopus* tail regeneration. *Development*, 131(11), 2669-2679. <https://doi.org/10.1242/dev.01155>
- Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., . . . Treutlein, B. (2018). Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science*, 362(6413). <https://doi.org/10.1126/science.aag0681>
- Ghosh, S., Roy, S., Seguin, C., Bryant, S. V., & Gardiner, D. M. (2008). Analysis of the expression and function of Wnt-5a and Wnt-5b in developing and regenerating axolotl (*Ambystoma mexicanum*) limbs. *Dev Growth Differ*, 50(4), 289-297. <https://doi.org/10.1111/j.1440-169X.2008.01000.x>
- Goss, R. J. (1969). *Principles of Regeneration*. Academic Press.
- Han, M. J., An, J. Y., & Kim, W. S. (2001). Expression patterns of Fgf-8 during development and limb regeneration of the axolotl. *Dev Dyn*, 220(1), 40-48. [https://doi.org/10.1002/1097-0177\(2000\)9999:9999<::AID-DVDY1085>3.0.CO;2-8](https://doi.org/10.1002/1097-0177(2000)9999:9999<::AID-DVDY1085>3.0.CO;2-8)
- Hay, E. D. (1958). The Fine Structure of Blastema Cells and Differentiating Cartilage Cells in Regenerating Limbs of *Amblystoma* Larvae. *Journal of Biophysical and Biochemical Cytology*, 4(5), 583-+. <https://doi.org/DOI 10.1083/jcb.4.5.583>
- Hay, E. D. (1959). Electron Microscopic Observations of Muscle Dedifferentiation in Regenerating *Amblystoma* Limbs. *Developmental Biology*, 1(6), 555-585. [https://doi.org/Doi 10.1016/0012-1606\(59\)90018-1](https://doi.org/Doi 10.1016/0012-1606(59)90018-1)
- Hayashi, S., Kawaguchi, A., Uchiyama, I., Kawasumi-Kita, A., Kobayashi, T., Nishide, H., . . . Yokoyama, H. (2015). Epigenetic modification maintains intrinsic limb-cell identity in *Xenopus* limb bud regeneration. *Developmental Biology*, 406(2), 271-282. <https://doi.org/10.1016/j.ydbio.2015.08.013>

- Herrera-Rincon, C., Golding, A. S., Moran, K. M., Harrison, C., Martyniuk, C. J., Guay, J. A., . . . Levin, M. (2018). Brief Local Application of Progesterone via a Wearable Bioreactor Induces Long-Term Regenerative Response in Adult *Xenopus* Hindlimb. *Cell Rep*, 25(6), 1593-1609 e1597. <https://doi.org/10.1016/j.celrep.2018.10.010>
- Horton, J. D. (1969). Ontogeny of the immune response to skin allografts in relation to lymphoid organ development in the amphibian *Xenopus laevis* Daudin. *J Exp Zool*, 170(4), 449-465. <https://doi.org/10.1002/jez.1401700407>
- Imokawa, Y., & Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proceedings of the National Academy of Sciences of the United States of America*, 94(17), 9159-9164. <https://doi.org/DOI> 10.1073/pnas.94.17.9159
- Khattak, S., Schuez, M., Richter, T., Knapp, D., Haigo, S. L., Sandoval-Guzman, T., . . . Tanaka, E. M. (2013). Germline transgenic methods for tracking cells and testing gene function during regeneration in the axolotl. *Stem Cell Reports*, 1(1), 90-103. <https://doi.org/10.1016/j.stemcr.2013.03.002>
- Kirsche, K., & Kirsche, W. (1964). [Regenerative Processes in the Telencephalon of *Ambystoma Mexicanum*]. *J Hirnforsch*, 7, 421-436. <https://www.ncbi.nlm.nih.gov/pubmed/14248567> (Regenerative vorgaenge im telencephalon von ambystoma mexicanum.)
