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Spotlight

Tracing the Origins of Axolotl Limb Regeneration

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The mechanisms underlying limb regeneration in axolotl have remained elusive due to limitations in isolating and tracking the cells that replenish lost tissues. In recent work, Elly Tanaka and Barbara Treutlein unite their expertise in axolotl limb regeneration and single-cell analysis to reveal cellular mechanisms underpinning regeneration.

One of the oldest scientific observations is that many organisms have the capacity to regenerate, although the mechanisms that drive this process have proved particularly difficult to elucidate. Organisms such as the planarian, axolotl, zebrafish, and mouse have been the focus of intense study given their wide-ranging regeneration potential and diverse cellular mechanisms that support the restoration of lost tissue and structures. Cellular mechanisms underlying regeneration in these organisms include the existence of pluripotent cells in planaria and the emergence of stem or progenitor cells with limited potential in axolotl (Tanaka and Reddien, 2011).

The axolotl model of limb regeneration is one of the most remarkable examples of repair proficiency found in vertebrates. Elly Tanaka is a contemporary pioneer in this field, having previously demonstrated that complete limb regeneration in axolotl is achieved and supported by formation the ''blastema,'' a structure comprising a pool of restricted progenitors that retain a ''memory'' of the lost appendage (Kragl et al., 2009). Connective tissue cells represent the most abundant cell type found within the blastema, guiding limb regeneration post-injury. However, up until now, isolating and tracking this pivotal cell population had presented an intractable technical challenge. Thus, the molecular mechanisms underlying how mature connective tissue cells produce a blastema to support limb regeneration have proven enigmatic.

Technical limitations leading to a lack of cellular resolution have hampered discovery across many cell biological disciplines. In this respect, single-cell technologies are enabling new observations by allowing the heterogeneity of complex biological systems to be deconstructed. Here, Barbara Treutlein has been leading recent advances in the application of single-cell technologies to dissect complex biological phenomena. Notably, she has applied these approaches to disentangle differentiation and lineage reprogramming systems, revealing key mechanisms of how mammalian cell identity is established, and how it can be reprogrammed in the context of regenerative medicine (Camp et al., 2017; Treutlein et al., 2016).

In a study in *Science*, Gerber et al. (2018) unites the expertise of Tanaka and her postdoc Prayag Murawala, with Treutlein, to reveal powerful new observations in the context of vertebrate regeneration. In this study, the authors employ a potent combination of lineage tracing and single-cell analysis to dissect the role of connective tissue in regeneration, at unprecedented resolution. First, they developed new axolotl transgenics, based on inducible Cre-loxP fluorescence, to genetically label and isolate connective tissue in the adult limb. Single-cell RNA-sequencing (scRNA-seq) was then employed to dissect connective tissue heterogeneity in (1) the adult limb; (2) across developmental stages; and (3) in a dense time course following limb amputation, subsequent blastema formation, and outgrowth of the regenerated arm. The elegant genetic labeling of connective tissue in combination with scRNA-seq enabled derivatives of this lineage to be longitudinally tracked and deconstructed throughout the regeneration process (Figure 1).

Single-cell profiling of connective tissue cells before and during regeneration did not support the existence of a pre-existing blastema-like population that ''seeds'' the regenerating limb. Rather, Gerber et al. (2018) demonstrate that the heterogeneous mature connective tissue population funnels into a relatively homogeneous blastema progenitor state, unique to regeneration, that transitions into an embryonic limb bud-like state. This de-differentiation is then followed by the re-emergence of differentiated connective tissue lineages via multipotent connective tissue progenitors. The initial broad labeling of connective tissue, via *Prrx1* expression, was followed by labeling of a more specific connective tissue subpopulation, including periskeleton, via *Col1a2* expression. Regeneration of this subpopulation commenced via funneling into the same homogeneous progenitor state, although the resulting cells were subsequently biased to extend pre-existing bone rather than more distal structures. Thus, a division of labor appears to exist in the regenerating connective tissue, with de-differentiating cells retaining a memory of their previous identity and function. Similarly, in a mouse model of skin regeneration, epithelial stem cells rearrange their chromatin following injury to retain a chromatin conformation memory that allows rapid reactivation of key genes responsible for repair (Naik et al., 2017). Considering these findings, chromatin accessibility assays applied to this axolotl model are poised to reveal further molecular detail on limb regeneration. Finally, Gerber et al. (2018) confirmed their findings using Brainbow image-based clonal analysis, which raises further exciting possibilities for future analyses; with lineage tracing entering the single-cell area, powerful

