CRITICAL COMMENTARY

Rebuilding limbs, one cell at a time

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1 | INTRODUCTION

The regeneration of salamander limbs has been a special fascination among scientists and keen observers for centuries. Perhaps due to how closely the salamander's limb anatomically mirrors our own, a grand aspiration of regenerative medicine has been to provoke such a process following injury or loss of human limbs. Research in the last century has focused on understanding the blastema, a proliferative cell mass that develops after limb amputation (see Box 1 "A primer on limb regeneration" and reviews for discussion of foundational knowledge[1-3]). The first micrographs of limb blastemas (examples in Thornton[4] and Hay[5]) brought limb regeneration to a cellular level and ushered in a new era of questions centered around the origin, potency, and processes of regenerative cells that has occupied the field ever since. Within this commentary, we will outline some of these persistent questions underlying limb regeneration, and how new technologies and approaches are paving the way toward a cellular understanding of complex tissue regeneration.
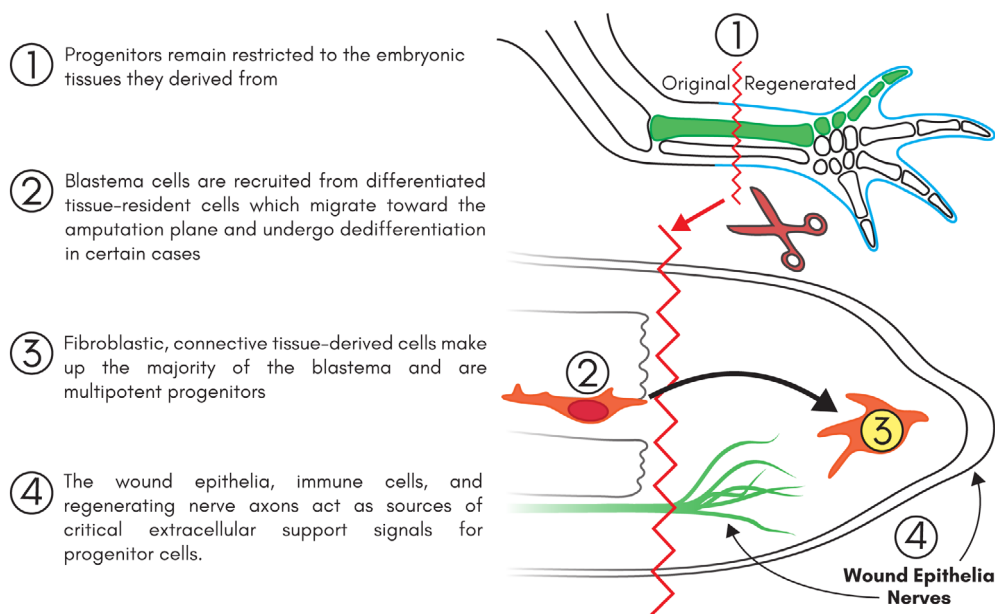
As with most enduring research questions, studying regeneration has not been without challenges. Compared to the uniform initiation and choreographed movements of limb development, regeneration is initiated by a variable and chaotic injury that obfuscates tissue structure and processes. Tissue compartments found at the injury site also represent complex, postdevelopmental structures that have distinct embryonic origins and heterogeneous contributions to the overall regeneration

process.[6-8] Tissue complexity is also compounded by the size of the salamander limb, encompassing thousands of cells within millimeter to centimeter diameters. Although comparisons to limb development have been a natural place to start when studying regeneration, the scale of the postdevelopmental limb dwarfs commonly used embryos and embryonic limbs. This size, particularly of the amphibian limb, has historically limited the feasibility to image and trace processes. In addition, the availability of genetic tools to study molecular and cellular processes in salamanders has lagged behind more common research models.

Despite challenges, the last decade has seen a rapid emergence of tools and technologies that have largely outstripped the pace at which they can be applied toward questions of regeneration (Table 1). The most significant advances have been in three general arenas. First, the emergence of high quality and (more) cost-efficient transcriptomic techniques for bulk tissue and single cells have provided a deeper understanding of gene expression changes across different tissues and time points. Generation of robust transcriptomic timelapse datasets have also increased the ability for comparative studies across an increasing number of limbed species.[6,9,26-28] Second, advances in deep tissue imaging (confocal, two-photon, and light sheet microscopy) combined with genetically encoded fluorescent markers and tissue clearing methods have made it possible to probe large samples without losses in cellular resolution.[7,29] Finally, organism-agnostic methods such as CRISPR/Cas genome

① Progenitors remain restricted to the embryonic tissues they derived from

② Blastema cells are recruited from differentiated tissue–resident cells which migrate toward the amputation plane and undergo dedifferentiation in certain cases

③ Fibroblastic, connective tissue–derived cells make up the majority of the blastema and are multipotent progenitors

④ The wound epithelia, immune cells, and regenerating nerve axons act as sources of critical extracellular support signals for progenitor cells.

BOX 1   A primer on limb regeneration. Four key concepts of salamander limb regeneration that form a foundation for some of the large unanswered questions in the field. (1) Regenerative cells remain restricted to reconstitute the tissues they derived from embryonically. For example, epithelia (blue) will only reconstitute epidermis, while connective tissue-derived cells (depicted as green skeleton) will only give rise to the tissue types that were derived from embryonic limb lateral plate mesoderm. (2) Cells for the regenerative cell mass, the blastema, are derived from tissue-resident cells which migrate toward the amputation plane to form the nascent blastema. (3) Connective tissue-derived cells, which make up the majority of the blastema, undergo a dedifferentiation process to give rise to multipotent progenitors. (4) The specialized wound epithelia, regenerating nerve axons, and immune cells (not pictured) are thought to produce essential trophic factors to support regeneration and are considered key tissues for creating the blastema cell microenvironment

engineering[30,31] and transgenesis[32] have expanded the functional toolkit in salamander models.