- Klein, A. M., Mazutis, L., Akartuna, I., Tallapragada, N., Veres, A., Li, V., . . . Kirschner, M. W. (2015). Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells. *Cell*, 161(5), 1187-1201. <https://doi.org/10.1016/j.cell.2015.04.044>
- Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., & Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature*, 460(7251), 60-65. <https://doi.org/10.1038/nature08152>
- Kumar, A., Velloso, C. P., Imokawa, Y., & Brookes, J. P. (2000). Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. *Developmental Biology*, 218(2), 125-136. <https://doi.org/10.1006/dbio.1999.9569>
- Kurabuchi, S. (1992). Outgrowth dependency of forelimb regeneration on nerves in adult African clawed frog, *Xenopus laevis*. *Roux's Arch Dev Biol*, 201(6), 376-382. <https://doi.org/10.1007/BF00365125>
- Lin, G., Chen, Y., & Slack, J. M. (2007). Regeneration of neural crest derivatives in the *Xenopus* tadpole tail. *BMC Dev Biol*, 7, 56. <https://doi.org/10.1186/1471-213X-7-56>
- Lin, G., Chen, Y., & Slack, J. M. (2013). Imparting regenerative capacity to limbs by progenitor cell transplantation. *Dev Cell*, 24(1), 41-51. <https://doi.org/10.1016/j.devcel.2012.11.017>
- Liversage, R. A., Anderson, M. J., & Korneluk, R. G. (1987). Regenerative response of amputated forelimbs of *Xenopus laevis* froglets to partial denervation. *Journal of Morphology*, 191(2), 131-144. <https://doi.org/10.1002/jmor.1051910204>
- Lo, D. C., Allen, F., & Brookes, J. P. (1993). Reversal of muscle differentiation during urodele limb regeneration. *Proc Natl Acad Sci U S A*, 90(15), 7230-7234. <https://doi.org/10.1073/pnas.90.15.7230>
- Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., . . . McCarroll, S. A. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*, 161(5), 1202-1214. <https://doi.org/10.1016/j.cell.2015.05.002>

- Masaki, H., & Ide, H. (2007). Regeneration potency of mouse limbs. *Dev Growth Differ*, 49(2), 89-98. <https://doi.org/10.1111/j.1440-169X.2007.00909.x>
- McLaughlin, D. S., & Liversage, R. A. (1986). Effects of denervation and delayed amputation on forelimb regeneration in *Xenopus laevis* froglets. *Anat Rec*, 214(3), 289-293. <https://doi.org/10.1002/ar.1092140308>
- Mercader, N., Tanaka, E. M., & Torres, M. (2005). Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. *Development*, 132(18), 4131-4142. <https://doi.org/10.1242/dev.01976>
- Mescher, A. L. (1976). Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *Journal of Experimental Zoology*, 195(1), 117-128. <https://doi.org/10.1002/jez.1401950111>
- Mitogawa, K., Makanae, A., & Satoh, A. (2018). Hyperinnervation improves *Xenopus laevis* limb regeneration. *Developmental Biology*, 433(2), 276-286. <https://doi.org/10.1016/j.ydbio.2017.10.007>
- Morrison, J. I., Borg, P., & Simon, A. (2010). Plasticity and recovery of skeletal muscle satellite cells during limb regeneration. *Faseb Journal*, 24(3), 750-756. <https://doi.org/10.1096/fj.09-134825>
- Mullen, L. M., Bryant, S. V., Torok, M. A., Blumberg, B., & Gardiner, D. M. (1996). Nerve dependency of regeneration: the role of Distal-less and FGF signaling in amphibian limb regeneration. *Development*, 122(11), 3487-3497. <https://doi.org/10.1242/dev.122.11.3487>
- Muneoka, K., Fox, W. F., & Bryant, S. V. (1986). Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. *Developmental Biology*, 116(1), 256-260. [https://doi.org/10.1016/0012-1606\(86\)90062-x](https://doi.org/10.1016/0012-1606(86)90062-x)
- Murugan, N. J., Vigran, H. J., Miller, K. A., Golding, A., Pham, Q. L., Sperry, M. M., . . . Levin, M. (2022). Acute multidrug delivery via a wearable bioreactor facilitates long-term limb regeneration and functional recovery in adult *Xenopus laevis*. *Sci Adv*, 8(4), eabj2164. <https://doi.org/10.1126/sciadv.abj2164>
- Nacu, E., Glausch, M., Le, H. Q., Damanik, F. F., Schuez, M., Knapp, D., . . . Tanaka, E. M. (2013). Connective tissue cells, but not muscle cells, are involved in establishing the proximo-distal outcome of limb regeneration in the axolotl. *Development*, 140(3), 513-518. <https://doi.org/10.1242/dev.081752>
- Nacu, E., & Tanaka, E. M. (2011). Limb regeneration: a new development? *Annu Rev Cell Dev Biol*, 27, 409-440. <https://doi.org/10.1146/annurev-cellbio-092910-154115>
- Nowoshilow, S., Schloissnig, S., Fei, J. F., Dahl, A., Pang, A. W. C., Pippel, M., . . . Myers, E. W. (2018). The axolotl genome and the evolution of key tissue formation regulators. *Nature*, 554(7690), 50-55. <https://doi.org/10.1038/nature25458>
- Ohgo, S., Itoh, A., Suzuki, M., Satoh, A., Yokoyama, H., & Tamura, K. (2010). Analysis of *hoxa11* and *hoxa13* expression during patternless limb regeneration in *Xenopus*. *Developmental Biology*, 338(2), 148-157. <https://doi.org/10.1016/j.ydbio.2009.11.026>
- Roensch, K., Tazaki, A., Chara, O., & Tanaka, E. M. (2013). Progressive Specification Rather than Intercalation of Segments During Limb Regeneration. *Science*, 342(6164), 1375-1379. <https://doi.org/10.1126/science.1241796>
- Ryffel, G. U., Werdien, D., Turan, G., Gerhards, A., Goosses, S., & Senkel, S. (2003). Tagging muscle cell lineages in development and tail regeneration using Cre recombinase in

- transgenic *Xenopus*. *Nucleic Acids Res*, 31(8), e44.