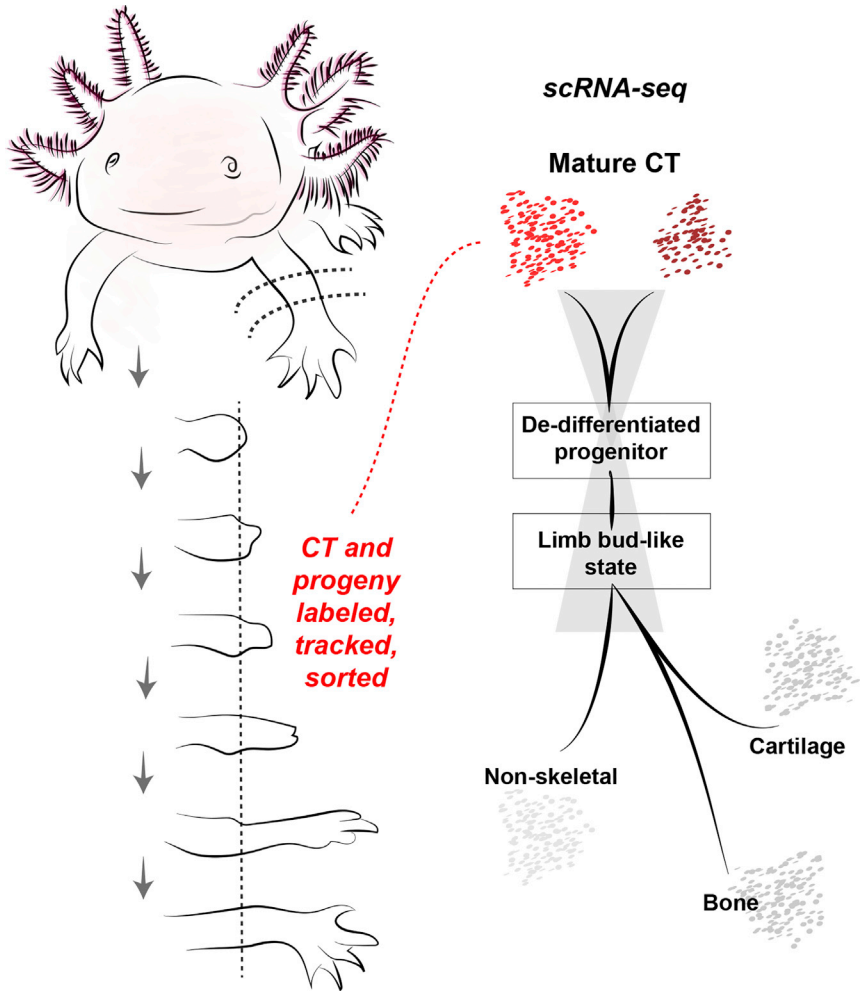

Figure 1. Tracking Cell Lineage and Identity at Single-Cell Resolution in the Regenerating Axolotl. Limb

Connective tissue (CT) cells are labeled via the inducible Cre-loxP system. Connective tissue cells and their derivatives are isolated throughout post-injury limb regeneration and profiled via single-cell RNA-sequencing (scRNA-seq). These experiments demonstrated that mature, heterogeneous connective tissue cells "funnel" via a de-differentiated blastema progenitor before transitioning to an embryonic limb bud-like state. From this state, the heterogeneity of mature connective tissue is re-established in the regenerated limb.

approaches to simultaneously track lineage and identity within individual cells has the potential to unlock regenerative processes at even greater resolution (Kester and van Oudenaarden, 2018).

The findings of Gerber et al. (2018) shed light on one of the most interesting regeneration phenomena in vertebrates and also facilitate a better understanding of how axolotl limb regeneration is achieved. Moreover, it permits the comparison of regenerative mechanisms across different organisms. For example, recent planaria regeneration studies have shown, via single-cell analysis, that within neoblasts (the cells that drive regeneration in planaria), there is functional heterogeneity. Moreover, a neoblast subpopulation was identified that, after a single cell was transplanted, could repopulate an entire animal following lethal irradiation (Zeng et al., 2018). Relative to axolotl limb regeneration, planaria regeneration does rely on pre-existing stem cells that are activated following injury, suggesting that there are many paths that can lead to regeneration even within the same organism. For example, regeneration in mice widely varies depending on the tissue. Whereas endothelial regeneration of large arteries in mice occurs when cells adjacent to the injury site rewire their transcriptional program to initiate proliferation in a two-stage process (McDonald et al., 2018), regeneration in the olfactory epithelium after injury has been recently shown to involve the transient de-differentiation of progenitor cells that revert back to more primitive or poorly differentiated states. This mechanism involves the activation of *Sox2*, enhanced by the inhibition of the Polycomb chromatin regulator Ezh2 (Lin et al., 2017). The different mechanisms present in each tissue and organism not only support comparison between common pathways and molecular mechanisms but can also be used to implement new approaches absent in target tissues to promote efficient regeneration.

In summary, new technologies such as scRNA-seq, combined with established approaches such as lineage tracing, transplant assays, and *in vitro* modeling, are beginning to reveal the specific cellular and molecular mechanisms driving regeneration programs across different tissues and organisms. This, coupled with the exploration of new regeneration models, will widen our knowledge of the different biological strategies employed in nature. This, in turn, has the potential to enable the development of tailored methods to promote regeneration for the benefit of human health.

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