The application of each of these new methods for gene expression analysis, imaging, and genome editing have brought the limb regeneration field to an analogous moment in time as that of Elizabeth Hay's blastema micrographs[5]: newly unveiled wonders into the dynamics and life histories of single cells during limb regeneration. Perhaps the biggest question will be whether we can effectively catalyze the information gleaned from new methodologies into a mechanistic understanding of limb regeneration. In the following pages, we lay out some of the most persistent questions in regeneration and posit that new, cell-based methodologies can bring fresh insight into mechanisms of limb regrowth.

- **How do we define multipotent blastema progenitor cells?**
- **How do cell behaviors and lineage decisions influence limb reconstitution?**
- **Are there conserved principles of limb regeneration across vertebrates?**
- **Is there cellular redundancy in limb regeneration?**
- **What nonprogenitor cell types are required to support regeneration?**

- **How similar or different are limb development and regeneration?**

Answering the above questions will require regeneration researchers to integrate classic studies of both limb development and regeneration, massive datasets, and molecular perturbation into a holistic understanding of regenerative cell biology. It will also require the field to wrestle with ideas of cell states and cell types that are currently playing out in the larger developmental and stem cell fields. Finally, it will require many more contexts of regeneration—partial or complete, across numerous species—to identify conserved and divergent principles that drive and maintain reconstitution of the limb.

## 2 | HOW DO WE DEFINE MULTIPOTENT BLASTEMA PROGENITOR CELLS?

At the heart of the blastema is a mass of multipotent, dermal and connective tissue-derived progenitor cells (see Box 1 "A primer on limb regeneration" and reviews for discussion of foundational knowledge[1-3]). Their unique formation from tissue-resident cells via dedifferentiation

TABLE 1 Current and future uses of single cell techniques in limb regeneration

Technology	Use	Future uses	Drawbacks	References
scRNAseq	Cell type/state characterization, marker gene identification	Time resolved sequencing, coupled with protein expression	Snapshot in time, inferred not definitive cell trajectories	Gerber et al,[9] Leigh et al,[6] Li et al,[10] Rodgers et al,[11] Qin et al[12]
scRNAseq w/ barcoding	Definitive lineage information of blastema progenitors, developmental origins of cells	Linking development to regenerative lineages	Transgenic/virus and sophisticated computational approaches required	See reviews by McKenna and Gagnon[13] and Wagner and Klein[14]
Single-cell functional screening	Cell intrinsic gene functions of blastema progenitors	Broad screens of blastema-associated genes and cell types	Better genomic resources required, cell extrinsic functions difficult to interpret	Pertub-seq,[15,16] CRISP-seq,[17] Mosiac-seq,[18] CROP-seq[19]
Spatial transcriptomics	Spatial organization of blastema, cell–cell interactome	Coupled with cell barcoding for clonal and spatial information[20]	Still a nascent technology, unproven outside of mice and human	See review by Asp et al[21]
Spatially and temporally controlled (i.e., clonal) CRISPR mutagenesis	Functional information of cell types states required for regeneration	In vivo characterization of blastema progenitor cell lineage decisions	Accessing single blastema cells or deep tissue difficult	See chapter by Mathony et al[22]
Multicolor (Limbow) based clonal analysis	Origin to destination lineage tracing	Combine w/ perturbation to visualize lineage decisions	Limited to fluorescent combinations	Currie et al[7]
In vitro/ex vivo reconstitution	Controlled conditions to recreate regenerative pathways	Complex composite tissues and 3D ECM scaffolds	May not fully recreate aspects of in vivo regeneration	Ferris et al,[23] Currie et al,[7] Aztekin et al,[24] Athippozhy et al[25]

and the clear applicability of such a process toward regenerative therapies make them a central focus of limb regeneration studies. The similar morphology of cells within the blastema suggested to earlier researchers that blastema cells were universally pluripotent; able to give rise to nearly any tissue required in the regenerate.[5] The use of cell labeling techniques and tissue transplantation methods solidified this was not the case; that blastema cells were restricted to unipotent or multipotent fates that align with the fates of the embryonic limb precursors from which they derived.[8,33,34] While experimental evidence for regenerative cell contributions has been made via grafting experiments,[8,33-37] a molecular definition of these multipotent cells had remained beyond our grasp. This was likely due to a dearth of single-cell resolution and multiplex-able technologies.