<https://doi.org/10.1093/nar/gng044>
- Sandoval-Guzman, T., Wang, H., Khattak, S., Schuez, M., Roensch, K., Nacu, E., . . . Simon, A. (2014). Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell*, 14(2), 174-187.
<https://doi.org/10.1016/j.stem.2013.11.007>
- Satoh, A., Graham, G. M., Bryant, S. V., & Gardiner, D. M. (2008). Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (*Ambystoma mexicanum*). *Developmental Biology*, 319(2), 321-335.
<https://doi.org/10.1016/j.ydbio.2008.04.030>
- Satoh, A., Ide, H., & Tamura, K. (2005). Muscle formation in regenerating *Xenopus* froglet limb. *Dev Dyn*, 233(2), 337-346. <https://doi.org/10.1002/dvdy.20349>
- Satoh, A., Makanae, A., & Wada, N. (2010). The apical ectodermal ridge (AER) can be re-induced by wounding, wnt-2b, and fgf-10 in the chicken limb bud. *Developmental Biology*, 342(2), 157-168. <https://doi.org/10.1016/j.ydbio.2010.03.018>
- Shimokawa, T., Yasutaka, S., Kominami, R., & Shinohara, H. (2013). Lmx-1b and Wnt-7a expression in axolotl limb during development and regeneration. *Okajimas Folia Anat Jpn*, 89(4), 119-124. <https://doi.org/10.2535/ofaj.89.119>
- Singer, M. (1954). Induction of Regeneration of the Forelimb of the Postmetamorphic Frog by Augmentation of the Nerve Supply. *Journal of Experimental Zoology*, 126(3), 419-471.
[https://doi.org/DOI 10.1002/jez.1401260304](https://doi.org/DOI%2010.1002/jez.1401260304)
- Spallanzani, L. (1768). *Prodromo di un' opera da imprimersi sopra le riproduzioni animali* [An essay on animal reproduction.]. In Modena : Nella Stamperia di Giovanni Montanari, 1768. <https://doi.org/https://doi.org/10.5962/bhl.title.158642>
- Steen, T. P. (1968). Stability of chondrocyte differentiation and contribution of muscle to cartilage during limb regeneration in the axolotl (*Siredon mexicanum*). *J Exp Zool*, 167(1), 49-78. <https://doi.org/10.1002/jez.1401670105>
- Steen, T. P. (1970). Origin and differentiative capacities of cells in the blastema of the regenerating salamander limb. *Am Zool*, 10(2), 119-132.
<https://doi.org/10.1093/icb/10.2.119>
- Stocum, D. L. (2017). Mechanisms of urodele limb regeneration. *Regeneration (Oxf)*, 4(4), 159-200. <https://doi.org/10.1002/reg2.92>
- Stopper, G. F., Richards-Hrdlicka, K. L., & Wagner, G. P. (2016). Hedgehog inhibition causes complete loss of limb outgrowth and transformation of digit identity in *Xenopus tropicalis*. *J Exp Zool B Mol Dev Evol*, 326(2), 110-124.
<https://doi.org/10.1002/jez.b.22669>
- Stopper, G. F., & Wagner, G. P. (2007). Inhibition of Sonic hedgehog signaling leads to posterior digit loss in *Ambystoma mexicanum*: parallels to natural digit reduction in urodeles. *Dev Dyn*, 236(1), 321-331. <https://doi.org/10.1002/dvdy.21025>
- Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H., & Tamura, K. (2006). Limb regeneration in *Xenopus laevis* froglet. *ScientificWorldJournal*, 6 Suppl 1, 26-37.