Defining blastema progenitors cells has remained elusive, though recent work imaging single cells during the process of digit tip regeneration revealed that a large portion of fibroblastic cells from periskeletal and dermal/interstitial spaces played an outsized role in creating the regenerated tissue.[7] These cells were multipotent and responsible for making new skeletal elements.[7] Subsequent pseudotime analysis using single-cell RNA sequencing (scRNAseq) could reconstruct multiple differentiation paths of fibroblastic cells.[9] While the previous studies were unable to identify a specialized blastema cell in unwounded tissue, parallel scRNAseq analysis suggests that there may be a mixture of broadly undifferentiated connective tissue cells and specialized tissue-resident stem cells that contribute to regeneration[6] (Figure 1). Several additional datasets have recently been generated that describe the blastema in salamanders at single-cell resolution.[6,9-12] These recent studies have shed light on novel cell states, such as the identification of cells with high mitochondrial content that may play a unique role in regeneration,[12] and that processes such as epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions contribute to limb regeneration.[10] Further work has characterized immune cells present in axolotls and the putative molecular identity of the initial fibroblast-like blastema cells.[11] Despite these impressive and insightful datasets, we still do not have a clear set of molecular or cellular characteristics that defines multipotent connective tissue-derived blastema progenitors. It is important to consider that there may be limited transcriptional attributes of a blastema progenitor cell that make it a distinct cell type with unique markers and clearly defined attributes. Instead, thinking about these cells in terms of their functionality (highly proliferative and migratory) and lineage relationships (multipotency) will move us toward a more stable definition. In addition, there may be several classes of progenitors within the
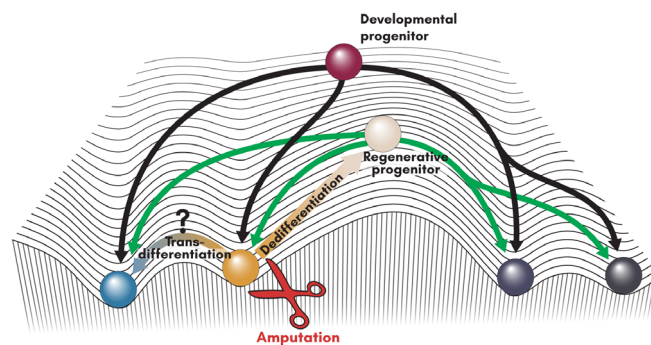
FIGURE 1 Potential differentiation pathways for regenerative cells. Schematic for possible states cells acquire through the course of regeneration. After amputation (red scissors), connective tissue-derived cells (gold) are thought to undergo dedifferentiation to a multipotent progenitor (pearl). Blastema progenitors are transcriptionally unique from the developmental progenitors they derived from (burgundy). Cells could regenerate tissue through a transdifferentiation process, although there is no current evidence that this occurs

blastema with varying degrees of differentiation potential or propensities toward clonal expansion or migration. Getting at these different classes of cells could be difficult due to the complexity of the limb and injury-initiating environment. Drawing inspiration from in vitro organoid and culture models that are being applied to stem cell and developmental biology questions may provide a more tenable means to address some of these questions. One such example could be high throughput live-imaging of isolated blastema cells ex vivo to determine differentiation potential or migratory capabilities, which could be connected to subsequent single-cell sequencing. Such a rigorous investigation of cell behavior coupled to gene expression would aid in determining the functional extent of heterogeneity in the blastema as well as potentially identify markers for cytometry-based sorting of specific cell types or states during regeneration. The ability to select for cells at particular stages of (de)differentiation would be a useful tool in dissecting how cell state transitions occur as well as provide a cell source for in vivo cell transplants to better understand lineage relationships.

While current gene expression-based measurements have proven vital and will continue to be used, we are also entering an age in which epigenomic and proteomic tools will be readily accessed in limb regeneration. The increase in multiomic techniques will only aid in defining the cells of the blastema and their relationships. In fact, it is already possible to pair scRNA-seq with chromatin accessibility techniques like Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)[38] which provides information on gene regulation and transcription factor binding, an extra layer of

information that may aid in defining blastema progenitors. Excitingly, there has already been a first foray into bulk ATAC-seq in limb regeneration[39] and other models of multitissue regeneration.[40] In addition, examining open chromatin regions throughout the genome pre- and postinjury may provide vital clues about how salamander cells are able to retain the ability to revert to a progenitor state as well as keep a positional memory of their origins in relation to injury. Finally, the development of single-cell proteomics, an improving but nascent technology,[41] would help to fill in the gaps of cellular responses during early, postamputation timepoints before limb progenitor cells have formed and may provide more definitive markers and targets that aid in multiplexed cell sorting. Possibly even more exciting will be the ability to evaluate phosphorylation and other protein modifications, allowing for detailed information about cell dynamics.[42] In the future, information on genomic regulation, transcription, and final protein products will likely be feasible to simultaneously probe at the single-cell level.

3 | HOW DO CELL BEHAVIORS AND LINEAGE DECISIONS INFLUENCE LIMB RECONSTITUTION?

A major focus of research in limb regeneration has been on the origin of cells that give rise to the regenerative blastema and the extent of their contributions to reconstitute missing tissues and structures. scRNAseq, and the growing technologies that can be paired with it, open the door to address several unresolved questions about how blastema progenitors are made, what cells they derive from and what cells they eventually regenerate. For instance, when do the first connective tissue-derived blastema progenitors arise and how long are they present? A growing list of options exists for scRNAseq-based lineage tracing and these approaches can answer different questions (see two excellent recent reviews[13,14]). Based on the throughput and resolution of these new lineage-based approaches, we can provide a more refined definition of the potency of various blastema progenitor cells. To understand lineage relationships CRISPR/Cas9-based techniques targeting exogenous (ScarTrace) loci,[43] endogenous genomic loci,[44] or barcodes introduced via viral transduction (CellTag)[45] provide a means to test clonal relationships between cells of interest. This would provide definitive information about how many different types of differentiated cells may arise from a single progenitor/origin. CRISPR/Cas-based lineage sequencing has been performed during axolotl limb regeneration and demonstrated strong reconstitution of clone sizes between the original and regenerated limbs.[46] An

extension of CRISPR/Cas barcoding are dynamic lineage tracing approaches which combine CRISPR/Cas barcodes with single-cell sequencing to reconstruct branching lineage trees, such as scGESTALT and LINNEAUS.[43,47,48] This would allow for the identification of progenitors and related progeny identities, a major step toward reconstructing the de- and redifferentiation that is required for blastema function.

While understanding lineage relationships during regeneration is critical, it must be connected to cell phenotypes and spatial coordination of building the blastema. Such analysis requires imaging-based methodology to observe and quantify the timing and location of individual cell behaviors that create the collective effect of tissue regeneration. Techniques such as intMEMOIR[20] have been developed to visually read out barcodes in situ and could be applied to create "maps" of where progenitors arise, where they are seeded in the blastema, gene expression in relation to lineage relationships, and if position within the blastema influences clonal and lineage outcomes. Other techniques that are able to resolve temporal changes in gene expression would further refine cell state transitions and give unprecedented information about the transitions to and from blastema progenitor cell states.[49] The question remains if movements into the blastema are molecularly choreographed, or if the regenerative process is largely stochastic, such that a cell only needs to find the "right place and time" to secure a different outcome to its starting neighbors. Another extension of barcoded or clonally labeled tissue would be to sample a single limb prior to and during repeated rounds of regeneration. This would allow one to determine clone behaviors and lineage connections over repeated amputations which should, in principle, allow one to determine if clone behaviors are heritable or are largely the product of location and proximity to extrinsic cues for migration, proliferation, and so forth.[45,46,50,51] The limb regeneration model is a challenging yet ideal in vivo model to query the heterogeneity of lineage decisions during morphogenesis. There are some recent insights into the role of kinetics of blastema cell recruitment on the contribution of cells to the regenerate. Cells that arrive early to the blastema are biased to form skeleton (the innermost regenerated tissue), while late arriving cells predominantly form soft tissue.[7,52] These findings suggest a tissue-level blueprint, but how decisions are made at the cellular level remains elusive. Spatial transcriptomics, which are now readily accessible and becoming ever more sophisticated in regards to resolution and sensitivity, promise to provide a detailed view of blastema organization throughout regeneration. Coupling spatial transcriptomics with clonal analysis using methods such as molecular barcoding or multicolor clonal imaging (i.e., Limbow/Brainbow) will be essential to connect any

compartmentalized gene expression to the outcomes of cells during regeneration. Spatial gene expression datasets can also likely be paired with new machine learning tools to generate testable hypotheses of potential regulators of lineage decisions. Which holds promise to uncover meaningful connections within large, complex datasets.[53]

As we learn more about the coordination of gene expression and cell behavior necessary to build and pattern the regenerated limb, functionally testing hypotheses will be critical to determine the underlying mechanisms of regeneration. Fortunately, we now possess molecular tools such as CRISPR/Cas and transgenesis to perturb gene expression and manipulate cells within regenerating tissue. In addition, new molecular tools can work synergistically with classical techniques that have been hallmarks of salamander limb experiments. Recent work in axolotls took advantage of two classic techniques, embryonic transplantation and the generation of haploid embryos, together with CRISPR/Cas gene knockout to generate single allele mutations in a single axolotl forelimb.[54] The development of new optogenetic transgenic tools is also a promising means to manipulate cells during the process of regeneration. Light-controlled transgenes could be used as a means to simply label cells with high temporal and spatial precision using photo-activatable fluorophores or light-inducible Cre recombinase.[55] Other sensors would have the ability to perturb intracellular pathways required for cell behaviors and fate decisions.[56] One key advantage of such methods would be the ability to compare locally altered cells to their unperturbed neighbors. Finally, techniques such as in vivo and in vitro CRISPR/Cas screens will also be useful to identify molecular determinants of lineage decisions. The application of approaches that allow for the identification of gene modules associated with cell types and states, such as Perturb-seq,[15] may provide a more refined means to define the cellular behaviors of blastema progenitor cells. In addition to developing functional assays, computational approaches applied to new and existing scRNAseq data will be essential to generate targeted predictions of gene networks that control lineage paths during the repatterning of regenerated tissue.

4 | ARE THERE CONSERVED PRINCIPLES OF LIMB REGENERATION ACROSS VERTEBRATES?

The question of why salamanders are the only adult vertebrates that are capable of complete limb regeneration remains unanswered. Comparisons to the regeneration-competent periods of the larval frog limbs and fish fins, the evolutionary precursors of limbs, are a natural starting place to draw comparisons across evolutionary time. In particular, *Xenopus laevis* has been a historically studied species, having been shown half a century ago that a short window of regenerative capacity exists in larval animals.[57] This also provides an ideal model to investigate reasons behind regenerative failure, given the transition between competent and refractory stages of development. With zebrafish emerging as a popular model species, there has also been increasing traction in comparisons of limb and fin regeneration.[58] These more distant comparisons, both evolutionary and morphologically, are still of interest. A particular advantage of the zebrafish model are the advanced genetic tools, which have provided a wealth of information about appendage regeneration.[59]

Cross-species comparisons of regenerative molecular processes have emerged as a powerful, and now highly accessible means to evaluate the regenerative response through the lens of evolutionarily conservation.[60-63] The most common comparison in mammals to salamander limb regeneration is digit tip regeneration in mice, which has also entered the age of single-cell characterization.[64,65] Further comparisons have been drawn to the blastemas formed in skin and ear hole regeneration of *Acomys* (African spiny mouse).[66,67] Recent examples in amphibians have combined classic transplantation and ex vivo culture techniques with single cell-based approaches to uncover potential cell intrinsic and extrinsic factors associated with the loss of regenerative ability in *Xenopus*.[24,68] Even among closely related species of salamander, the strategies used to accomplish limb regeneration may exhibit divergences, exemplified by differences between newt and axolotl muscle regeneration.[35,69] Therefore, even comparisons among salamanders will be valuable to identify the full repertoire of regenerative mechanisms.