<https://doi.org/10.1100/tsw.2006.325>
- Tanaka, H. V., Ng, N. C. Y., Yang Yu, Z., Casco-Robles, M. M., Maruo, F., Tsonis, P. A., & Chiba, C. (2016). A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*, 7, 11069.
<https://doi.org/10.1038/ncomms11069>

- Tata, P. R., Mou, H. M., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B. M., . . . Rajagopal, J. (2013). Dedifferentiation of committed epithelial cells into stem cells in vivo. *Nature*, 503(7475), 218-+. <https://doi.org/10.1038/nature12777>
- Thornton, C. S. (1938). The histogenesis of muscle in the regenerating fore limb of larval *Amblystoma punctatum*. *Journal of Morphology*, 62(1), 17-47. <https://doi.org/DOI.10.1002/jmor.1050620104>
- Thornton, C. S. (1957). The effect of apical cap removal on limb regeneration in *Amblystoma* larvae. *J Exp Zool*, 134(2), 357-381. <https://doi.org/10.1002/jez.1401340209>
- Thornton, C. S. (1960). Influence of an eccentric epidermal cap on limb regeneration in *Amblystoma* larvae. *Developmental Biology*, 2, 551-569. [https://doi.org/10.1016/0012-1606\(60\)90054-3](https://doi.org/10.1016/0012-1606(60)90054-3)
- Thornton, C. S., & Thronton, M. T. (1965). The regeneration of accessory limb parts following epidermal cap transplantation in urodeles. *Experientia*, 21(3), 146-148. <https://doi.org/10.1007/BF02141984>
- Torok, M. A., Gardiner, D. M., Izpisua-Belmonte, J. C., & Bryant, S. V. (1999). Sonic hedgehog (shh) expression in developing and regenerating axolotl limbs. *J Exp Zool*, 284(2), 197-206. <https://www.ncbi.nlm.nih.gov/pubmed/10404648>
- Van Stone, J. M. (1955). The relationship between innervation and regenerative capacity in hindlimbs of *Rana sylvatica*. *J Morph*, 97(2), 345-391. <https://doi.org/https://doi.org/10.1002/jmor.1050970206>
- Vieira, W. A., & McCusker, C. D. (2018). Regenerative Models for the Integration and Regeneration of Head Skeletal Tissues. *Int J Mol Sci*, 19(12). <https://doi.org/10.3390/ijms19123752>
- Yakushiji, N., Suzuki, M., Satoh, A., Ide, H., & Tamura, K. (2009). Effects of activation of hedgehog signaling on patterning, growth, and differentiation in *Xenopus* froglet limb regeneration. *Dev Dyn*, 238(8), 1887-1896. <https://doi.org/10.1002/dvdy.22011>
- Yakushiji, N., Suzuki, M., Satoh, A., Sagai, T., Shiroishi, T., Kobayashi, H., . . . Tamura, K. (2007). Correlation between Shh expression and DNA methylation status of the limb-specific Shh enhancer region during limb regeneration in amphibians. *Developmental Biology*, 312(1), 171-182. <https://doi.org/10.1016/j.ydbio.2007.09.022>
- Yao, Y., & Wang, C. (2020). Dedifferentiation: inspiration for devising engineering strategies for regenerative medicine. *NPJ Regen Med*, 5, 14. <https://doi.org/10.1038/s41536-020-00099-8>
- Yokoyama, H., Endo, T., Tamura, K., Yajima, H., & Ide, H. (1998). Multiple digit formation in *Xenopus* limb bud recombinants. *Developmental Biology*, 196(1), 1-10. <https://doi.org/10.1006/dbio.1998.8856>
- Yokoyama, H., Ide, H., & Tamura, K. (2001). FGF-10 stimulates limb regeneration ability in *Xenopus laevis*. *Developmental Biology*, 233(1), 72-79. <https://doi.org/10.1006/dbio.2001.0180>
- Yokoyama, H., Yonei-Tamura, S., Endo, T., Izpisua Belmonte, J. C., Tamura, K., & Ide, H. (2000). Mesenchyme with fgf-10 expression is responsible for regenerative capacity in *Xenopus* limb buds. *Developmental Biology*, 219(1), 18-29. <https://doi.org/10.1006/dbio.1999.9587>