To create cross-appendage/organs models of tissue regeneration will also require researchers to wrestle with concepts of cell type evolution and cellular orthology; driving down into the question of whether special regeneration-promoting cells only exist in regeneration-competent animals. The more species and blastema types sampled will only enhance our ability to define conserved molecular pathways employed by regenerative cells. Currently, only a few salamander species have had detailed transcriptomic datasets collected over the course of limb regeneration,[26-28,31,70-76] but comparing regenerative gene expression across dozens of salamander species may provide a better understanding of true signal versus noise in regard to pathways that are necessary to build the blastema. In addition to sampling more regeneration-

competent salamanders, the continued sampling of new species will likely uncover animals with intermediate regenerative capacity. The ability or inability to regenerate paired appendages in closely related species provides a valuable and tenable means to compare recent evolutionary losses of regenerative ability. Perhaps more important than gene expression profiles will be the ability to link the phenotypic functionality of regenerative cells to determine the orthology of cells across species.

An added tool in interspecies comparisons of regeneration are the numerous methods for genome engineering, which will potentially allow for the sophisticated and large-scale insertion, deletion, or replacement of chromosomal segments. This would include replacing multiple genes, promoters, or enhancers to determine essential functional components of the transcriptional regulation of regeneration. This could lead to interesting gain- and loss-of-function experiments to, for instance, break regeneration in salamanders by replacement with other non-regenerating species′ genetic material. More specifically, one could "humanize" (or "*Xenopus*-ize") salamander genomes to understand the critical components to the regenerative process. At the same time, introduction of salamander genes and noncoding elements into imperfect regenerating organisms would test the sufficiency of genomic elements or salamander proteins to boost regenerative outcomes. Such techniques are already being tested in regard to regeneration-specific enhancers that might steer injury outcomes toward repair in non-regenerative species.[62,77] These hybrid genomes could provide a new lens to evaluate the regenerative process.

5 | IS THERE CELLULAR REDUNDANCY IN LIMB REGENERATION?

An important and almost untouched question is how much redundancy and potential compensation is programmed into the process of limb regeneration. Due to a lack of conditional deletion tools, this question has been difficult to approach. We know that phagocytic cells that take up liposomes (i.e., macrophages) are one clear example of a required cell type in limb regeneration.[78] However, it is also evident that limbs can regenerate in the complete absence of certain limb tissues. A beautiful example for this scenario are *pax3*^{−/−} newts, which develop muscle-less limbs which can in turn regenerate muscle-less limbs.[31] It is thus clear that some cells are essential for regeneration and may act to coordinate the process at large, while others are largely dispensable. We know relatively little about how to draw these distinctions and which cells are essential coordinators of the

entire regenerative process. We have evidence from surgical,[79] gene-targeting,[28,80,81] and pharmacological[7] loss-of-function experiments that regeneration can be obstructed, paused or delayed without an impact on the end point regeneration once the perturbation has been removed. These phenotypes may be worth further unpacking to glean information on the source cells, their targets, and possible compensatory mechanisms that affect the regenerative process. Several instances of experimentally induced delays seem to be due to decreases in proliferation or an increase in cell death, both of which are tantamount to a cell depletion.[28] Through this lens, the ability of the regenerative process to recover from such insults suggests a certain level of redundancy. It will be of great interest to further investigate growth factor and survival signals in the blastema and their roles on certain cell types. This will lead to questions such as how the blastema is able to sense deficiencies created by lower cell proliferation or cell death. At what point of progenitor cell to differentiated cell status are cells still able to proliferate and compensate for cell loss? Answers to these questions will drive at the sensitive rheostat-like functions of the blastema that are capable of rapid, on the fly adjustments to the regenerative process.

One explanation for observed delays in regeneration could be due to incomplete penetrance across the blastema for morpholino or mosaic F_0 CRISPR loss-of-function. However, acute morpholino-mediated knockdown of von Willebrand Factor D and EGF domains (Vwde) in the blastema resulted in dramatic end point phenotypes.[63] This suggests that experiments in which regeneration was merely delayed could be due to compensatory mechanisms and not a result of incomplete targeting. This result with *Vwde* suggests that there may exist essential checkpoints within the regenerative process, where cells require a stimulus at the correct time, without which successful regeneration is impossible. It will be important to decipher whether the emergence or disappearance of certain cell types/states and their products dictates certain checkpoints during regeneration. Sophisticated conditional depletion systems will be paramount to tease apart these contributions.
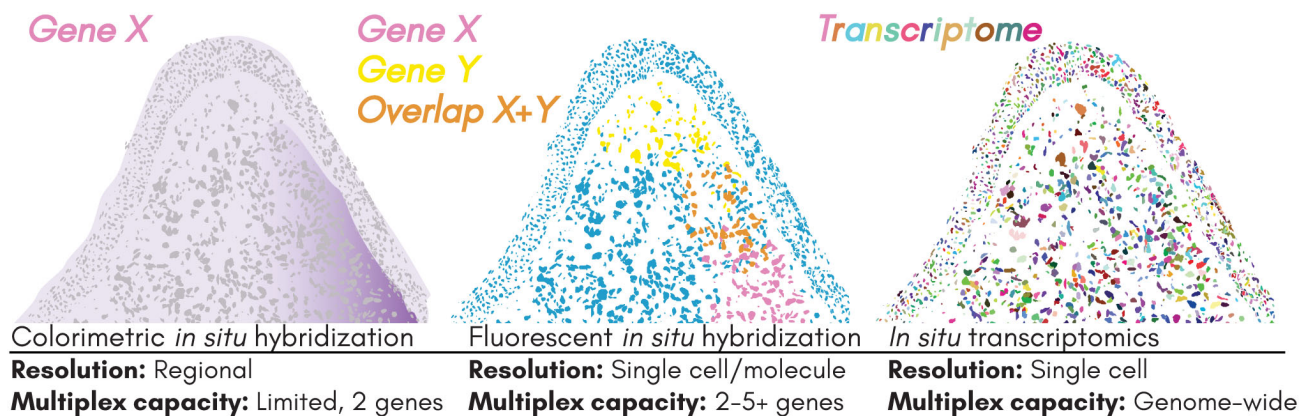
6 | WHAT NONPROGENITOR CELL TYPES ARE REQUIRED TO SUPPORT REGENERATION?

Although the lineage relationships and behavior of blastema progenitors have long intrigued researchers, progenitor cells do not form or function in isolation. Rather, they are supported by a proregenerative microenvironment that plays essential roles during limb regeneration.

This regenerative "niche" is (1) transient and dynamic, (2) spatially distinct, and (3) may support multiple progenitor types in unique ways. First, the proregenerative niche likely changes through regeneration to support the sequential processes of wound resolution, blastema formation, and limb patterning. Of particular interest are the early steps which happen primarily within the stump tissue to transition from the initial injury to the regenerative blastema, since they may provide insights into the molecular determinants that provoke regeneration over a nonregenerative outcome. More detailed comparisons to nonregenerative healing, across various species, anatomical structures, and cell types will be crucial to identify common features that precede and lead to a regenerative versus nonregenerative wound niche.[26,82] Second, as cells migrate from the stump to the amputation plane and accumulate in the blastema, there are likely spatially-distinct microenvironments that support progenitors proximal to and within the blastema. Nonprogenitor cells such as macrophages, which are essential for blastema formation, are thought to release matrix metalloproteinases to liberate connective tissue cells from their resident matrices and secrete a variety of cytokines.[78] Within the blastema, limb nerves are required for regeneration[83] and produce essential signals that stimulate limb regeneration.[84,85] In addition, the wound epidermis has long been regarded as an essential component of the regenerative process, with its removal resulting in a block in regeneration.[79,86-89] This specialized wound epidermis plays a key role during regeneration,[90] although it lacks certain signals that are known to be essential for mammalian and chick limb development (eg, *fgf8, fgf9, fgf17*).[91,92] Recent work using transcriptomic approaches has now shed light on the gene expression profiles that define this unique structure and will help contextualize the more historic studies of this tissue.[6,90] This paints an already complicated picture of multiple tissue types that would not be considered blastema progenitor cells playing an instrumental role in the regenerative process. Understanding how this niche is created and contributes to the overall regenerative process is vital, not only our understanding of the fundamental aspects of regeneration, but also as a foundation for translational therapies.

Unlike homeostatic tissue niches, the regenerative niche is formed following injury and encompasses a blastema that can support multiple progenitor types. One intriguing possibility is that certain niche factors signal to subsets of progenitor types in the blastema and may have distinguishable and separable effects on the overall regenerative process. In this regard, single-cell approaches would excel at making predictions on progenitor cell-niche cell interactions and elucidating cellular crosstalk in regeneration. Recent sequencing-based efforts have evaluated potential receptor-ligand interactions between different cell types in the axolotl limb.[12] These kinds of studies will allow for functional follow-up to identify cellular communication networks in limb regeneration. In the future, these predictions can be further refined with the variety of techniques that provide additional spatial information, including single-cell spatial transcriptomics.[21] The increase in granularity from colorimetric RNA in situ hybridizations, to fluorescent in situ (FISH), and finally single-cell resolution spatial transcriptomics will provide an entirely new way to query communication within the regenerate (Figure 2). Spatial transcriptomic techniques will allow high throughput mapping of potential progenitor-niche signaling based on

Colorimetric *in situ* hybridization	Fluorescent *in situ* hybridization	*In situ* transcriptomics
Resolution: Regional	**Resolution:** Single cell/molecule	**Resolution:** Single cell
Multiplex capacity: Limited, 2 genes	**Multiplex capacity:** 2-5+ genes	**Multiplex capacity:** Genome-wide

FIGURE 2 Increasing spatial information of gene expression through new in situ technologies. (Left) Traditional colorimetric in situ hybridization can provide qualitative, regional gene expression information. (Middle) Fluorescent in situ hybridization (FISH) can provide quantitative (up to single molecule counting) measurements of mRNA levels in single cells. FISH can be multiplexed, which is limited by the spectral separation of probe-associated fluorophores. (Right) Most spatial transcriptomics techniques use spatially barcoded capture oligos and NGS sequencing to map mRNA expression information to in situ position within tissue

proximity to a putative signal or reciprocal receptor/ligand expression. Such an approach has the advantage of being predictive, largely unbiased (although in need of a priori signaling pathway knowledge), and able to make more powerful inferences than the typical "highest fold expression hits" derived from bulk RNA sequencing studies. Spatial transcriptomic approaches can also be paired with cell labeling information (eg, EdU pulse, mitotic pH3 staining, transgenic label, or hematoxylin and eosin), which would allow one to link cell morphology and behavior with gene expression. Creating digital in situ maps across the entire transcriptome will also be useful for identifying spatial zones within the blastema that relate to particular progenitor behaviors or cell states.

It is important to keep in mind that the regenerative niche is transient and likely highly dynamic, meaning that in addition to sequencing efforts, high-resolution imaging-based techniques will play an important role in transforming macroscopic phenotypes observed from genetic and pharmacological perturbations into detailed cellular mechanisms underlying regeneration. Live imaging of single cells can capture physical interactions between progenitor cells and nonprogenitor cells; especially transient interactions that would be missed or underrepresented by fixed snapshots. Many of the progenitor-supporting cell types such as immune cells and nerve cells are highly dynamic during regeneration, thus imaging can provide key insights into how progenitor support signals change in space and time. This has important implications when thinking about how rapidly support signals arise or are differentially regulated in space and time between regenerative and non-regenerative scenarios.[93,94] In addition to transient support signals is the concept of transient cell types that arise to foster regeneration, but do not remain in the finally regenerated structure. For example, senescent cells are thought to act as signaling centers during regeneration but are cleared from the newly regenerated limb.[95] Such transient cell types would be missed with traditional endpoint lineage tracing techniques, but potentially detected and validated with a combination of scRNAseq snapshots and live cell imaging techniques.

Finally, as the field begins to reconstruct the regenerative blastema niche it will be vital to tease apart cell autonomous and noncell autonomous effects of niche signals as well as correctly characterize the direct or indirect effects they have on progenitor cells. Undertaking such work will absolutely require rigorous methodology to manipulate and follow individual or subsets of cells. Traditional electroporation approaches or inducible stable transgenes that express CRISPR/Cas or dominant negative constructs can be used in conjunction with live cell imaging or FACS-assisted sequencing to identify cell autonomous effects of perturbations to putative niche

signaling pathways. As an example, it is not entirely clear what types of blastema cells (connective tissue-derived, muscle progenitors, etc.) are acted on by neurotrophic signals as well as what indirect signals might be triggered as a consequence of nerve-derived Nrg1 expression.[85,96] Although in vivo systems can be applied, establishing ex vivo tissue culture and defined composite tissue explant systems[97] will greatly accelerate our understanding of niche-progenitor interactions. A key advantage of such systems would be the ability to define what aspects of regeneration are progenitor cell autonomous versus niche-derived. With the overarching goal of characterizing the regenerative niche, we can create a systems-level overview of signals that recruit, instruct, and maintain progenitor cells. Such knowledge would ultimately aid in identifying deficits in nonregenerating species and reconstituting regenerative environments for regenerative therapies.

7 | HOW SIMILAR OR DIFFERENT ARE LIMB DEVELOPMENT AND REGENERATION?

A common theme of limb regeneration is the redeployment of molecular pathways that were crucial for growth and patterning of the embryonic limb. A primary example of this is the initiation of a positive feedback loop between sonic hedgehog and FGF8 during axolotl limb regeneration in a similar fashion as is present in the axolotl limb bud.[91] In a similar manner, it is currently thought that the patterning of regenerated tissue follows the same morphogenetic programs as limb development.

Though there are similarities as noted previously, the origins of progenitors responsible for development versus regeneration are clearly separable (Figure 3). Limb bud cells are formed from delaminated lateral plate mesoderm cells[98,99] and follow a path from naive progenitor to specialized tissue-resident cells within fully developed tissues. Connective tissue blastema cells, on the other hand, are derived from differentiated tissue and are mostly thought to follow a lineage-restricted dedifferentiation to form a multipotent progenitor within the blastema. The ability to assess both regeneration and development at single cell resolution has generated new possibilities for elucidating key differences and similarities between the two processes.

Most of the work comparing limb development to limb regeneration has been performed under the premise that limb development is a highly conserved process across species. Surprisingly, recent work suggests that salamander limb development may deviate from classically studied mouse and chick limbs.[92,100] Efforts have already been made to compare limb development in mouse and
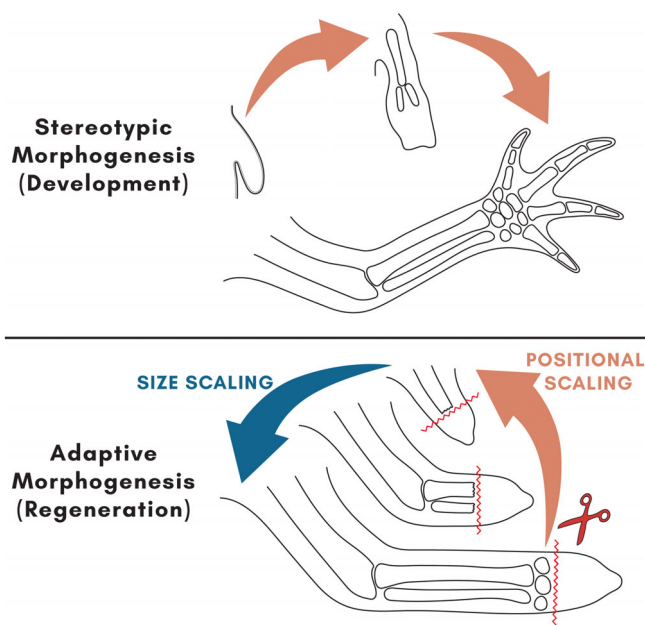
FIGURE 3 Tissue-scale differences between limb development and regeneration. Limb development (top) proceeds through stereotypic proximal to distal patterning and appendage size scale to sculpt progenitor cells into mature limb structures. In contrast, limb regeneration (bottom) must adapt to positional changes in the amputation position (red line) at either the upper, lower limb, or wrist/hand, as well as changes in the overall size of the limb, which continues growing throughout the animal's life

chick using scRNA-seq data[101] and undoubtedly more of these important comparisons are underway. This suggests the potential for how a limb develops may influence its future regenerative capacity. There are also fundamental differences in fully developed limbs, such as the axolotl's pseudostratified epidermis compared to the stratified epidermis of mice.[102] Given the foundational impact of limb development on limb regeneration, it will be prudent to expand our definitions of limb development to reflect what may include a variety of unique mechanisms across limbed species. Characterizing variations in limb development is more accessible than ever using single-cell techniques. This type of work will undoubtedly provide us with a more comprehensive understanding of how cell types and the signals they respond to are varied across species, as well as differential lineage outcomes. Such a multispecies index of developmental strategies should provide a more refined baseline to evaluate similarities and differences between limb development and regeneration.

Even within our current understanding of limb development, it is clear that for every similarity between development and regeneration there are just as many differences. The most notable divergences during regeneration are the injury-related initiation of regeneration and the differentiated cell source that seeds the blastema. We can generally compare regeneration and limb development by two metrics – (1) How molecularly similar are limb bud cells and blastema cells? and (2) how similar are the cues that coordinate and pattern the developing limb versus the regenerating limb? On a single-cell transcriptional level, connective tissue progenitors of the limb bud and blastema are similar, yet distinct, suggesting that the blastema is not simply a recreated limb bud, but instead an analogous and distinct structure.[9] We know that common lineage markers for chondrogenic commitment markers such as a SOX9 are present in the limb bud and blastema, but it is unclear whether SOX9 is induced in the same way and what regulates its expression in both systems.[7,92,103] Lineage tracing suggests that progenitors in both cases can create clones which contribute to multiple differentiated tissue types, but the full range of differentiation potential has not been completely tested or compared.[9,104] There remain several open questions about how limb bud cells and blastema cells may operate differently to achieve the same outcome. For instance, do blastema progenitors and limb bud cells have the same multipotent potential? Likewise, are the same pathways used to specify cell types during patterning? Further studies using transplantation assays between developing and regenerating contexts or in vitro assays to directly compare differentiation potential will shed light on the key intrinsic differences between cell types.

Another unexplored comparison between regenerative and developmental progenitors is the differences in metabolism and cell cycle kinetics needed to undergo regeneration. Based on the rapid onset of proliferation during limb regeneration from a largely quiescent source population, understanding how the cell cycle is regulated is a key question of how regenerative growth is maintained. In particular, live imaging using cell cycle sensors or modeling will help to determine how cell cycle speed and proliferation are sustained over time.[105-107] In addition to changes in proliferation, cell metabolism may be another aspect that differentiates limb development from regeneration. The rapid proliferation of the blastema likely has higher metabolic demands than the rest of the organism. Given the lack of vasculature in the early blastema,[108] it has historically been thought to be a relatively hypoxic environment. This has given rise to the hypothesis that normal aerobic metabolism may be harder to maintain for rapidly dividing cells. Although some evidence exists for glycolytic metabolism during other contexts of regeneration,[109,110] new methods for measuring metabolic activity in single cells may be an important next step to understand how regeneration can rapidly reconstitute new tissue.[111,112]

The most apparent difference in the microenvironment between limb development and regeneration is the surrounding context of injury that initiates regeneration and drives the formation of the blastema. Several injury-related signals have been described during regeneration without corresponding expression during development.[113] Such signals could be crucial in activating quiescent cells or linking injury-related signals to morphogenetic/developmental pathways. In vitro studies may help to elucidate the cell autonomous effects of individual factors outside of a complex in vivo milieu. In addition, transplantation of cells between the limb bud and blastema will help identify similarities and differences of the microenvironment as well as intrinsic properties of limb bud and regenerative cells.[68]

One additional element of the regenerative environment that deviates from development is the ability of signals and processes to scale to replace differently sized limbs and limb segments. Very little is known about how regenerative scaling works. For larger adult animals, the blastema and initial regenerated limb are significantly smaller than the original amputated limb but undergo a continuous scaling growth over several months to eventually match the stump size and contralateral limb.[114] The observed regeneration followed by scaling suggests that there may be an upper limit to the size of the blastema that can successfully pattern limb segments and a "catch up" growth period is subsequently used to match organismal size. Even with an initially smaller adult limb regenerate, the scale difference between larval and adult limbs is millimeters to centimeters in diameter, respectively. Two nonexclusive hypotheses are that (1) blastema patterning cues are able to scale to accommodate an order of magnitude change in size (reviewed by Čapek and Müller[115]) or (2) as mentioned previously (see "Does origin predispose destination?"), there is a small "prepattern" of the limb that arises at an early and small stage of blastema formation that propagates and persists to a greater extent than in small limb regenerates to produce a larger regenerate structure.[116] Quantitative and computational methods matched with cellular and molecular-level measurements of signaling gradients and cell cycle kinetics will be crucial to test theories of regenerative scaling. In addition, quantitative and high sensitivity methods such as single molecule FISH will be helpful to visualize patterning signals over different scales of regeneration.

8 | CONCLUSION

In our estimation, the limitations to understanding limb regeneration are no longer due to technical hurdles, but rather limitations in the creative design and adaptation of tools, thoughtful analysis of new and existing datasets, and in designing experiments that functionally test cellular and molecular mechanisms. What insights does the next decade hold toward understanding regeneration at cellular resolution? We expect that testable models will emerge that describe the essential molecular circuits needed to create dedifferentiated limb progenitors. As such models emerge, there will be concomitant cross-species comparisons that characterize conservation or deficiencies within regenerative pathways. The ability to visualize and characterize cell types and lineage decisions of regenerative cells will become more refined, with increasingly sophisticated methods to manipulate them as more transgenic tools become available. Finally, increasing collaboration between multiple groups and disciplines such as biophysics, engineering, and computational modeling will bring more perspectives into the limb regeneration field. The allure of this fascinating process will hopefully encourage groups with complementary expertise to join in the quest to uncover the cellular requirements for regeneration.

AUTHOR CONTRIBUTIONS
Nicholas D. Leigh: Conceptualization (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Joshua D. Currie:** Conceptualization (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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